# Molecular Characterisation of Selected Gastrointestinal Microbiota in South African HIV-Positive Patients during HAART

**By**

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# CERTIFICATION OF SUPERVISORS

In terms of the "General rules for the degree of Doctor of Philosophy (PhD)", paragraph GP5.2, we, the supervisors of the candidate Sarah Jane du Plessis, support the submission of this thesis for examination.



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# CONTENTS

# ABSTRACT

The human gastrointestinal tract harbours a rich microbial community made up of an estimated 500 different bacterial species. This complex community plays an integral role in intestinal development and maintenance and functions as a protective barrier against invading pathogens. HIV/AIDS is a global epidemic, severely affecting the health systems of many countries, especially South Africa. Progression of the HIV disease is characterised by a massive depletion of $CD_4^+$ T cells and it has been shown that patients living with a more advanced HIV infection have a higher risk of developing diarrhoea due to the disruption of the gastrointestinal microbiota caused by either the HIV-infection or the use of antibiotics and drugs such as highly active antiretroviral therapy (HAART). An imbalance in the microbial composition, attributable to a disturbed mucosal barrier, as well as increased permeability and inflammation caused by HIV, can influence the metabolic (carbohydrate fermentation) and protective functions provided by the microbiota. The effect of HIV on the intestinal microbiota has not been widely examined and those studies that have focused on HIV and the gastrointestinal tract, have investigated it mainly from a virological perspective. Consequently, the aim of the study was to ascertain whether the diversity and/or abundance of the endogenous intestinal microbiota of South African HIV-positive patients was disrupted on account of HIV within the gastrointestinal tract. An additional aim was to determine whether the administration of HAART affected the microbiota during a 6 month longitudinal study.

The diversity of the intestinal microbial composition was characterised with respect to the total bacteria, *Bifidobacterium* and *Lactobacillus* species using PCR-DGGE. qPCR was used to determine the abundance of total bacteria, *Bifidobacterium*, *Lactobacillus*, *Escherichia coli*, the *Bacteroides/Prevotella*, *Clostridium coccoides* and *Clostridium leptum* groups. The study cohort for DGGE studies consisted of 12 South African HIV-negative and 12 HIV-positive donors (females $n = 8$ and males $n = 4$). During HAART, in a 6 month longitudinal study the cohort was reduced to 8 subjects in each group. In addition, three potential intestinal pathogens (*Clostridium difficile*, *Campylobacter jejuni* and *Salmonella enterica*) were monitored by qPCR during this period, to determine their prevalence in the HIV-positive patients. The recruited HIV-positive donor group comprised mostly Black African females between the ages of 20 and 45, all of whom had blood $CD_4^+$ T cell counts of less than 200 cells/$mm^3$. PCR-DGGE analyses revealed host specific banding profiles for all the donors.

Before the initiation of HAART, the microbial diversity of the total bacteria, as determined by PCR-DGGE and using the Shannon-Wiener diversity index ($H'$), showed that it was significantly lower in the HIV-positive donors ($H'$= 1.80) when compared to the HIV-negative donor group ($H'$= 2.27) ($p$ = 0.009). Although the diversity of *Bifidobacterium* spp. appeared higher in the faecal samples of the HIV-positive donors ($H'$ = 0.65), relative to the HIV-negative group ($H'$= 0.37), this was not statistically significant ($p$ = 0.10). No significant difference was seen in the *Lactobacillus* group. The *Bifidobacterium* and *Lactobacillus* populations were both monitored over a 6 month longitudinal period and showed general temporal stability. However, the most noticeable change in the dynamics of these groups was seen when multiple antibiotics were administered, which drastically affected the diversity of the species present.

The results generated using quantitative real-time PCR (qPCR) provided additional insight into the effect that HIV infection has on the abundance of other intestinal bacterial groups. Comparative analysis of selected groups within the gastrointestinal tract of HIV-positive patients and HIV-negative donors, showed a significant reduction in the abundance of the *C. leptum* group ($p$ = 0.02) in the HIV-positive donors prior to the initiation of HAART. The *Bacteroides* group was also reduced in the HIV-positive patients, but this did not attain statistical significance ($p$ = 0.08). There was no significant difference between the two donor groups with respect to any of the other groups tested. After 6 months of HAART, significant differences in the abundance of the *C. leptum* ($p$ = 0.05) and *Bacteroides* ($p$ = 0.004) groups as well as the total bacteria ($p$ = 0.03) were seen relative to the HIV-negative donors. No detectable levels of *Enterococcus* spp. were found in either the HIV-positive or HIV-negative donor groups, while *S. enterica* and *Clostridium* cluster XI species were detected in both donor groups.

The *Bifidobacterium* and *Lactobacillus* populations were not majorly affected by the persistence of HIV within the gastrointestinal tract, and as a result of this, prescribing these bacteria as probiotics may not necessarily aid HIV recovery. Conversely, the significant reduction in the abundance of the *C. leptum* and *Bacteroides* groups, in the HIV-positive cohort after 6 months of HAART, suggests that probiotic supplementation with specific bacteria from within these groups may assist in normalising the gut microbiota. Species belonging to these dominant bacterial groups could be considered as possible probiotic

candidates, as increasing the abundance of these groups within the intestinal tract, may counteract and reduce the disruption and inflammation caused by the replication of HIV.

This pilot study is the first to report on the differences in the diversity and abundance of bacterial species within the gastrointestinal tracts of South African HIV-positive patients. The findings of this study have shed some light on the impact that HIV has on the intestinal microbiota, but also emphasise the need for more in-depth analysis that will expand further on the data generated in this thesis. Finally, successful monitoring of the status of the gastrointestinal microbiota of HIV-positive patients, along with the identification of potential probiotic candidates may open the way to more efficient and targeted probiotic supplementation in these patients as a supportive care during HAART.

# ABBREVIATIONS

| | | |
|---|---|---|
| AIDS | : | acquired immunodeficiency syndrome |
| ARV | : | antiretroviral |
| bp | : | base pairs |
| BSA | : | bovine serum albumin |
| CFU | : | colony forming units |
| DGGE | : | denaturing gradient gel electrophoresis |
| DNA | : | deoxyribonucleic acid |
| dNTP | : | deoxynucleotide triphosphate |
| EDTA | : | ethylenediaminetetra-acetic acid |
| FISH | : | fluorescent in situ hybridisation |
| g | : | gram(s) |
| gDNA | : | genomic DNA |
| GALT | : | gut associated lymphoid tissue |
| GC | : | guanine and cytosine |
| $H'$ | : | shannon wiener diversity index |
| HAART | : | highly active antiretroviral therapy |
| HIV | : | human immunodeficiency virus |
| L | | litre |
| LAB | : | lactic acid bacteria |
| log | : | logarithmic |
| M | : | molar |
| mg | : | milligram |
| ml | : | millilitre |
| mM | : | millimolar |
| min | : | minute(s) |
| *n* | : | number of samples |

| | | |
|---|---|---|
| ng | : | nanogram |
| nm | : | nanometre |
| *p* | : | *p*-value, indicating significance |
| PCR | : | polymerase chain reaction |
| qPCR | : | quantitative real time PCR |
| QR | : | quartile ranges |
| RNA | : | ribonucleic acid |
| rRNA | : | ribosomal ribonucleic acid |
| sec | : | second(s) |
| spp. | : | species |
| STI | : | sexually transmitted infections |
| TAE | : | tris-acetate-EDTA electrophoresis buffer |
| TB | : | tuberculosis |
| Tris | : | tris(hydroxymethyl)aminomethane |
| µl | : | microlitre |
| µg | : | microgram |
| µM | : | micromolar |
| UV | : | ultraviolet |
| V | : | volts |
| v/v | : | volume per volume |
| w/v | : | weight per volume |
| α | : | alpha |
| % | : | percent |
| $^{o}$C | : | degree(s) Celsius |

# CHAPTER ONE

## General Introduction

### Contents

## 1.1 THE HUMAN GASTROINTESTINAL MICROBIOTA

The human gastrointestinal tract (GIT), which includes the stomach, small and large intestines, is densely populated by a highly complex community of obligate and facultative anaerobic microbial species (Nielsen *et al*., 2003, Tannock, 2001; Zoetendal *et al*., 2002). The gastrointestinal microbiota of humans can be divided into two distinct groups namely, transient and endogenous (Gerritsen *et al*., 2011; Isolauri *et al*., 2004). The latter comprises bacterial species that originate and are established within the gastrointestinal tract, while the former refers to microorganisms that are not members of the endogenous intestinal microbiota, but have been introduced from the environment, for example, through the intake of food. Transient bacterial species may also become endogenous if they become established within the gastrointestinal tract (Alvarez-Olmos and Oberhelman, 2001; Alderberth, 2000).

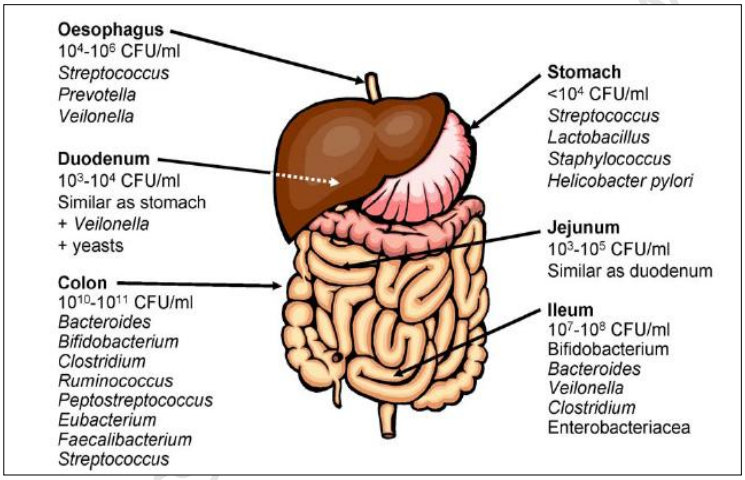


**Figure 1.1:** **Distribution of the microbiota** commonly found in the gastrointestinal tract of a healthy, human adult (Ouwehand and Vesterlund, 2003).

The diversity and composition of the endogenous intestinal microbiota varies considerably along the gastrointestinal tract (Figure 1.1). The upper gastrointestinal tract, namely the stomach and small intestine, is characterised by short transit times of contents, relatively low pH, and a range of digestive enzymes from the stomach, liver and pancreas, all of which contribute to a small microbial population size ($10^1$–$10^4$ CFU/ml) (Booijink *et al*., 2007; Dethlefsen *et al*., 2006). However, within the lower section of the gastrointestinal tract, the flow of contents becomes slower. Fewer inhibitory and antimicrobial compounds as well as more anaerobic conditions are present, consequently favouring the establishment of a more complex and dynamic microbiota (Isolauri *et al*., 2004; Rastall, 2004; Holzapfel *et al*., 1998). The large intestine harbours a rich

microbial community with typical numbers of about $10^{10}$-$10^{12}$ CFU/ml (Booijink *et al*., 2007; Dethlefsen *et al*., 2006).

Both culture-dependent and culture-independent studies have demonstrated that the vast majority of the gastrointestinal microbiota, found in healthy individuals, belongs to two major phyla, namely the Gram-positive Firmicutes and the Gram-negative Bacteroidetes (Flint, 2011; Mariat *et al*., 2009; Eckburg *et al*., 2005). These two dominant phyla together account for approximately 90% of the total bacteria based on 16S rRNA molecular analyses (Flint, 2011). The Bacteroidetes phylum consists of three classes, of which the genera *Bacteroides* and *Prevotella* are the most well studied. The Firmicutes is the largest phylum, containing over 200 genera, with majority of the Firmicutes detected in the gastrointestinal tract falling into two main groups, namely, the *Clostridium coccoides* group (also known as *Clostridium* cluster XIVa) and the *Clostridium leptum* group (referred to as *Clostridium* cluster IV) (Mariat *et al*., 2009). Some of the predominant Firmicutes identified include: *Faecalibacterium prausnitzii*, *Eubacterium rectale/Roseburia* spp. and *Eubacterium hallii* (Walker *et al*., 2011; Louis and Flint, 2009).

In addition to the Bacteroidetes and Firmicutes phyla, species belonging to other phyla such as Proteobacteria, Actinobacteria, Fusobacteria and Spirochaetes have also been detected within the gastrointestinal tract (Walker *et al*., 2011; Tap *et al*., 2009; Zoetendal *et al*., 2008; Rajilić-Stojanović *et al*., 2007). Culture-independent analysis of the fungal diversity within the gastrointestinal tract revealed that the majority of fungal species belong to the fungal phyla Ascomycota (*Candida* and *Saccharomyces*) and Basidiomycota (Ott *et al*., 2008).



### 1.1.1 Functions of the gastrointestinal microbiota

The ultimate role of the endogenous intestinal microbiota is to positively benefit the host, by carrying out various metabolic, nutritional, physiological and immunological processes in the human body (Gerritsen *et al*., 2011; Wang *et al*., 2005). Examples of some of these key functions include specific metabolic activities, regulation and maintenance of the intestinal epithelium and immune system, and protection against opportunistic pathogens (Guarner and Malagelada, 2003).

*Metabolic functions*

One of the major metabolic functions carried out by the endogenous microbiota is the fermentation of otherwise indigestible dietary polysaccharides such as, resistant starch and dietary fibres, to produce short chain fatty acids (SCFA) including butyrate, acetate and propionate and various gases (e.g. carbon dioxide and hydrogen) (Gerritsen *et al*., 2011; Laparra and Sanz, 2010). These short chain fatty acids regulate epithelial cell growth and differentiation in the small and large intestine (Canny and McCormick, 2008; Frankel *et al*., 1994), play an important role in the maintenance of the mucosal layer and in providing the colonic cells with additional energy, as well as stimulating the proliferation of the epithelium (Turroni *et al*., 2008; Marchesi and Shanahan, 2007; Anuradha and Rajeshwari, 2005; Eckburg *et al*., 2005; Isolauri *et al*., 2004; Guarner and Malagelada, 2003; Brouns *et al*., 2002; Salminen *et al*., 1998).

This diverse microbial community within the colon is estimated to have 100 times more total genes than the human host, which means that a variety of enzymes and biochemical pathways, distinct from the host's own enzymes, are available and can be used to the host's advantage (Keeney and Finlay, 2011; Guarner and Malagelada, 2003). Genomic and physiological studies have demonstrated that the gut microbiota provides enzymes specialised in the utilisation of non-digestible carbohydrates and host-derived glycoconjugates (mucin) (Laparra and Sanz, 2010). Gut bacterial enzymes are also involved in the metabolism of cholesterol and bile acids. The endogenous microbiota is capable of reducing cholesterol to coprostanol, which is then secreted and removed in the faeces (Laparra and Sanz, 2010). *Bacteroides intestinalis*, *Bacteroides fragilis* and *Escherichia coli* have all been described, in the literature, as potentially being involved in the generation of secondary bile acids in the colon (Fukiya *et al*., 2009). Primary bile acids, cholic acid and chenodeoxycholic acid, which are both synthesised from cholesterol in the liver, are readily converted into secondary bile acids (deoxycholic acid and lithocholic acid) by the intestinal bacteria. The conversion of these primary bile acids into secondary bile acids assists in limiting the solubilisation and absorption of dietary lipids throughout the intestine (Ridlon *et al*., 2006).

Other important metabolic contributions of the intestinal microbiota include biosynthesis of essential vitamins, such as vitamin B and K (Salminen *et al*., 1998), production of amino acids required by humans (Laparra and Sanz, 2010; Hamer *et al*., 2008; Wong *et al*., 2006), and the absorption of calcium, magnesium and iron (Younes *et al*., 2001).

*Immune functions*

Another important function carried out by the intestinal microbiota is the establishment of communication between the host and the microbiota at the mucosal barrier, thereby allowing the development and regulation of the host's immune system (Sanz and De Palma, 2009; Canny and McCormick, 2008). The intestinal epithelium constitutes a physical barrier that regulates the transit of exogenous substances and prevents the entry of luminal antigens. This barrier is further strengthened by the presence of the mucus layer (comprising mucins) and the synthesis of antimicrobial peptides and other secretions such as bile acids and enzymes (Laparra and Sanz, 2010). The endogenous microbiota form part of the primary line of defence and are capable of regulating the expression of mucin genes found within the goblet cells, as well as the secretion of specific antimicrobial peptides (e.g. α-defensins) by the paneth cells (Laparra and Sanz, 2010). Furthermore, the intestinal microbiota plays an integral role in the postnatal development of the host's immune system by influencing the number of T cells found within the lamina propria, the number of immunoglobulin A producing B cells, as well as intraepithelial T cells (Laparra and Sanz, 2010; Tlaskalovà-Hogenovà *et al*., 2004). There is a strong indication that microbial colonisation of the gastrointestinal tract also functions in regulating the gut associated lymphoid tissue (Macdonald and Monteleone, 2005).

*Protective functions*

A further important function of the intestinal microbiota is to protect the host from invading pathogens and prevent damage to the epithelial cells by serving as a protective barrier (Keeney and Finlay, 2011; Guarner and Malagelada, 2003; Rolfe, 2000). Several different mechanisms whereby the endogenous microbiota exerts a protective effect against potential pathogens have been proposed. For example, many species of the endogenous microbiota are capable of producing antimicrobial compounds that inhibit the growth of both Gram-positive and negative bacteria. These inhibitory substances include hydrogen peroxide, organic acids and microbial proteins such as bacteriocins (Louis and O'Byrne, 2010; Rastall *et al*., 2005; Rolfe, 2000). A second possible mechanism involves the intestinal microbiota binding to the intestinal epithelial surfaces and competitively inhibiting the attachment or toxin binding sites of pathogenic bacteria such as *Salmonella* and *Clostridium* species (Rastall *et al*., 2005; Rolfe, 2000). Lastly, direct competition for nutrient availability is another strategic mechanism employed, whereby a number of species of the intestinal microbiota actively utilise nutrients required by the pathogen (Keeney and Finlay, 2011).

### 1.1.2 Factors influencing the composition of the gastrointestinal microbiota

Even though the endogenous microbiota of the intestinal tract is generally temporally stable, factors such as the age of an individual, diet and nutrition, and antibiotic treatments are all capable of influencing the composition and diversity of this community.

#### *1.1.2.1 Age of an individual*

The age of an individual has been shown to influence and affect the composition of the intestinal microbiota. During adult life, the intestinal microbial community is relatively stable (Costello *et al*., 2009), unlike the intestinal tract of infants which appears to be in a continuous state of change, with an extremely low microbial diversity (Roger *et al*., 2010; Blaut *et al*., 2002). The microbial diversity of the gastrointestinal tract of infants is influenced by several factors such as mode of delivery (natural birth or caesarean section), gestational age, infant hospitalisation and antibiotic use (Penders *et al*., 2006). Colonisation of the gastrointestinal tract commences immediately after birth and, by the time a child turns 2 years old, an adult-like microbiota has become well established with a strong predominance of *Bacteroides* and *Clostridium spp*. (Tiihonen *et al*., 2010; Frank and Pace, 2008; Isolauri *et al*., 2004; Alvarez-Olmos and Oberhelman, 2001).

On the other hand, a significant shift and modification of the intestinal microbiota has also been shown to occur within the elderly (usually defined as people over the age of 65). In the elderly, there are marked physiological changes that occur, all of which have an impact on the composition and functionality of the intestinal microbiota (Tiihonen *et al*., 2010; Woodmansey *et al*., 2007). Many elderly humans suffer from decreased intestinal motility, which can result in prolonged intestinal transit times and faecal retention. In addition, age-related deterioration of the immune system, as well increased use of laxatives, antibiotics and other medication have also been shown to be associated with a change in the microbiota composition (Gerritsen *et al*., 2011; Schiffrin *et al*., 2010). The gastrointestinal microbiota of the elderly is characterised by low *Bifidobacterium*, *Lactobacillus* and *Bacteroides* numbers, along with a significant increase in *Clostridium* and *Enterobacteriaceae* species (*E. coli*) compared to healthy, younger controls (Gerritsen *et al*., 2011; Zwielehner *et al*., 2009; Marchesi and Shanahan, 2007; Woodmansey, 2007; Bartosch *et al*., 2004).

*1.1.2.2 Diet and Nutrition*

Modulation of the intestinal microbiota of humans can also occur as a result of diet (Barnett *et al*., 2012), and the influence of diet and lifestyle habits on the structure and dynamics of the endogenous microbiota has been widely investigated. The diet of an individual serves as the main source of energy for the intestinal microbiota, and, therefore, plays an integral role in determining which bacterial species will persist within the large intestine (Louis and Flint, 2009).

During the first few months of development, a particularly high number of *Bifidobacterium* spp., up to 90% of the total faecal bacteria, have been detected in breast-fed infants while, after only 6 months, increasing levels of species belonging to the *Bacteroides* and *C. coccoides* groups were identified (Laparra and Sanz, 2010; Hopkins *et al*., 2005). This significant change in microbial diversity has been directly linked to weaning, on account of the introduction of alternative carbohydrate-based substrates (Scott *et al*., 2011). However, it is important to note that the *Bacteroides* to Firmicutes ratio is still significantly lower than that present in adults (Mariat *et al*., 2009). In contrast, infants receiving a formula-based diet tended to exhibit a more mixed microbiota, consisting of larger numbers of *Lactobacillus*, *Bacteroides*, *Clostridium*, *Ruminococcus* and *Streptococcus* species and relatively few *Bifidobacterium* species (Laparra and Sanz, 2010; Alvarez-Olmos and Oberhelman, 2001; Salminen *et al*., 1998).

In a study conducted by Ley *et al*., (2006), the effect of diet on the gastrointestinal microbiota was evaluated, using culture-independent techniques. The results of this study showed that the microbial communities of 12 obese individuals were significantly enriched in Firmicute species and depleted in *Bacteroides* species, relative to a non-obese control group. Moreover, consumption of a low-calorie diet by the obese study group led to a shift in their intestinal microbial community back towards a healthy, lean state (Ley *et al*., 2006). However, since the publication of the findings by Ley *et al*., (2006), the majority of other investigations have been unable to confirm these results, with all showing varying results with respect to the diversity and composition of the intestinal microbiota of obese people (Zhang *et al*., 2009; Duncan *et al*., 2008).

Plant cell wall polysaccharides such as cellulose and pectins, and storage polysaccharides such as inulin and resistant starch are all important for the growth of endogenous intestinal microbiota. Diets rich in nondigestible carbohydrates often lead to high levels of bacterial

fermentation in the large intestine. Dietary components, such as resistant starch have been shown to increase the production of butyrate in the large intestine (Louis and Flint, 2009; Louis *et al*., 2007). In a recent study conducted by Walker *et al*., (2011), rapid (within 3 days) and reversible changes in the abundance of specific dominant bacterial groups were observed following specific dietary changes. The most noticeable response was the significant increase in the abundance of *Ruminococcus bromii* and *Eubacterium rectale* in the volunteers who had been receiving a diet consisting of resistant starch (Walker *et al*., 2011). In a different study, Duncan *et al*., (2007) reported that decreasing the carbohydrate intake in a diet resulted in a significant decrease in detectable numbers of the *E. rectale*/*Roseburia* group, as well as butyrate production. This distinct relationship between the numbers of the *E. rectale*/*Roseburia* group, butyrate production and carbohydrate intake has been shown in other human studies (Abell *et al*., 2008).

*1.1.2.3 Antibiotic treatments*

The administration of antibiotics results in the disturbance of the intestinal microbiota and increases the risk of opportunistic pathogenic species becoming established and colonising the intestinal tract (Tiihonen *et al*., 2010). Broad-spectrum antibiotics are routinely administered, in an attempt to counteract the spread and colonisation of invading pathogenic microorganisms. However, the use of antibiotics is not always desirable in that, not only are the targeted pathogens affected, but so too are members belonging to the endogenous microbiota, significantly altering the structure and diversity of the microbial community (Gerritsen *et al*., 2011; Donskey *et al*., 2003). In spite of this, normalisation of the microbiota to a stable, regulated state has been shown to occur following the cessation of the antibiotic treatment (Dethlefsen and Relman, 2010). An additional problem associated with the widespread use of antibiotics is the increased prevalence of antibiotic resistance resulting from the transfer of resistances between microorganisms (Jernberg *et al*., 2010).

### 1.1.3 Physiology of the gastrointestinal mucosal barrier

The gastrointestinal mucosal barrier is the complex mucous layer coating the stomach, small intestine and large intestine and is the main barrier separating the host's epithelial cells and underlying tissues from the microbiota present in the lumen (Johansson *et al*., 2011; McGuckin *et al*., 2011). Thickness of the mucosal layer varies throughout the gastrointestinal tract, ranging from 700 µm in the stomach, where it provides protection against the strong acidic conditions,

to 150-300 µm and 800 µm in the small and large intestines respectively (Atuma *et al*., 2001). On the whole, the structure of the mucosal barrier consists of a single layer of columnar epithelial cells covered with a layer of mucous that is composed of two separate layers, namely, a thin internal layer and a thicker outermost layer (Figure 1.2A). The innermost layer remains firmly attached to the epithelium, whilst the outermost mucus layer is continuously removed on the lumen side and replenished by secretion from the underlying epithelium (Derrien *et al*., 2010; Linden and McGuckin, 2010). Goblet cells are largely responsible for the production of mucins, as well as several other proteins. Mucins are high molecular weight glycoproteins and are the major structural components of the mucous layer, responsible for giving it its viscous nature. A total of 15 human mucins have been identified so far (McGuckin *et al*., 2011; Derrien *et al*., 2010; Linden and McGuckin, 2010). The main mucin constituent, identified in both of the mucus layers within the large intestine, was MUC2 while MUC5AC and MUC6 are present in the stomach (McGuckin *et al*., 2011; Derrien *et al*., 2010). Paneth cells, however, produce non-specific antimicrobial compounds such as α-defensins, lysozymes, lactoferrin, Trefoil factors and specific immunoglobulins that are secreted and retained within the mucous matrix (Figure 1.2B) (McGuckin *et al*., 2011).

The gastrointestinal mucosal barrier has several functions. These include facilitating the transit of food along the gastrointestinal tract by acting as a lubricant, and providing a nutrient source for the growth of certain bacterial species. Mucin glycans are an excellent energy source for the endogenous microbiota that are capable of utilising glycan-degrading enzymes to digest the glycans, resulting in the release of monosaccharides. Bacteria then use these monosaccharides to produce SCFA (Johansson *et al*., 2011). Commensal microbiota utilise the outermost mucosal layer for attachment (Derrien *et al*., 2010). The mucosal barrier also functions as a mechanical barrier, separating the commensal microbiota present in the lumen from the epithelium (Leser and Mølbak, 2009) and protecting the commensal microbiota present in the lumen of the gastrointestinal tract (acts as first line of defence) as well as providing the starting point for host-microbe interactions (Derrien *et al*., 2010).

Mucosa associated bacteria are less susceptible to elimination, have access to a wider range of nutrient sources provided by the mucous and are in close proximity to the epithelial cells, thus potentially influencing these cells (Derrien *et al*., 2010). The number of bacterial species associated with the mucosal layer is lower in number than those present within the lumen (Zoetendal *et al*., 2008). Maintenance of the integrity of the mucosal layer is essential to

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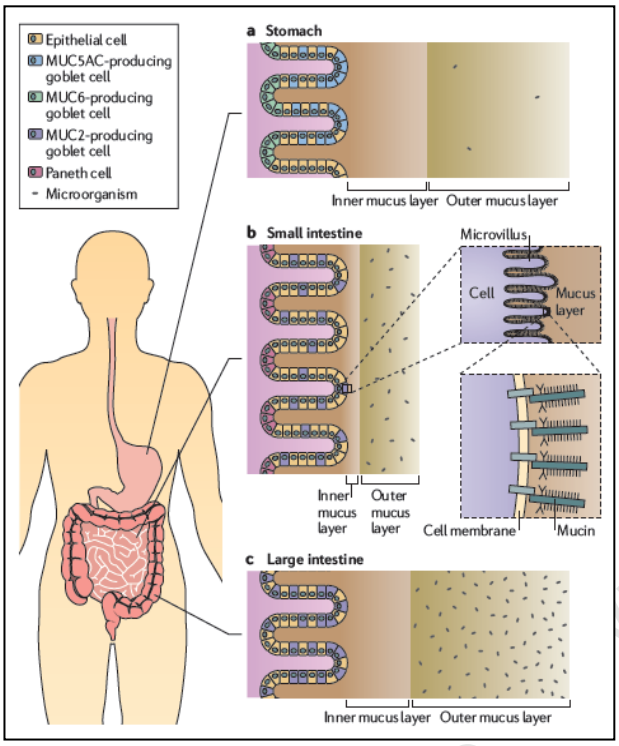


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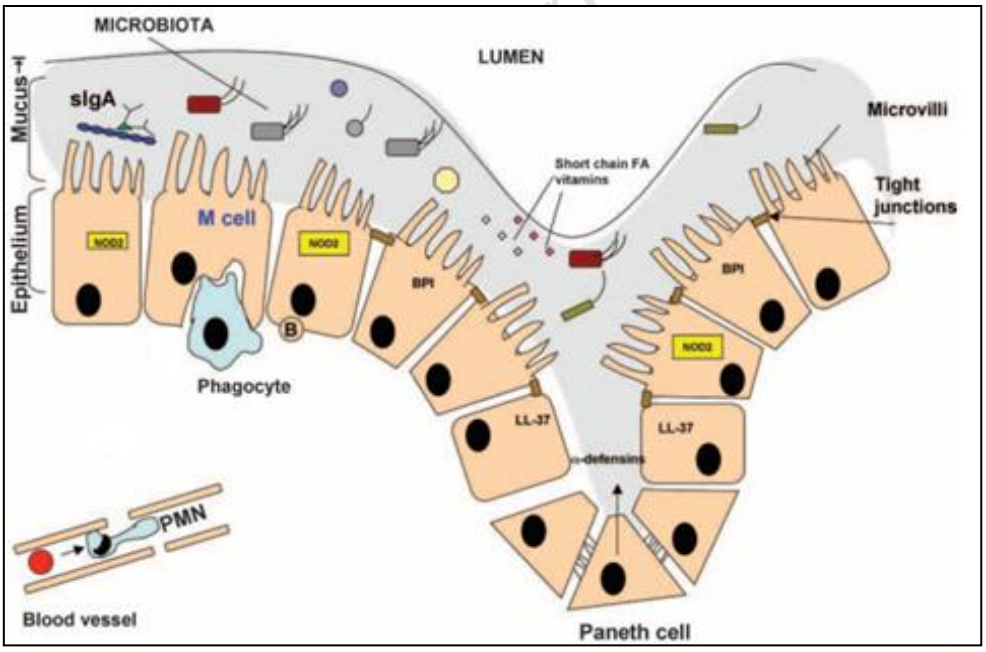



**Figure 1.2:** **Basic structure of the gastrointestinal mucosal barrier**. (A) Schematic representation of the gastrointestinal mucosal barrier (McGuckin *et al.*, 2011). (B) Illustration of the mucosa in a healthy gastrointestinal tract (Canny and McCormick, 2008). Abbreviations: [sIgA] – Immunoglobulin A; [M cell] – epithelial microfold cell; [Nod 2] – nucleotide oligomerisation domain 2; [BPI] – bactericidal increasing protein; [LL 37] – cathelicidin and [PMN] – polymorphonuclear leukocyte migration.

ensure that the underlying epithelial cells are protected. A disruption to the integrity of the mucosal barrier can be an indication of the development of a disease condition, for example Crohn's disease or ulcerative colitis (Derrien *et al*., 2010).

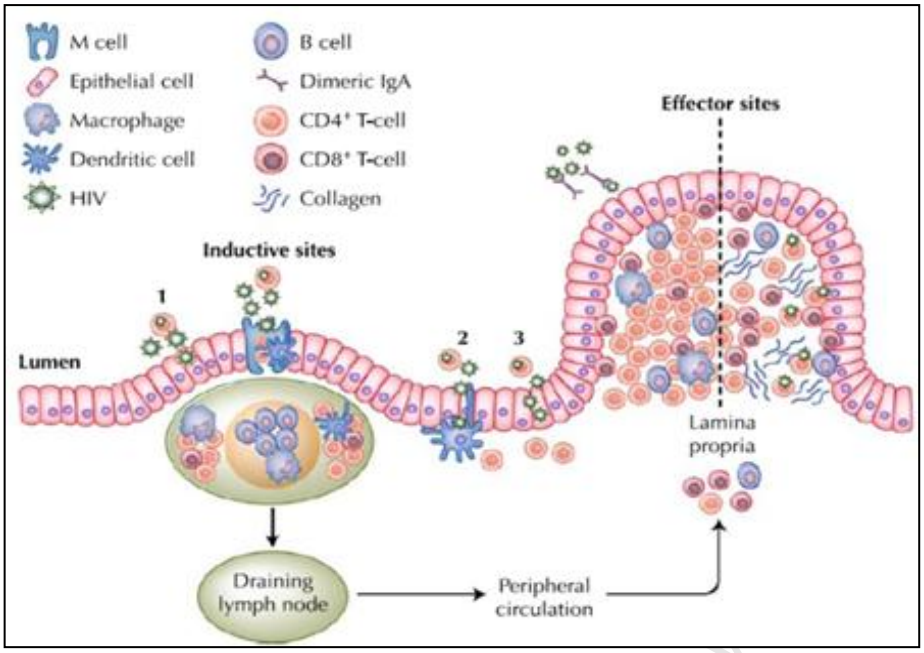
## 1.2 THE GASTROINTESTINAL TRACT AND HIV

### 1.2.1 HIV infection and the gastrointestinal tract

The mucosal surfaces of the human gastrointestinal tract play a vital role in the overall pathogenesis of HIV (Smith *et al*., 1997). The progression of HIV is characterised by a massive depletion of $CD_4^+$ T cells, which occurs during all the stages of HIV infection. The intestine has been shown to be the earliest site of $CD_4^+$ T cell depletion in HIV-positive patients, with approximately 70-95% depletion of these cells occurring within the first few weeks after exposure to HIV (Gori *et al*., 2008; Veazey and Lackner, 2004; Veazey *et al*., 1998). T helper cells are a type of white blood cell that plays an integral role in the human immune system. Mature T helper cells express the surface protein $CD_4$ and are then classified as $CD_4^+$ T cells. $CD_4^+$ T cells play a central role in immune protection through their capacity to help B cells make antibodies, the induction of macrophages, the production of cytokines and the recruitment of neutrophils, eosinophils, and basophils to sites of infection and inflammation (Zhu and Paul, 2008). The CCR5 protein belongs to the beta chemokine receptor family of integral membrane proteins, and is predominantly expressed on the surface of T cells, macrophages and dendritic cells (Samson *et al*., 1996).

The mucosal immune system is directly involved in the generation of innate and adaptive immune responses in order to protect the host from external factors such as invading opportunistic pathogens (Paiardini *et al*., 2008). The largest component of this immune system is the gut associated lymphoid tissue (GALT), present throughout the gastrointestinal tract and containing approximately more than $10^6$ lymphocytes/g of tissue (Paiardini *et al*., 2008; Hein, 1999; Salminen *et al*., 1998). Lymphocytes populating the gastrointestinal tract can be divided into two categories, namely those present in the epithelium and those in the underlying inductive (Peyer's patches, lymphoid follicles) and effector (diffuse lamina propria) gut-associated lymphoid tissues (Figure 1.3) (Shacklett and Anton, 2010). The lamina propria is rich in plasma cells, granulocytes and mast cells, while both the inductive and effector sites possess macrophages and dendritic cells (Shacklett and Anton, 2010; Hein, 1999; Salminen *et al*., 1998).

A




B

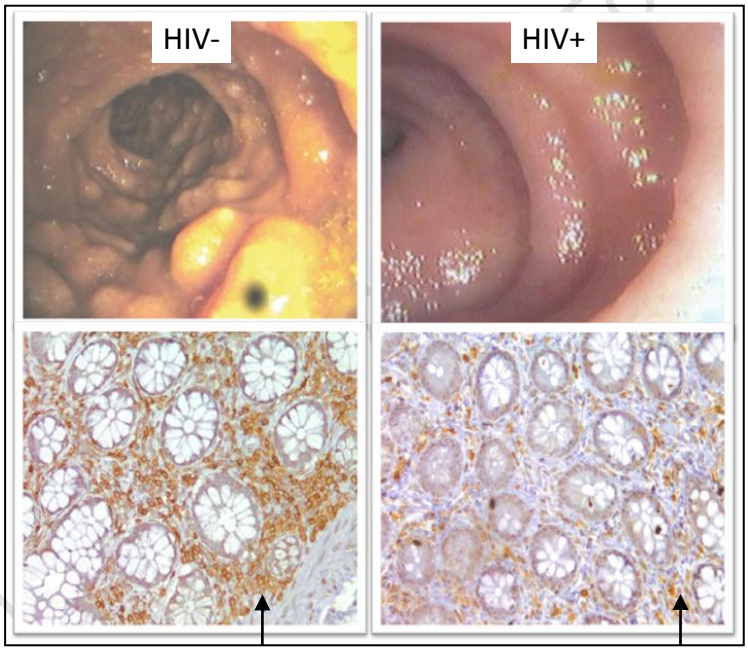



**Figure 1.3:** **HIV infection and the gastrointestinal tract. (A)** Intestinal inductive and effector sites in HIV-infection (Shacklett and Anton, 2010). Numbers indicate how HIV and/or infected cells may cross the epithelium: (1) transcytosis across epithelial or M cells; (2) adhering to dendrites of mucosal dendritic cells; (3) direct passage through epithelial breaches. **(B)** HIV-negative individual shows large aggregates of lymphoid tissue and large numbers of $CD4^+$ T cells, while a newly infected HIV-positive individual shows a complete lack of this tissue and significant $CD_4^+$ T cell depletion in the gastrointestinal tract (Brenchley *et al.*, 2004). Arrows indicate $CD_4^+$ T cells.

The preferred targets of HIV are the mucosal memory $CD_4^+$ T cells, expressing the CCR5 HIV co-receptor. As many as 70% of the $CD_4^+$ T cells found within the gastrointestinal tract possess the CCR5 HIV co-receptor (Douek, 2007; Brenchley *et al*., 2004; Veazey and Lackner, 2004). In contrast, only 20% of the peripheral blood lymphocytes are found to express the CCR5 co-receptor (Anton *et al*., 2000). Inductive lymphoid tissue sites are characterised by the presence of resting $CD_4^+$ and $CD_8^+$ T cells, which are capable of migrating to effector lymphoid tissue sites upon exposure to a variety of antigens (Veazey and Lackner, 2004).

Immediately after exposure, HIV is capable of penetrating and initiating infection within the intestinal epithelial lining through a number of different routes. HIV can access the lamina propia directly, either through small mucosal breaks within the epithelium or by simply adhering to the mucosal dendritic cells or specialised transport cells, known as M cells (Figure 1.3A), which transfer the virus to susceptible $CD_4^+$ T cells present in the underlying inductive sites (Shacklett and Anton, 2010). Once inside the intestinal lamina propria, viral replication is initiated, resulting in a rapid depletion of $CD_4^+$ $CCR5^+$ T cells in as little as 21 days from infection (Figure 1.3B) (Lackner *et al*., 2009; Veazey and Lackner, 2004). Following replication newly infected cells move towards the lymph draining nodes, where they are then disseminated throughout the body to infect new target cells (Lackner *et al*., 2009).The rapid depletion of $CD_4^+$ T cells from the gut associated lymphoid tissues, results in an increase in gut mucosa permeability, significant impairment of the mucosal immune system, translocation of microbial products such as lipopolysaccharides and, increased activity of opportunistic and pathogenic intestinal microorganisms (Paiardini *et al*., 2008; Veazey and Lackner, 2004).



### 1.2.2 Highly Active Antiretroviral Therapy (HAART)

The elucidation of virus-specific processes during the virus replication cycle (viral binding, entry into host cells, reverse transcription and viral DNA and RNA synthesis), provides a number of suitable targets for antiviral drugs, ultimately resulting in a reduction in viral replication without any detrimental effects to the host (De Clercq, 2001). The introduction of highly active antiretroviral therapy (HAART) into local hospitals has resulted in a considerable improvement in the quality of life for the patient and a decrease in the morbidity and mortality rates (Call *et al*., 2000). HAART is the term used to describe the combination of at least three different antiretroviral (ARV) drugs used in the treatment of HIV. These combinations are generally made up of two nucleoside/nucleotide analogue reverse transcriptase inhibitors

(NRTIs) and, either a non-nucleoside reverse transcriptase inhibitor (NNRTIs) or a protease inhibitor (PI) (Warnke *et al*., 2007; Tramarin *et al*., 2004). To date, a total of 30 ARV drugs have been approved for use in HIV-positive patients, and are grouped into six different drug classes, based on their mechanism of action (Table 1.1) (Kim and Read, 2010; Chen *et al*., 2007; Warnke *et al*., 2007). For the purpose of this thesis, the three most important drug classes used in first-line ARV regimes will be discussed further.

**Table 1.1** ARV drugs currently administered to HIV-positive patients

| ARV class | Selected Examples | ARV class | Selected Examples |
|---|---|---|---|
| Nucleoside/nucleotide analogue reverse transcriptase inhibitors (NRTIs) | Didanosine (ddi)<br>Stavudine (d4T)<br>Lamivudine (3TC)<br>Tenofovir (TDF)<br>Zidovudine (ZDV/AZT) | Fusion Inhibitors | Enfurvitide (T-20) |
| Non-nucleoside reverse transcriptase inhibitors (NNRTIs) | Nevirapine (NVP)<br>Efavirenz (EFV)<br>Delavirdine (DLV) | CCR5 Inhibitors | Maraviroc (MVC)<br>Viciviroc (VCV) |
| Protease Inhibitors | Ritonavir (RTV)<br>Lopinavir (LPV)<br>Aluvia (INNs) | Integrase Inhibitors | Raltegravir (RAL)<br>Elvitegravir |

*Nucleoside/Nucleotide Analog Reverse Transcriptase Inhibitors:*

Nucleoside/Nucleotide Analog Reverse Transcriptase Inhibitors (NRTIs) are an important component of the ARV regimen and are used in the majority of first-line treatments. NRTIs are incorporated into the viral DNA and the mechanism of action involves inhibiting viral DNA synthesis by preventing reverse transcription (Figure 1.4). As a result of this, viral replication is prematurely terminated and the infection of new $CD_4^+$ T cell targets is subsequently reduced. However, in order to carry out the abovementioned antiviral activity, NRTIs require intracellular phosphorylation by cellular kinases (Warnke *et al*., 2007). Currently, there are eight different NRTIs available, with Zidovudine (AZT) and Lamivudine (3TC) being two of the most frequently used NRTIs (Warnke *et al*., 2007). Interestingly, Lamivudine (3TC) has been shown to be effective against both HIV and a Hepatitis B co-infection (Chen *et al*., 2007).

*Non-Nucleoside Reverse Transcriptase Inhibitors:*

Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs) function at the same point of the HIV replication cycle as the NRTIs (Figure 1.4), but are capable of binding directly to the reverse transcriptase of HIV (Grob *et al*., 1992; Merluzzi *et al*., 1990). In contrast to the NRTIs, NNRTIs do not require intracellular phosphorylation and are not incorporated into the viral

DNA (Chen *et al*., 2007; Warnke *et al*., 2007). Nevirapine (NVP), Efavirenz (EFV) and Delavirdine (DLV) are examples of three specific NNRTIs currently used in the treatment of HIV. Nevirapine and Efavirenz are also both inducers of the hepatic cytochrome CYP450 3A4 isoenzymes (Chen *et al*., 2007).

*Protease Inhibitors:*

HIV-1 protease is a multifaceted enzyme responsible for the cleavage of large viral precursor polypeptide chains into smaller, functional proteins needed in the formation of new virions. This particular process takes place at the end of the HIV replication cycle (Figure 1.4) (Chen *et al*., 2007; Warnke *et al*., 2007). Inhibiting HIV-1 protease results in the release of disorganised, non-infectious virions (Pillay *et al*., 1995). Drug interactions are an important consideration with the use of Protease inhibitors. Protease inhibitors that are most commonly administered to HIV-positive patients include Ritonavir (RTV) and Lopinavir (LPV).

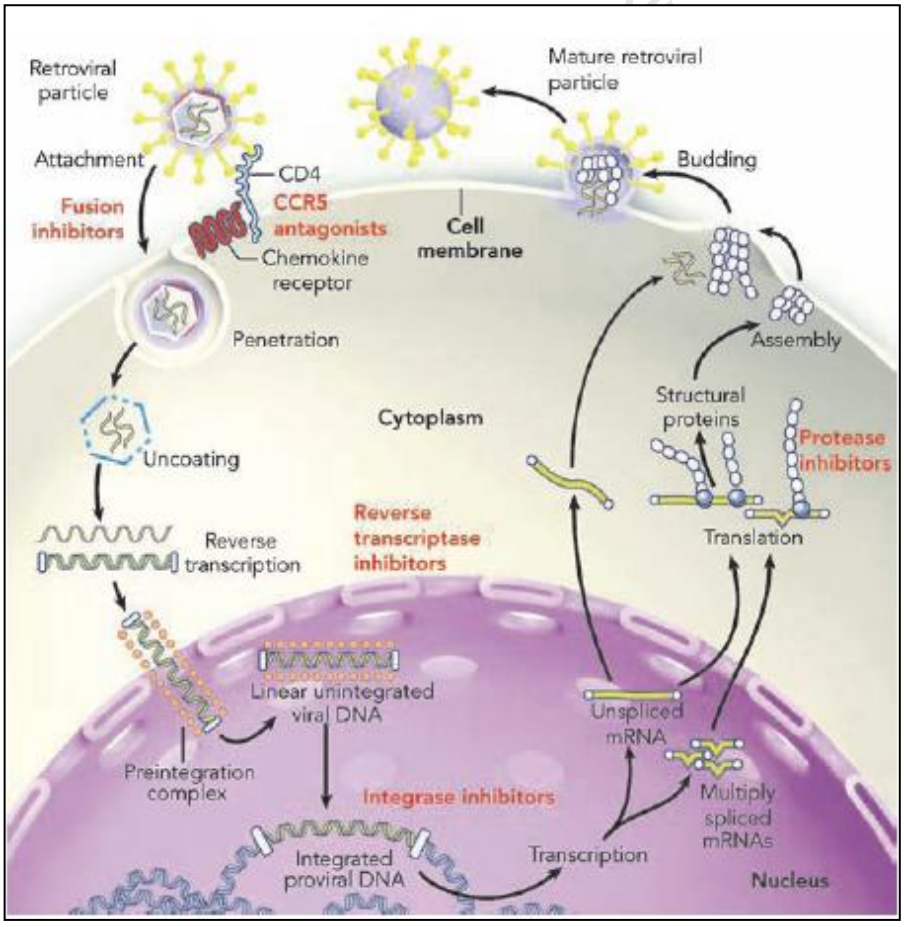



**Figure 1.4: HIV replication cycle and ARV drug targets.** (Chen *et al*., 2007).

The current trend in the treatment of HIV in South African Public Health Facilities is to delay the initiation of HAART until HIV-positive patients exhibit characteristic symptoms of AIDS-related infections or have significantly low $CD_4^+$ T cell levels in the blood (< 200 $CD_4^+$ T cells/µl). Before initiating any first-line HAART regime, factors such as the efficacy of the prescribed combination, possible drug interactions between ARV's, Hepatitis A and B co-infections, pregnancy and any adverse side effects, need to be considered. Moreover, lack of adherence to the HAART regime is a serious limitation encountered during the treatment and management of HIV, possibly resulting in heightening the risk of increased drug resistance, as well as reduced ARV activity and/or efficacy.

### 1.2.3 HIV-related diarrhoea

Diarrhoea is a recurrent gastrointestinal manifestation associated with HIV/AIDS, having a negative impact on the quality of life for the patient. Clinical symptoms of diarrhoea in HIV-positive patients can occur in either an acute or chronic form. Acute diarrhoea is characterised by irregular abdominal cramps and is generally short-lived, lasting only 2 to 3 weeks, while chronic diarrhoea, although being less painful, lasts for several months (Sestak, 2005; Tramarin *et al*., 2004). At present, the etiology of diarrhoea in HIV-positive patients can be separated into two categories, specifically, opportunistic infections and non-infectious causes.

The introduction of HAART has seen a subsequent improvement in the immune status of HIV-positive patients and, as a result, a significant reduction in the incidence of diarrhoea caused by opportunistic pathogens (Wilcox and Saag, 2008; Nannini and Okhuysen, 2002). However, prior to the use of HAART in the treatment of HIV, the most common opportunistic pathogens known to cause diarrhoea in HIV-positive patients included: *Cryptosporidium* and *Cyclospora* species, *Cytomegalovirus*, *Microsporidium*, *Mycobacterium avium* and *Giardia lamblia* (Sestak, 2005). Furthermore, pathogenic bacteria such as *Salmonella, Shigella, Escherichia, Campylobacter* and *Clostridium* species, have also been shown to be capable of colonising the compromised intestinal tract of HIV-positive individuals, resulting in the development of secondary infections that often require treatment with an antibiotic (Schroder *et al*., 2006; Sestak, 2005). In particular, *C. difficile* is fast becoming a commonly identified causative agent of antibiotic induced chronic diarrhoea in HIV-positive patients (Sanchez *et al*., 2005).

However, despite the significant reduction in the number of opportunistic pathogens causing diarrhoea in HIV-positive patients, the number of reported diarrhoea cases has not decreased significantly and this is thought to be as a result of drug-associated diarrhoea. Drug-induced diarrhoea is a common side-effect, seen particularly in patients receiving protease inhibitors, such as amprenavir, ritonavir and idinavir, as part of their HAART programme. Studies have shown that the incidence of diarrhoea associated with protease inhibitors, occurs in 33-50% of patients receiving nelfinavir and in up to 20% of those prescribed lopinavir/ritonavir and fosamprenavir/ritonavir (Guest *et al*., 2004). Given the relatively high prevalence of drug-induced diarrhoea in HIV-positive patients, prescribing a protease based HAART regime is not done regularly, as there remains a major concern that patients will discontinue the prescribed HAART regime. Consequently, an ARV regime, comprising two nucleoside/nucleotide analogue reverse transcriptase inhibitors and a non-nucleoside reverse transcriptase inhibitor, serves as a valuable alternative to protease inhibitors, as it is often associated with lower incidents of diarrhoea (Nannini and Okhuysen, 2002).

In light of the fact that diarrhoea continues to remain a frequent gastrointestinal complication in HIV-positive patients, targeted complementary therapy (in addition to HAART) that limits the incidence of diarrhoea and supports re-colonisation of the gastrointestinal tract in these patients is needed, and the use of probiotic agents provides an attractive solution that is worth exploring further.



## 1.3 PROBIOTIC AGENTS IN HUMANS

### 1.3.1 Probiotics and Prebiotics

Probiotics can be defined as viable, non-pathogenic microorganisms that, when ingested, beneficially influence the host, by exerting a positive effect on the health of the host and improving intestinal barrier function (Harish and Varghese, 2006). Probiotic formulations are usually composed of large numbers of one or more strains of a single species, or a mixture of several species, all of which are common components of the gastrointestinal microbiota (Chapman *et al*., 2011; Timmerman *et al*., 2004). Several studies have reported the successful incorporation of specific probiotic strains into food products such as yoghurt (Dols *et al*., 2011; Anukam *et al*., 2008; Hekmat and Koba, 2006), fermented milk products (Farnworth *et al*., 2007) and even ice cream (Akin *et al*., 2007). In spite of numerous improvements in treatments such as antibiotics and immunosuppressive therapies, gastrointestinal complications, together

with changes in the composition of the gut microbiota, continue to pose a major clinical problem (Rolfe, 2000). Probiotics are routinely used in the treatment of intestinal disorders, where the normal endogenous microbiota of the GI tract has been disrupted, making the host susceptible to disease and colonization by pathogens. These include antibiotic-associated diarrhoea (AAD), irritable bowel syndrome (IBS), Crohn's disease, colon cancer, obesity and small bowel bacterial overgrowth (Gerritsen *et al*., 2011; Gareau *et al*., 2010; Iannitti and Palmieri, 2010). The desired outcome of administering probiotics is to increase the number and activity of the health-promoting microorganisms, until such time that a healthy microbiota composition is re-established (Gerritsen *et al*., 2011). Research has shown that the numerous beneficial health effects attributed to probiotic strains can be classified into three distinct categories. Firstly, all probiotic species are capable of directly interacting with the gastrointestinal tract, for example by interacting with the endogenous intestinal microbiota or through enzymatic activities (Gerritsen *et al*., 2011; Rijkers *et al*., 2010). Secondly, probiotic species can interact directly with the intestinal mucus layer and epithelium, and thirdly they can be responsible for stimulating a range of specific and non-specific host immune responses (Gerritsen *et al*., 2011; Rijkers *et al*., 2010). These may include enhancing phagocytic and natural killer (NK) cell activity (Rastall *et al*., 2005).

Currently, the best-studied probiotics are species that belong to the *Lactobacillus* and *Bifidobacterium* genera, both of which are integral components of the gastrointestinal microbiota (Kleerebezem and Vaughan, 2009; Felis and Dellaglio, 2007; Rolfe, 2000). In addition to these genera, other commercially available probiotics contain species from other genera such as *Streptococcus*, *Enterococcus*, *Bacillus* and *Propionibacterium*. Interestingly, some Gram-negative bacteria for example *E. coli* and yeast (*Saccharomyces*) are also being used as probiotic microorganisms (Gareau *et al*., 2010; Iannitti and Palmieri, 2010).

The use of prebiotics has been proposed as an alternative or simultaneous treatment strategy in improving the composition of gastrointestinal microbiota. Prebiotics can be defined as a non-digestible food component, which beneficially affects the host, by stimulating the growth and/or activity of one or more of the normal intestinal microbiota that confer positive health benefits to the host (Roberfroid *et al*., 2010). Prebiotics are carbohydrate substrates which are not hydrolysed or absorbed in the small intestine, and become available to serve as a substrate for the endogenous microbiota of the large intestine (Laparra and Sanz, 2010). Prebiotics, commonly used in human nutrition, can range from small disaccharides to large

oligosaccharides, examples of which are lactulose, galacto-oligosaccharides (GOS), fructo-oligosaccharides (FOS), xylo-oligosaccharides (XOS), lactosucrose and inulin-derivatives (Laparra and Sanz, 2010; Harish and Varghese, 2006). The main end product of carbohydrate metabolism is the production of short chain fatty acids, which then are made available to the host as an additional energy source (Laparra and Sanz, 2010; Harish and Varghese, 2006; Isolauri *et al*., 2004). Studies have shown that the use of prebiotics, such as galacto-oligosaccharides and inulin, are capable of increasing the *Bifidobacterium* and *Faecalibacterium prausnitzii* concentration within the large intestine (Laparra and Sanz, 2010, Ramirez-Farias *et al*., 2009), and enhancing resistance to colonization of the intestine by *C. difficile* (Katz, 2006). Specific prebiotics can be administered simultaneously with probiotic species, to form a "synbiotic" (Gerritsen *et al*., 2011). A synbiotic functions through a distinct synergistic action where the probiotic will enhance the composition of the endogenous intestinal microbiota by colonising the epithelial lining, whilst the prebiotic serves as a substrate that the probiotic can utilise once established, thus beneficially affecting the host (Rolfe, 2000).



### 1.3.2 Probiotics in HIV therapy

HIV infection has a negative, disruptive effect on the gastrointestinal mucosal barrier and the mucosal immune system, resulting in the deterioration of gut homeostasis, an altered digestive function and increased permeability. Subsequent bacterial translocation may further stimulate the immune system, promoting HIV replication (Gori *et al*., 2011; Sestak, 2005; Veazey and Lackner, 2004). Probiotic supplementation, when administered in an appropriate dose in HIV-positive patients, may assist in restoring GALT homeostasis. Proposed mechanisms through which this could occur include: the stimulation of natural killer (NK) cell activity (Gill *et al*., 2001), re-establishment of essential functions performed by the mucosal barrier (Hummelen *et al*., 2010) and a substantial reduction in systemic inflammation (Kekkonen *et al*., 2008). A limited number of studies have evaluated and monitored the viability of probiotic supplementation in HIV-positive patients and some of these findings will be discussed briefly.

In a randomised controlled clinical (RCT) study based in Tanzania, 145 HIV-positive women, all receiving HAART, were supplied for 29 days with either plain yoghurt or specially formulated yoghurt supplemented with *L. rhamnosus* GR-1 (Dols *et al*., 2011). Although gut health was not the primary focus of this investigation, researchers did record an improvement in the vaginal microbiota of the women, a better appetite and an increase in energy. Dols *et al*.,

(2011) concluded that the results of this RCT study showed that yoghurt was a nutritious food component and a suitable probiotic delivery method that could be safely administered daily to HIV-positive patients on HAART. In a second clinical trial, conducted by Anukam *et al*., (2008), a similar finding was recorded, when yoghurt containing *L. rhamnosus* GR-1 and *L. reuteri* RC-14 was administered to 24 HIV-positive patients and resulted in a significant decrease in diarrhoea as well as diarrhoea-related conditions, along with a small increase in the $CD_4^+$ T cell count. More recently, Hummelen *et al*., (2011) assessed whether long-term probiotic supplementation in HIV-positive patients would lead to a maintained and consistent immune function. Immune compromised HIV-positive patients (> 200 $CD_4^+$ T cells/µl) were given oral capsules comprising 2 x $10^9$ CFU/capsule of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 for 25 weeks. For the duration of the longitudinal study, $CD_4^+$ T cell counts were recorded prior to probiotic supplementation and at 10 and 25 weeks after starting probiotics. After 10 weeks of probiotic use, $CD_4^+$ T cell counts increased on average 50 cells/µl while, after 25 weeks of probiotic administration, the $CD_4^+$ T cell count showed an increase of 46 cells/µl. Hummelen *et al*., (2011) concluded that *L. rhamnosus* GR-1 and *L. reuteri* RC-14 could both be safely administered at a concentration of 2 x $10^9$ CFU/day, but that these probiotic species did not appear to have a significant impact on the immune function.

Other treatment strategies have involved supplementing the diet with components such as soluble fibers, calcium supplements and L-Glutamine, but this has resulted in only small improvements in gastrointestinal symptoms associated with HIV-infection (Huffman and Walgren, 2003). In a study carried out by Gori *et al*., (2011), 57 HIV-positive, HAART naïve patients supplemented their diet for 12 weeks with a unique oligosaccharide mixture, made up of galacto-oligosaccharides, fructo-oligosaccharides and pectin hydrosylate-derived oligosaccharides. The results of this study showed changes in the microbial composition upon prebiotic supplementation specifically, an increase in *Bifidobacterium* species and a decrease in the *Clostridium coccoides*/*Eubacterium rectale* and *Clostridium lituseburense*/*Clostridium histolyticum* groups. Another significant finding of this study was the increased natural killer cell activity seen in the treatment group when compared to the control group. Therefore, administration of a prebiotic mixture to HIV-positive patients resulted in an improvement in the composition of the intestinal microbiota and possible re-establishment of homeostasis within the gastrointestinal tract (Gori *et al*., 2011).

Finally, a concern associated with probiotics in HIV therapy, is the risk of bacteraemia, bacterial translocation and sepsis. Thus far, only 5 cases of lactobacillemia have been reported in severely immune compromised HIV-positive patients. Moreover, all of these patients had extremely low $CD_4^+$ T cell counts (< 55 cells/µl) and were suffering from a secondary infection (Rogasi *et al*., 1998; Abgrall *et al*., 1997; Horwitch *et al*., 1995). Nevertheless, no specific safety concerns have been raised preventing probiotic use in HIV-positive patients, but close monitoring and establishing what the most advantageous point during the HIV infection might be for probiotic intervention to provide the best results, is strongly recommended.

## 1.4 PROFILING OF COMPLEX MICROBIAL POPULATIONS

In light of the importance of the gut microbiota in human health, it is important to be able to monitor its composition and abundance in assessing the roles of its various members in disease. A variety of analytical techniques have been employed in the study of the gastrointestinal microbiota of humans. The availability and use of these techniques has provided researchers with considerable insight into the composition, function and dynamics of the endogenous intestinal microbiota. Several methods currently used in the profiling and analysis of large, complex microbial communities, will be reviewed in detail below.

### 1.4.1 Culture-dependent analysis of the intestinal microbiota

The composition of the intestinal microbiota of humans has been extensively studied and examined using mostly traditional culture-based methods. These methods have many advantages including a reduced cost, enumeration and isolation of various bacterial species and further biochemical and phenotypical investigations. Unfortunately, these techniques are also limited in that they are time consuming, labour intensive, require complex selective and differential media as well as specific anaerobic growth conditions, and have a poor discriminatory index (Mohania *et al*., 2008; Turroni *et al*., 2008; Tannock, 2002; Heilig *et al*., 2002; Tannock, 2001). Previously published reports have estimated that approximately 60 to 80% of the endogenous intestinal bacterial population could not be successfully cultured within the laboratory, highlighting the importance of alternative detection methods (Gerritsen *et al*., 2011; Eckburg *et al*., 2005; Nielsen *et al*., 2003; Tannock, 2002). However, results generated in a recent study by Walker *et al*., (2011), indicated that this particular conclusion may no longer be valid. Walker *et al*., (2011) showed that the percentage of phylotypes having > 98% sequence identity to a cultured bacterial species, increased proportionally with phylotype abundance. The four most

abundant phylotypes detected were *E. rectale*, *F. prausnitzii*, *Clostridium aerofaciens* and *Bacteroides vulgates*. All four of these species corresponded with the most abundant species detected by anaerobic culture, in an investigation performed by Moore and Moore, (1995). These findings tend to suggest that the inability to culture gastrointestinal bacterial species may be due to insufficient anaerobic isolation work rather than inherent non-culturability (Walker *et al*., 2011).

### 1.4.2 Culture-independent analysis of the intestinal microbiota

In order to overcome the limitations associated with culture work, novel culture-independent or molecular-based methods have been developed and applied in investigations targeting the diversity of the intestinal microbiota. A variety of different molecular-based techniques have been used to study the intestinal microbial community and have led to both a more detailed insight into the intestinal microbiota and the identification of a large number of new bacterial species. Culture-independent techniques that have been developed and utilised in analysing complex microbial communities such as the intestinal microbiota, include: PCR together with denaturing gradient gel electrophoresis (PCR-DGGE), Fluorescent *in situ* hybridization (FISH), %GC profiling, quantitative real time PCR (qPCR), the human intestinal tract chip (The HITChip) and metagenomics (Turroni *et al*., 2008).

#### *1.4.2.1 16S rRNA Sequencing*

The 16S ribosomal RNA (rRNA) gene has been the most frequently used genetic marker in studying bacterial phylogeny and diversity (Janda and Abbott, 2007; Pernthaler and Amann, 2005; Amann *et al*., 1990). The reasons for this include its universal presence in almost all bacteria, the fact that the function of the 16S rRNA gene has not changed significantly over time, and that the 16S rRNA gene readily allows group and species specific primers to be designed (Patel, 2001). Since the development and implementation of automated DNA sequencing and subsequent work on 16S rRNA sequencing of bacteria, a vast amount of sequence information has been accumulated (Woo *et al*., 2008). Comparisons of these sequences have indicated that the 16S rRNA gene is conserved within organisms belonging to the same genus and species, but differ between organisms of other genera and species. The use of 16S rRNA sequencing has resulted in numerous bacterial genera and species being re-classified or re-named, novel bacterial species have been discovered and classified, and intricate phylogenetic relationships determined (Woo *et al*., 2008).

*1.4.2.2 PCR amplification and Denaturing gradient gel electrophoresis*

PCR-DGGE is an example of a DNA fingerprinting tool that essentially generates a DNA profile of the microbial community present in each sample and allows comparison between different samples, based on any differences evident between the two fingerprints (Sekirov *et al*., 2010). The fundamental principle of PCR-DGGE involves the electrophoresis of PCR amplified 16S rRNA gene fragments through a polyacrylamide gel composed of a linear, chemical (urea and formamide) denaturing gradient (Mohania *et al*., 2008; Tannock, 2002; Muyzer *et al*., 1993). Bacterial DNA is extracted from the faecal sample before undergoing PCR amplification with species-specific primers, one of which contains a 5' GC-clamp (Nielsen *et al*., 2003). The double-stranded DNA fragments migrate through the polyacrylamide gel according to their G+C content (the most stable DNA will migrate further) until the sample is partially denatured, resulting in the formation of a distinctive banding pattern (Sekirov *et al*., 2010). Complete denaturation of the PCR fragment does not occur on account of the GC clamp and overall migration of the product is also significantly slowed (Tannock, 2002; Muyzer *et al*., 1993). The banding pattern generated is representative of the microbial diversity present within the samples screened (Sekirov *et al*., 2010). PCR-DGGE makes it possible for samples to be evaluated over time (temporal variation) or different treatments (antibiotics/probiotics) (Ben Amor *et al*., 2007). PCR-DGGE is, in general, efficient in terms of time, labour and reproducibility and can be considered a semi-quantitative method, due to the fact that the differences in the relative intensities of the bands produced, reflect the relative number of amplicons present (Heilig *et al*., 2002; Muyzer *et al*., 1993). Additional benefits associated with PCR-DGGE, include the excision of specific DGGE bands that can be re-amplified using PCR and sequenced directly, eliminating the need for a cloning step (Muyzer *et al*., 1993). Unfortunately, PCR-based analytical techniques are limited as different types of bias, such as inefficient cell lysis during DNA extraction, and preferential amplification during PCR are introduced, which might lead to an inaccurate representation of the microbial diversity present within the tested sample (Wang *et al*., 2005).

*1.4.2.3 Fluorescent in situ hybridisation*

Fluorescent *in situ* hybridisation (FISH) is a relatively new technique that has been widely used for the quantitative analysis of intestinal microbiota. FISH makes use of fluorescently labelled oligonucleotide probes that have been designed to a specific region within the 16S rRNA gene of the targeted bacterial group. Probes can be designed in such a way that relatively large groups of bacteria can be detected. Alternatively, more specific predominant bacterial groups can be

targeted (Sekirov *et al*., 2010). Cells are first permeabilised either with paraformaldehyde or ethanol, allowing the probes to reach their target and causing the cell to fluoresce (Blaut *et al*., 2002). Individual cells are then counted, using fluorescence microscopy or flow cytometry (Amann *et al*., 1990). A major advantage of FISH is being able to detect specific targeted bacterial species within a complex sample mixture and not requiring the use of culture work (Blaut *et al*., 2002). FISH has an added advantage of being able to utilise multiple fluorescent probes within the same sample, to differentiate between different bacterial targets (Bartlett, 2003). However, FISH is associated with certain limitations, such as low sensitivity (Rinttilä *et al*., 2004; Schwiertz *et al*., 2000), low probe permeability, especially through the gram-positive cell wall (Cleusix *et al*., 2010; Rinttilä *et al*., 2004; Franks *et al*., 1998), possible nonspecific detection (Schwiertz *et al*., 2000; Franks *et al*., 1998) and is time-consuming when used in conjunction with fluorescence microscopy (Cleusix *et al*., 2010). The latter limitation has been improved since combining FISH with flow cytometry (Rigottier-Gois *et al*., 2003).

*1.4.2.4 Percent Guanine Cytosine (% GC) profiling*

Percent GC profiling is a tool which, when used in conjunction with other molecular profiling techniques is able to improve the microbial diversity detected by enriching for bacterial groups of a known GC composition. The foundation of G+C profiling is based on the fact that all bacterial species present within an unknown sample, contain a unique percent G+C content in their chromosomal DNA, which will affect its density. In other words, bacterial species having a high G+C content will migrate differently during density gradient centrifugation, compared to species with low percent G+C content. To enrich the microbial diversity obtained, total bacterial genomic DNA is extracted from the microbial rich community and, thereafter, samples are fractionated using density gradient centrifugation (Kassinen *et al*., 2007). Following ultracentrifugation, all of the DNA-containing fractions are quantified, using UV absorbance (280 nm) (Dicksved *et al*., 2008; Apajalahti *et al*., 1998). A unique advantage of percent G+C profiling is its ability to examine a large DNA pool acquired from a complex unknown microbial community and is not influenced by external biases, such as primer inefficiency or PCR inhibitors (Dicksved *et al*., 2008). It also allows bacterial species that are not present in high numbers to be amplified during later downstream processing (Kassinen *et al*., 2007; Holben and Harris, 1995). It should be noted however, that G+C profiling is not extensively used, due to its limited application (Mühling *et al*., 2008) and because researchers tend to favour a more sequencing-based approach when investigating complex microbial communities.

*1.4.2.5 Quantitative real-time PCR*

Quantitative real-time PCR (qPCR) is another molecular-based technique that monitors the amplification of a DNA target through changes in the fluorescence signal and can be used to target specific bacterial groups. qPCR is generally used either in conjunction with FISH, to confirm the results recorded, or as an alternative detection method to FISH (Cleusix *et al*., 2010; Sekirov *et al*., 2010). During qPCR, a fluorescence signal reflecting the amount of PCR product present is generated during the initial cycles of amplification. The fluorescent signal is weak and cannot be differentiated from the background but, as amplification continues, the amount of product generated begins to accumulate, and the fluorescent signal being produced increases exponentially before levelling off (Mohania *et al*., 2008; Kubista *et al*., 2006; Malinen *et al*., 2003). Currently, there are numerous options available to measure fluorescence during qPCR and include hydrolysis probes, molecular beacons and double-stranded DNA intercalating dyes (D'haene *et al*., 2010).

Real time qPCR has several advantages over conventional PCR, in that it focuses on product accumulation within the exponential phase of the amplification cycle rather than end point analysis, thereby achieving more accurate and non-biased results. The elimination of post-PCR manipulation ensures a reduction in crossover contamination and fast turnaround time of results (D'haene *et al*., 2010; Mohania *et al*., 2008; Kubista *et al*., 2006; Malinen *et al*., 2003). Finally, real time qPCR is also associated with a high sensitivity due to the significant reduction in the production of false positives (99.9%) that can be observed in conventional PCR protocols (Mohania *et al*., 2008; Song *et al*., 2004).

*1.4.2.6 The HITChip*

The human intestinal tract chip (The HITChip) is a powerful phylogenetic microarray, enabling high throughput analysis of intestinal microbial communities and capable of targeting over 1000 different bacterial species found within the human gastrointestinal tract (Rajilić-Stojanović *et al*., 2007). The fundamental principle of phylogenetic arrays relies on the hybridisation of isolated microbial DNA to a large set of specific oligonucleotides, immobilised onto a secure platform (Mohania *et al*., 2008). The HITChip is a highly reproducible phylogenetic fingerprinting tool that can be used for determining the diversity and relative quantification of microbial groups (Rajilić-Stojanović *et al*., 2009). Moreover, the HITChip is extremely versatile and can be utilised in a variety of applications, for example, monitoring the temporal stability of the endogenous intestinal microbiota or, simply determining the intestinal microbiota

composition (Rajilić-Stojanović *et al*., 2009). The utilisation of a gastrointestinal specific microarray, such as the HITChip, is associated with many benefits, in that it is cost-effective, time efficient and provides an alternative to 16S rRNA gene sequencing methods. Nonetheless, there are still concerns regarding detection limits and possible hybridisation bias, especially in situations where certain bacterial species are capable of hybridising more efficiently than other species (Sekirov *et al*., 2010). The reliability and accuracy of the results generated when using the HITChip was recently tested, in an experiment where the stability of the intestinal microbiota was analysed in a longitudinal study of five healthy adults, using both PCR-DGGE and the HITChip microarray. Data generated, using the HITChip, resulted in a consistent clustering of all five donors throughout the study, whereas clustering of only three of the five donors was seen when using PCR-DGGE (Rajilić-Stojanović *et al*., 2009).

*1.4.2.7 Metagenomics and High Throughput Sequencing*

Metagenomics is one of the most recent methods to be used in the profiling of microbial communities and serves as a powerful alternative to rRNA based sequencing and analysis of complex communities (Qin *et al*., 2010). The current definition of metagenomics is the study of a collection of genomes obtained from a complex ecosystem which is capable of providing information regarding the phylogenetic and physical properties, as well as potential functions of the present microbial communities (Gosalbes *et al*., 2011; Handelsman, 2004). The principle of metagenomics involves extracting and sequencing the total genomic DNA from the microbial community (Zoetendal *et al*., 2008). A metagenomic library can be constructed by cloning the DNA fragments into a suitable host such as *E. coli*, which can then be used for genetic diversity analysis of the microbial ecosystem. This is known as sequence-driven analysis. Alternatively, the library may be screened for any novel enzymes or functions (function-driven analysis) (Zoetendal *et al*., 2008). The use of metagenomics for either sequence or function-driven analysis has recently led to the characterisation of novel genes from uncultured bacterial species and comparison of bacterial communities present within different environments. The development of other function focused analyses, such as metaproteomics (protein targets), metabolomics (metabolite targets) and metatranscriptomics (RNA targets) are being implemented more often, in attempting to assess the dynamics of key microbial functions within the gastrointestinal tract, as well as determining how these functions affect both the gut microbiota and the host (Sekirov *et al*., 2010).

Recent advances in high-throughput DNA sequencing have provided researchers with an innovative tool for quickly and affordably obtaining DNA sequences (Jones, 2009). At the end of 2008, three different High-throughput sequencing platforms were available commercially: the Applied Biosystems SOLiD, Illumina Genome Analyser II/IIx, and the Roche/454 GS FLX titanium sequencer (Kircher and Kelso, 2010; Jones, 2009). The Applied Biosystems SOLiD and Illumina Genome Analyser systems both use short reads (25-75 bp read length); while the Roche/454 GS FLX platform produces average reads of 400 bp (Kircher and Kelso, 2010). An advantage of this technique is that it does not require cloning the DNA before sequencing, removing one of the main biases in environmental sampling (Kircher and Kelso, 2010). Recently, Qin *et al*., (2010) successfully compiled and described a microbial "gene catalogue" of the human intestinal tract, using the Illumina Genome analyzer. The metagenomic approach results in the production of large amounts of sequence data (around 3 gigabases (Gb)) which require a significant amount of data management and computational analysis (Qin *et al*., 2010; Zoetendal *et al*., 2008). Such facilities may not currently be available to all research institutes.

Nevertheless, the use of metagenomics, in conjunction with any of the other methods reviewed in this thesis, has the potential of providing a detailed analysis of the intestinal microbiome. Profiling methods have continued to develop over the last few years, and all of the different techniques, discussed in this chapter, have their own unique benefits and drawbacks. The particular method used in research will depend upon the questions being addressed. It is important therefore, that these methods be utilised in combinations, as well as expanding and increasing the number of cultured species from the gastrointestinal tract, enabling more phenotypical and biochemical investigations to be conducted under controlled experimental settings.

## 1.5 AIMS AND RESEARCH OBJECTIVES OF THIS STUDY

The primary aim of this project was to determine and identify changes in the composition, diversity and abundance of specific members of the gastrointestinal microbiota within HIV-positive patients. In order to address the above-mentioned aim, and to broaden the understanding of how HIV and HAART influence the microbiota, the following research objectives were formulated:

***Objective 1:***

To establish if the microbial community of HIV-positive donors differed from HIV-negative donors in terms of diversity and composition. In order to do this, a baseline comparison (prior to HAART initiation) would be undertaken to analyse the microbial condition of the intestinal tract in HIV-positive and HIV-negative donors, with respect to total bacteria, as well as the *Bifidobacterium* and *Lactobacillus* groups using PCR-DGGE.

***Objective 2:***

To determine the effect of HIV and HAART on *Bifidobacterium* and *Lactobacillus* species in HIV-positive donors during a 6 month longitudinal analysis. The *Bifidobacterium* and *Lactobacillus* communities would be selected because they are well characterised probiotic groups that provide a beneficial and protective function to the host. If these groups were reduced in HIV-positive patients receiving HAART, then probiotic supplementation with these species might be of value.

***Objective 3:***

To establish if HIV replication within the gastrointestinal tract and HAART have an effect on the abundance of the endogenous microbiota in HIV-positive patients. Real time qPCR would be employed to determine the abundance of the total bacterial population, as well as four dominant (*Bacteroides/Prevotella*, *Clostridium coccoides*, *Clostridium leptum*, and *Bifidobacterium*) and three sub-dominant (*E. coli*, *Lactobacillus* and *Enterococcus*) gastrointestinal bacterial groups, in HIV-positive donors prior to and 6 months after the initiation of HAART.

***Objective 4:***

To determine the presence of three intestinal pathogens, namely *Campylobacter jejuni*, *Salmonella enterica* and *Clostridium difficile* in the faeces of HIV-positive patients not suffering

from diarrhoea. A significant alteration to the structure of the endogenous intestinal microbiota may result in the loss of the protective barrier function provided by these species, which could then lead to an increase in pathogen colonisation.

# CHAPTER TWO

**Stool sample collection and recruitment of HIV-positive and HIV-negative South African donors residing in Cape Town**

**Contents**

## 2.1 SUMMARY

HIV/AIDS is a global epidemic severely affecting the health systems of many countries, especially South Africa. HIV-positive patients have a high risk of developing diarrhoea, due either to the HIV-infection or the use of antibiotics disrupting the gastrointestinal microbiota. This study aimed to establish whether there were changes in the diversity or abundance of specific gastrointestinal bacterial groups present in the faeces of HIV-positive patients during highly active antiretroviral treatment (HAART). Twelve HIV-positive donors attending Cape Town's Groote Schuur AIDS clinic, as well as 12 age-, race- and gender-matched HIV-negative donors (females $n$ = 8 and males $n$ = 4) were recruited for this investigation. The majority of faecal samples received were from Black, female donors, aged 20-45, and represents a demographic group that has been shown to have the highest risk of HIV infection within South Africa.

## 2.2 INTRODUCTION

South Africa has been most heavily affected by the HIV epidemic, having the highest number of HIV-positive people of any other country, and an estimated HIV prevalence rate of approximately 10.6% (Statistics South Africa, 2011; Long *et al*., 2010). Despite global trends indicating an overall decrease in the number of new HIV infections, the number of people living with HIV in South Africa continues to increase steadily. Recent reports have shown that the total number of South Africans living with HIV, has increased from 4.21 million in 2001 to 5.38 million people in 2011, with 316 900 new HIV infections being recorded in 2011 alone (Statistics South Africa, 2011; UNAIDS, 2010).

Interestingly, the annual number of AIDS-related deaths has begun to show a substantial decrease from 2006 to 2011, with approximately 43.6% of all recorded deaths in South Africa for 2011 being as a result of AIDS-related illnesses (Statistics South Africa, 2011). This decrease is thought to be accredited to the fact that antiretroviral treatment is now being initiated earlier in HIV-positive patients, thus successfully reducing the viral load and improving the $CD_4^+$ T cell count. The national HIV/AIDS and sexually transmitted infections (STI) strategic plan for South Africa (2007-2011) was implemented by the Department of Health (DOH) to increase the number of people receiving ARV medication at public sector hospitals across South Africa. The national first-line ARV regime currently administered to new HIV-positive patients includes a combination of two nucleotide/nucleoside analogues, such as Didanosine (ddi), Lamivudine (3TC), Stavudine (d4T) or Tenofovir (TDF), together with a non-nucleoside reverse transcriptase inhibitor such as Efavirenz (EFV) or Nevirapine (NVP). At the beginning of 2005, only 101 416 HIV-positive patients were on HAART but, by the end of 2010, a total of 1 058 399 HIV-positive patients were receiving HAART (Statistics South Africa, 2011; Rehle *et al*., 2010). However, despite the success and progress of this strategic plan, many South African HIV-positive patients still require HAART at public sector hospitals.

The use of faecal material to characterise the gastrointestinal microbiota is a useful tool to generate knowledge regarding the effect of antibiotics and disease on the gut microbiome. Most of the current knowledge regarding the diversity of the human intestinal microbiota is as a result of numerous investigations and analysis of faecal material, whereas fewer studies identifying the microbiota associated with the mucosal wall have been carried out (Turroni *et al*., 2009; Ahmed *et al*., 2007; Nielsen *et al*, 2003). Faecal samples are more readily obtained

from a donor, but the exact representation of the intestinal microbiota is a contested issue, as the microorganisms present within the sample will be mainly composed of those present within the lumen of the colon (Isolauri *et al*., 2004; Zoetendal *et al*., 2002). The use of biopsy samples has begun to generate more interest, due to the fact that specimens can be extracted under more controlled conditions and it allows sampling at different locations along the gastrointestinal tract (Chandra *et al*., 2010; Ott *et al*., 2004). However, despite these advantages, retrieval of the biopsy sample requires the donor to undergo an invasive procedure, where a small amount of sample is obtained and exposure to oxygen during the sampling procedure is high, potentially leading to a remarkable reduction in the number of strict anaerobes present (Zoetendal *et al*., 2002).

On this basis, faecal material was selected as the sampling material in this study, due to the fact that numerous studies have conducted similar investigations using faecal material and providing a stool sample would be much easier and safer for the HIV-positive donors. In order for biopsy samples to be obtained, the donors would have to have undergone an invasive endoscopic examination, giving rise to serious ethical concerns, such as the safety both of the patient during the procedure and those working with the HIV-infected biopsy sample.

Monitoring the diversity and abundance of bacterial species, within the gastrointestinal tracts of HIV-positive patients could identify any significant changes in the microbiota that may result from HIV replication within the intestinal tract. Establishing if the endogenous microbiota is affected and whether, after commencing with HAART, it returns to an HIV-negative state, may identify an appropriate probiotic strain which could be administered as a supportive care to patients receiving HAART. This research study comprised two important stages, the first of which was to successfully recruit and acquire faecal samples from HIV-positive patients prior to and during HAART. Following successful patient recruitment, the second stage involved determining the diversity and abundance of several different gastrointestinal bacterial groups using specific molecular techniques.

## 2.3 MATERIALS AND METHODS

### 2.3.1 Study design, selection criteria of donors and ethical considerations

This research study was conducted as a collaboration between the Department of Molecular and Cell Biology (MCB) and the Groote Schuur AIDS Clinic. HIV-positive patients attending this clinic were assessed and recruited by physicians working within the clinic, based on specific inclusion and exclusion criteria (Table 2.1). Participation in this study was entirely voluntary and patients were free either to refuse participation in this study or withdraw from it at any point. All relevant demographic and clinical information for each donor was recorded and provided by the clinic.

**Table 2.1:** Selection criteria used in the recruitment of HIV-positive and HIV-negative donors

| Inclusion Criteria for HIV-positive donors | Inclusion Criteria for HIV-negative donors | Exclusion Criteria for all participants |
|---|---|---|
| Must be HIV-positive, with no prior history of HAART. | Must be HIV-negative | |
| Donor may be male or female and between the ages of 18 and 55. | Donor may be male or female and between the ages of 18 and 55. | |
| Must be TB negative | Must be TB negative | TB positive donors will be excluded.<br>TB negative participants who develop TB during the study will also be excluded. |
| Must have no intestinal disorders or diarrhoea. | Must have no intestinal disorders or diarrhoea. | Donors who develop diarrhoea during the study will be excluded. |
| Must not be receiving any form of chemotherapy, radiation and/or probiotics. | Must not be receiving any form of chemotherapy, radiation and/or probiotics. | Exclude if any of these criteria change during the study. |
| Must not be receiving any antibiotics except Bactrim. | Must not be receiving any antibiotics at all. | Any additional antibiotics administered will be recorded. Donor remains in individual longitudinal study, but is excluded from general cohort analysis. |

Each individual has a unique microbiota in terms of diversity and composition that has been developed and influenced over time by a number of different intrinsic and extrinsic factors such as: antibiotic treatments, diet, and age. As a result of this, healthy, HIV-negative donors were recruited as an age, race and gender-matched control group for the HIV-positive donors. All of the HIV-negative donors were recruited through an established community care centre,

situated within Cape Town where the donors were aware of their HIV status. Donors were recruited through a general advertisement, in conjunction with active recruitment by the centre's staff. Exclusion criteria for the HIV-negative donors included a known history of gastrointestinal disorders, or the administration of antibiotics, probiotics or immune-suppressing medication within the last four months. In terms of the HIV-positive donors, the same exclusion criteria were used except that antibiotic usage could not be completely excluded, due to the fact that all patients attending the AIDS clinic were receiving Bactrim (Trimethoprim-sulfamethoxazole) as supportive therapy (Table 2.4).

HIV-positive patients were clinically assessed by physicians working at the AIDS clinic, and donors who met the above selection criteria, and were willing to participate in this study, were recruited. Once an adequate sample group had been established, a matched control group was recruited for each HIV-positive donor. All donors participating in this study were required to complete and sign an informed consent form in their home language (Appendix) outlining the objectives of this study. Ethical approval to perform this research study was obtained from the University of Cape Town's Human Ethics Research Committee, reference number 077/2008.



### 2.3.2 Collection and processing of faecal samples

During the period 2009 to 2011, a total of 12 HIV-positive patients attending the Groote Schuur AIDS Clinic and 12 HIV-negative donors were recruited. Each donor was provided with a sterile 2L tin, with a secure sealing lid, in which to collect their stool. Faecal samples were collected from each HIV-positive donor prior to them initiating their HAART (T0) and then at 2 (T1), 6 (T2), 12 (T3) and 24 (T4) weeks following the commencement of HAART. HIV-positive donors provided a stool sample on the day of their respective clinic visits and these samples were immediately collected and transported back to the laboratory for processing. The control HIV-negative group was similarly sampled. A portion of each sample was aliquoted into a sterile pathology tube and immediately frozen at -20°C, until required for DNA extraction. Processing of the faecal samples was carried out within a Class II biohazard safety cabinet (ESCO ® Airstream S-Series).

## 2.4 RESULTS AND DISCUSSION

### 2.4.1 Donor demographics and clinical characteristics

All donors participating in this study were assigned a specific donor ID, namely, P1-P12 (HIV-positive) or H1-H12 (HIV-negative), thus ensuring that all information received remained strictly confidential (Table 2.2). Of the 12 HIV-positive donors initially recruited, only eight completed the 6 month longitudinal study (discussed in Table 2.4 and section 2.4.2), with four of the longitudinal donors providing an additional 12 month faecal sample. A single stool sample was provided by all the HIV-negative donors, with the exception of donor H3, who also completed the 6-month longitudinal study, so that a comparison between the HIV-positive and HIV-negative donors could be established. Full patient data, treatment regimens and abbreviations of drug names are shown in Table 2.2.

The administration of HAART is mostly patient specific and influenced by criteria such as $CD_4^+$ T cell count, viral load, as well as the patient's individual history. All antiretroviral regimes were administered according to South Africa's National public sector HAART program. Thirty three percent (4/12) of the recruited HIV-positive donors received standard first-line ARV's (d4T, 3TC and EFV) while another 25% (3/12) received d4T, 3TC and NVP. Donors receiving the latter ARV treatment were all Black females, aged 21-38.

#### *2.4.1.1 Gender and population group*

The majority of faecal samples received during this study came from female donors (67%), while only 23% of the samples originated from male donors (Figure 2.1A). Most of the recruited HIV-positive donors were from the Black African population, accounting for 75% of the donors, while the remaining 17% and 8% were represented by the White and Coloured populations respectively (Figure 2.1B).

According to Statistics South Africa, the estimated population size of South Africa in 2011 was 50.59 million people, of which 52% (approximately 26.07 million) was female (Statistics South Africa, 2011; UNAIDS, 2010). Population statistics acquired during 2010 showed that, 53% of the South African HIV-positive population was female, of which 65% were receiving HAART, whilst males and children accounted for the remaining 38% and 9% respectively (Department of Health, 2010). Statistics South Africa further reported that the Black African population constitutes just more than 79.4% of the South African population, while the Asian,

**Table 2.2:** Demographic data of the HIV-positive and HIV-negative donors used in this study

| HIV-positive Donors (*n* = 12) | | | | ARV Regime | | | | | | | | Sampling Period* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Donor ID** | **Gender** | **Race** | **Age (Yr)** | **d4T** | **3TC** | **EFV** | **AZT** | **NVP** | **ddi** | **INNs** | **TDF** | |
| P1 | Female | Black | 26 | + | + | + | | | | | | S |
| P2 | Female | Black | 38 | | + | | + | + | | | | L |
| P3 | Female | Black | 37 | | | | + | | + | + | | L |
| P4 | Female | Black | 33 | + | + | | | + | | | | L |
| P5 | Male | Coloured | 43 | | + | + | | | | | + | L |
| P6 | Female | Black | 49 | + | + | + | | | | | | S |
| P7 | Female | Black | 21 | + | + | | | + | | | | L |
| P8 | Male | White | 50 | | + | + | | | | | + | L |
| P9 | Female | Black | 37 | + | + | | | + | | | | S |
| P10 | Male | Black | 30 | + | + | + | | | | | | L |
| P11 | Male | White | 43 | + | + | + | | | | | | L |
| P12 | Female | Black | 23 | | + | | | + | | | + | S |
| **HIV-negative Donors (*n* = 12)** | | | | | | | | | | | | **Sampling Period*** |
| **Donor ID** | **Gender** | **Race** | **Age (Yr)** | | | | | | | | | |
| H1 | Male | White | 52 | | | | | | | | | S |
| H2 | Female | Black | 22 | | | | | | | | | S |
| H3 | Female | Black | 39 | | | | | | | | | L |
| H4 | Female | Black | 24 | | | | | | | | | S |
| H5 | Female | Black | 30 | | | | | | | | | S |
| H6 | Female | Black | 26 | | | | | | | | | S |
| H7 | Female | Black | 50 | | | | | | | | | S |
| H8 | Male | Black | 33 | | | | | | | | | S |
| H9 | Female | Black | 42 | | | | | | | | | S |
| H10 | Female | Black | 44 | | | | | | | | | S |
| H11 | Male | Coloured | 43 | | | | | | | | | S |
| H12 | Male | White | 33 | | | | | | | | | S |

* [L] Longitudinal samples; [S] Single T0 sample

* [+] Prescribed ARV administered by physicians at the Groote Schuur AIDS Clinic

* [d4T] Stavudine; [3TC], Lamivudine; [EFV], Efavirenz; [AZT], Zidovudine; [ddi], Didanosine; [INNs], Aluvia; [NVP], Nevirapine; [TDF], Tenofovir

White and Coloured population groups are estimated to represent only 2.6%, 9.1% and 8.9% of the total population respectively (Statistics South Africa, 2011).

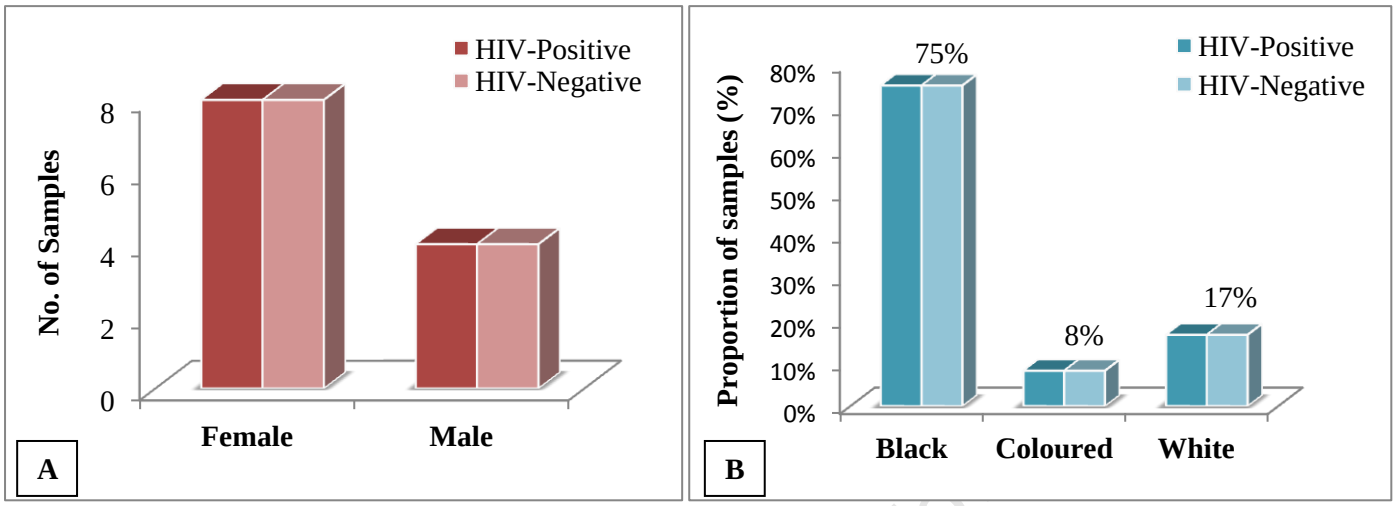



**Figure 2.1:** (A) **Gender distribution** of the HIV-positive and HIV-negative donors recruited for this study. (B) **Population group distribution** of the HIV-positive and HIV-negative donors recruited for this study

*2.4.1.2 Age*

Population statistical studies have shown that 16.6% of the South African population aged 15 to 49 years, are HIV-positive (Statistics South Africa, 2011; Rehle *et al*., 2010). In this study, this was reflected by the fact that the majority of the faecal samples were obtained from donors in the 30-45 year age group (Figure 2.2 and Table 2.3), with the second largest age group providing samples being the 20-29 year group. Interestingly, the latter age group consisted solely of black, female donors.

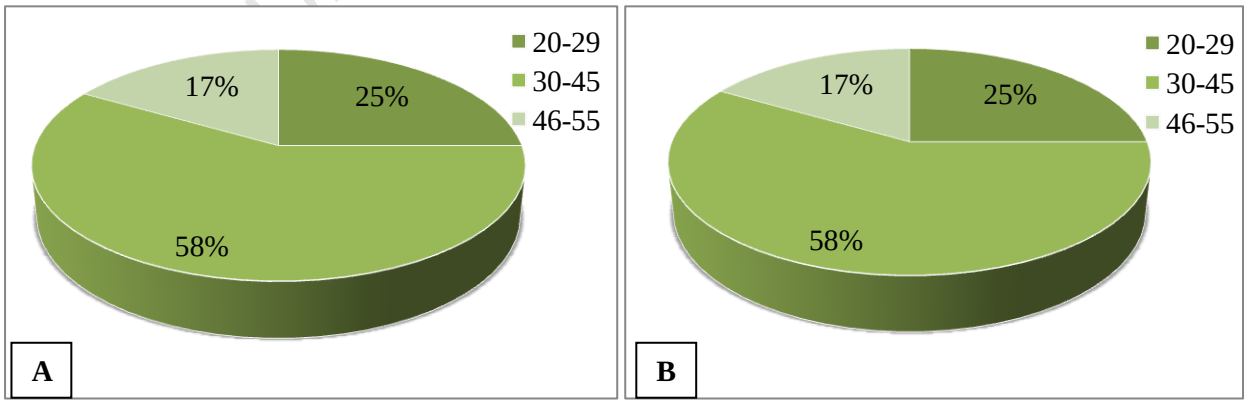



**Figure 2.2:** **Age distribution** of the HIV-positive (A) and HIV-negative (B) donors recruited for this study

The mean age of the HIV-positive study group was 36 years, while for the HIV-negative group, it was 37 years. When calculating the mean ages based on gender for the HIV-positive group (Table 2.3), it was evident that the female donors were nearly 10 years younger (± 33 years) than the male donors (± 42 years) participating in this study.

**Table 2.3:** Age and gender comparisons for both donor groups used in this study

| | **HIV-positive donors** | | **HIV-negative donors** | |
|---|---|---|---|---|
| **Age Group** | **Female** | **Male** | **Female** | **Male** |
| 20 – 29 | 3 | 0 | 3 | 0 |
| 30 – 45 | 4 | 3 | 4 | 3 |
| 46 – 55 | 1 | 1 | 1 | 1 |
| *Mean age* | *(33)* | *(42)* | *(35)* | *(40)* |

*2.4.1.3 Patient history*

All relevant clinical data and patient history for each of the 12 HIV-positive donors is summarised in Table 2.4. The South African Department of Health recommends that the administration of ARV medication be initiated in patients based on the clinical stage of the HIV-infection (WHO stage 1-4) or in those who have a $CD_4^+$ T cell count of < 350 cells/µl. The $CD_4^+$ T cell counts of the participating donors were taken at four specific time points, namely prior to the initiation of their HAART (T0) and at 12, 24 and 52 weeks after commencing with their HAART. In most instances, donors (7/12) initiated ARV treatment at $CD_4^+$ T cell counts lower than 200 cells/µl (median 138 cells/µl), while the remaining 5 donors were all started on HAART at slightly higher $CD_4^+$ T cell counts (median 289 cells/µl) (Table 2.4). Donor P6 in particular, started treatment at a $CD_4^+$ T cell count of 491 cells/µl, due to the fact that this donor was diagnosed with severe psoriasis that was thought to be HIV-related. Unfortunately, due to poor hospital follow-up, this donor could not be contacted and did not return to the clinic. $CD_4^+$ T cell response within HIV-positive patients is extremely variable, and according to physicians at the Groote Schuur AIDS clinic, a $CD_4^+$ T cell increase of 50-70 cells/µl over pre-treatment levels is usually expected after one month of HAART. Thereafter, an average increase of 70 cells/µl per year is considered to be a good indication of increasing $CD_4^+$ T cells attributable to viral suppression (Dr M Mendelson, personal communication). Overall, most of the donors tended to show an increase in their $CD_4^+$ T cell count as the ARV treatment progressed over the 6-months. An exception to this trend was donor P2, who initially showed a good rise in $CD_4^+$ T cell counts

**Table 2.4:** Clinical data and patient history for the 12 HIV-positive donors

| Donor ID | $CD_4^+$ Count at T0§ | $CD_4^+$ Count at 12 weeks§ | $CD_4^+$ Count at 24 weeks§ | $CD_4^+$ Count at 1 year§ | Viral Load at 6-months/1 year | Patient History (All patients were receiving Bactrim) |
|---|---|---|---|---|---|---|
| P1 | 142 | Patient moved to Lesotho in December 2008 | | | | No episodes of diarrhoea. |
| P2 | 95 | 255 | 304 | 165 | LDL/LDL | No episodes of diarrhoea.<br>Good $CD_4^+$ rise and viral suppression.<br>Patient became pregnant during study. |
| P3 | 90 | 293 | 331 | Left clinic in August 2009 | LDL | No episodes of diarrhoea.<br>Defaulted treatment after 6 months. |
| P4 | 215 | 323 | 305 | Left clinic in Dec 2009 | 380 | No episodes of diarrhoea.<br>Good $CD_4^+$ rise and viral suppression at 6 months.<br>Defaulted treatment after 6 months. |
| P5 | 167 | [-] | 310 | 287 | LDL/LDL | No episodes of diarrhoea.<br>Good $CD_4^+$ rise and viral suppression.<br>Treatment of recurrent urethritis using multiple antibiotics. |
| P6 | 491 | Patient lost to follow up after in hospital ARV initiation | | | | Severe psoriasis thought to be HIV-related and ARV's started at a higher $CD_4^+$ T cell count. |
| P7 | 123 | 213 | 334 | Transferred out of clinic | LDL | No episodes of diarrhoea.<br>Good $CD_4^+$ rise and viral suppression.<br>Transferred out of clinic after 6 months. |
| P8 | 186 | 212 | 281 | 328 | LDL/LDL | No episodes of diarrhoea.<br>Good $CD_4^+$ rise and viral suppression. |
| P9 | 204 | Patient withdrew from the study | | | | No episodes of diarrhoea. |
| P10 | 235 | 578 | 412 | 839 | LDL/LDL | No episodes of diarrhoea.<br>Good $CD_4^+$ rise and viral suppression. |
| P11 | 163 | [-] | 270 | No sample provided | LDL | No episodes of diarrhoea.<br>Good viral suppression. |
| P12 | 301 | Single sample | | | | No episodes of diarrhoea.<br>New patient to the clinic. HAART prescribed but patient not followed up due to termination of pilot study. |

* [LDL] Low detectable levels (< 50 copies/ml)

* [-] Patient missed appointment and no CD4+ T count was recorded

§ [CD4+ Cell Count] = cells/µl

but, at 52 weeks post-ARV initiation, the $CD_4^+$ T cell count had dropped considerably to 165 cells/µl. This reduction was thought to be as a result of her being pregnant and not as a result of viral replication (Burns *et al*., 1996; Biggar *et al*., 1989; Castilla *et al*., 1989; Sridma *et al*., 1982; Dr M Pandie, personal communication).

Standard HIV/AIDS treatment protocols followed within South African clinics advise that Bactrim (Trimethoprim-sulfamethoxazole) be administered to HIV-positive patients, in conjunction with HAART, when the $CD_4^+$ T cell count is below 200 cells/µl. The administration of Bactrim is to prevent the development and spread of opportunistic infections, such as *Pneumocystis* pneumonia (PcP). All HIV-positive donors recruited during this study received Bactrim prior to HAART on account of their low $CD_4^+$ T cell counts taken at T0 (Table 2.4). Several investigations have looked at the effect of Bactrim on the normal intestinal microbiota. Similar results were found in each of these studies, which clearly showed that Bactrim was highly effective against *Enterobacteriaceae*, but caused no alterations to the endogenous anaerobic gastrointestinal microbiota (Singer and Nash, 2000; Haase *et al*., 1984; Hargadon *et al*., 1981; Knothe, 1979).

In conjunction with the $CD_4^+$ T cell count, each donor's HIV viral load (plasma HIV-1 RNA levels) was recorded after 24 and 52 weeks, to ascertain whether the prescribed ARV regime was working (Table 2.4). An infective viral load is approximately $10^4/10^5$ copies/ml of blood and, with the commencement of HAART, it is anticipated that the viral load will be suppressed to low detectable levels (LDL), approximately < 50 copies/ml (Department of health and human services, 2011; Thompson *et al*., 2010; Griffith and Mayo, 2006; Chesebro and Everett, 1998). All eight donors who participated in the longitudinal study showed good viral suppression at both the 24 and 52 week clinic visits. The exception was, however, donor P4, who recorded a viral load count of 380 copies/ml and, shortly after this follow-up appointment, defaulted on the ARV treatment and subsequent follow-up visits.

In terms of secondary treatment, only donor P5 was receiving multiple antibiotics in order to treat a recurrent urethritis infection, as well as a Hepatitis B co-infection (Table 2.4). This donor received ciprofloxacin (250 mg), cefixime (400 mg), doxycycline (100 mg) and metronidazole (400 mg) administered at the 2, 6, 12 and 24 week clinic visits.

Throughout the entire course of this study, none of the 12 participating donors developed diarrhoea or any other gastrointestinal complications associated with an HIV infection.

### 2.4.2 Limitations of recruitment for this study

As with most clinical based investigations, there were several limitations encountered during the course of recruitment of patients for this study. Success of an investigation such as this lies in part in the recruitment of donors and the network established between the public sector clinic and the recruited donor. A noticeable limitation of this study was its small cohort size of 12 HIV-positive donors recruited over an 18 month period. Nonetheless, despite this sample size being fairly small, statistical analyses and the drawing of specific conclusions could still be carried out successfully (Nachar, 2008). Throughout this project, physicians at the Groote Schuur AIDS clinic attempted to recruit a larger sample size of approximately 20 HIV-positive donors, but the majority of the new patients, declined to participate when approached. Reasons cited by these patients were their reluctance to supply a faecal sample and having to commit to the longitudinal sampling time constraints. In addition, patient transportation continued to be a serious setback, in spite of it being supported financially by the study.

The second limiting factor of this study was patient compliance, specifically patient adherence to their HAART and committed attendance for the 6-month longitudinal study. Unfortunately, some donors missed specific follow-up appointments, stopped attending the clinic and, in some instances, donors even defaulted on their treatment after the 6 month clinic visit. This meant that extending the longitudinal study to 12 months after ARV initiation was not a feasible option for this investigation.

As previously mentioned, patient participation in this study was entirely voluntary and, at any point during the study, a donor could withdraw without affecting the administration of their HAART. Three of the recruited donors withdrew during the course of the study. One (donor P6) was lost as a result of poor hospital follow-up, and another donor (P1) could not participate in the longitudinal study because they transferred to another AIDS clinic located in a geographical region outside of Cape Town. Donor P9 voluntarily withdrew from the study. Patient P12 was only admitted to the study at the end of the pilot study period and, therefore, only contributed a sample at T0.

## 2.5 CONCLUSION

This clinical study is the first investigation to determine the effect of HIV and HAART on the actual levels of selected bacterial species in the gastrointestinal tracts of South African, HIV-positive donors. The first stage of this study involved the recruitment of donors for the HIV-positive and the control study groups and, despite several limitations encountered during this phase of the study, successful HIV-positive and HIV-negative donor recruitment was achieved. Even though the cohort size was small, it is an accurate representation of the socio-demographic profile of the South African population, as well as the HIV-positive population, and is amenable to statistical analyses. This sample group also reflects the fact that Black, South African women, aged 25-49, have the highest risk of HIV infection than any other demographic group within South Africa (Rehle *et al*., 2010; Rosen *et al*., 2010). Faecal material obtained from both donor groups could now be used for further microbiological and molecular investigations.

As discussed in Chapter 1, the human gastrointestinal tract is one of the earliest sites of HIV replication and $CD_4^+$ T cell depletion and is associated with elevated levels of intestinal inflammation and possible alterations in the microbial intestinal community. In order to determine how HIV replication affects the normal human gastrointestinal microbiome, a baseline comparison needs to be established between that of HIV-positive patients, prior to HAART initiation (T0) and a normal, healthy, HIV-negative population. To achieve this, the diversity of the total bacteria and the two probiotic genera, *Bifidobacterium* and *Lactobacillus*, was characterised in both study groups as described in Chapter 3.

# CHAPTER THREE

## PCR-DGGE analysis of total bacteria, *Bifidobacterium* and *Lactobacillus* species in South African HIV-positive and HIV-negative donors

**Contents**

## 3.1 SUMMARY

The human gastrointestinal tract houses a complex, commensal microbial community that assists in the development and maintenance of the gut associated lymphoid tissue (GALT), while simultaneously forming a protective barrier against invading opportunistic pathogens. In this study, it is hypothesised that the replication of HIV within the intestinal tract affects the endogenous microbiota in such a way that species belonging to genera, such as *Bifidobacterium* and *Lactobacillus* as well as other possible candidates, are no longer able to provide a beneficial and protective function to the host. The diversity of the total microbial population as well as the *Bifidobacterium* and *Lactobacillus* subpopulations present in the faecal material of 12 South African HIV-positive and HIV-negative donors was characterised using PCR-DGGE. Analysis of the PCR-DGGE results showed that each donor exhibited a unique and host specific profile for all of the investigated bacterial groups. There were no statistically significant differences between the diversity of the *Bifidobacterium* and *Lactobacillus* groups. However, the results showed that the diversity of the total bacterial population was significantly lower in the HIV-positive donors ($p$ = 0.009) compared to the control HIV-negative group. This finding may possibly be attributed to the replication of HIV within the GALT and the associated destruction of the gut mucosa. This is the first study to show a noticeable difference in the diversity of the total microbiota within the gastrointestinal tracts of South African HIV-positive patients compared to that of their healthy counterparts.

## 3.2 INTRODUCTION

The endogenous microbiota of the gastrointestinal tract is a large and complex community that plays an integral role in intestinal development and maintenance, homeostasis (Marchesi and Shanahan, 2007), stimulation of the host's immune system (Malinen *et al*., 2005), protection against epithelial cell injury (Eckburg *et al*., 2005), and protection against invading pathogens by functioning as a barrier (Marchesi and Shanahan, 2007).

*Bifidobacterium* and *Lactobacillus* species belong to the Lactic Acid Bacteria group (LAB) and are important constituents of the intestinal microbiota. The genus *Lactobacillus*, is comprised of Gram-positive, rod-shaped, catalase negative, non-sporing, microaerophilic species (Felis and Dellaglio, 2007). Although *Lactobacillus* species are estimated to make up less than 1% of the intestinal population, with *L. acidophilus, L. gasseri, L. fermentum* and *L. reuteri* being the most frequently isolated species in human faeces (Reuter, 2001; Sghir *et al*., 2000), these bacteria are actively involved in influencing and maintaining the gastrointestinal tract (Felis and Dellaglio, 2007; Rolfe, 2000). Certain *Lactobacillus* species are capable of stimulating macrophages and secreting antimicrobial substances which inhibit the growth and attachment of invading pathogens to the intestinal tract (Anuradha and Rajeshwari, 2005; Alvarez-Olmos and Oberhelman, 2001).

*Bifidobacterium* spp. are Gram-positive, non-motile bacteria with a high GC content, and represent some of the most common organisms found within the human intestinal tract (Turroni *et al*., 2008; Matsuki *et al*., 2004, Biavati and Mattarelli, 2001). In addition to protecting the host from invading opportunistic pathogens, *Bifidobacterium* species such as *B. adolescentis, B. bifidum, B. breve* and *B. longum* also contribute towards the maintenance and integrity of the epithelial lining (Felis and Dellaglio, 2007). *Bifidobacterium* species are capable of producing glutamine from $NH_4^+$ and glutamic acid, which serves as a supplement for the intestinal epithelium and maintains the integrity of the mucosal lining (Anuradha and Rajeshwari, 2005).

PCR-DGGE (discussed in Chapter 1) has been shown to be a rapid and effective tool in studying the diversity of complex microbial populations present within the intestinal tract, and in determining the effects that the administration of antimicrobials have on the host's gut microbiota (Turroni *et al*., 2008; Handschur *et al*., 2007). The fundamental principle of PCR-

DGGE relies on the sequence-specific separation of equally sized PCR fragments, generating a particular "population profile" for each microbial community (Muyzer *et al*., 1993).

Investigations into the diversity of the gastrointestinal bacterial community make use of the 16S rRNA gene which has a high discriminatory index and consists of several distinct conserved and variable regions pertaining to a range of phylogenetic levels such as, group, genus and species (Mohania *et al*., 2008; Satokari *et al*., 2003; Heilig *et al*., 2002). This particular feature has led to the development of group- and species-specific primers that can be utilized in the detection and identification of bacteria such as *Bifidobacterium* and *Lactobacillus* species (Satokari *et al*., 2003; Heilig *et al*., 2002). As a result of these differing sequences found within the 16S rRNA gene of bacterial species, PCR fragments will possess unique and different chemical stability properties causing them to migrate to different positions within the denaturing gradient across the gel. However, PCR fragments aligning at the same positions do not necessarily prove they are identical or derived from the same species, and therefore, sequencing of these fragments or the use of a narrower denaturing gradient to further enhance resolution is required (Muyzer *et al*., 1993). Muyzer *et al*., (1993) showed that PCR-DGGE was sensitive enough to detect a particular bacterial species representing only 1% of the total microbial population.

The primary objective of the research presented in this chapter was to examine the microbial condition of the gastrointestinal tract in HIV-positive patients, with respect to two well-known probiotic groups of bacteria and to the total microbial population. This would assist in determining whether the continuous replication of HIV, within the gut, causes a significant disruption to the endogenous microbiota and whether probiotic supplementation might help in stabilising conditions within the gut. Serious alterations and changes to the distribution of intestinal populations, such as the *Bifidobacterium* and *Lactobacillus* groups, could result in them no longer being able to provide a protective function to the host. Therefore, the major representatives of the total microbial population, as well as the *Bifidobacterium* and *Lactobacillus* groups, present in the faecal material of 12 South African HIV-positive and HIV-negative donors were analysed using PCR-DGGE, and the major species represented identified phylogenetically.

## 3.3 MATERIALS AND METHODS

### 3.3.1 Bacterial strains and plasmids

All bacterial strains and plasmids used in this section of the study are described in Table 3.1. *Enterococcus faecalis* (Juste-Poinapen, 2007) and all *Lactobacillus* type strains were cultured in de Man, Rogosa, Sharpe (MRS) [Biolab] media, while *B. longum* and *B. adolescentis* were cultured in Basal Yeast (BYG) medium containing 1% (w/v) glucose, as described by Degnan and Macfarlane, (1993). *B. fragilis* 638R was grown in a supplemented brain heart infusion (BHIS) [Difco] medium (Holdeman and Moore, 1972), whilst a *Clostridium* basal growth medium (CBM) was used to culture *C. perfringens* (O'Brien and Morris, 1971). All reference strains, with the exception of *E. coli,* were grown anaerobically in an anaerobic chamber (Forma Scientific Inc, Model 1024) under atmospheric conditions of 85% $N_2$, 10% $CO_2$ and 5% $H_2$. *E. coli* was grown aerobically at 37$^{o}$C in Luria Bertani (LB) broth or agar plates containing ampicillin (100 µg/ml) when required (Sambrook *et al*., 1989).

**Table 3.1** Bacterial strains and cloning plasmid used in this study

| Bacterial strains and plasmid | Relevant characteristics | Source or reference* |
|---|---|---|
| *Bifidobacterium longum* NCIMB 702259$^{T}$ | Type strain of *B. longum* | NCIMB |
| *Bifidobacterium adolescentis* ATCC 15703$^{T}$ | Type strain of *B. adolescentis* | ATCC |
| *Lactobacillus casei* DSM 20011$^{T}$ | Type strain of *L. casei* | DSM |
| *Lactobacillus brevis* DSM 20054$^{T}$ | Type strain of *L. brevis* | DSM |
| *Lactobacillus reuteri* DSM 20016 $^{T}$ | Type strain of *L. reuteri* | DSM |
| *Lactobacillus gasseri* ATCC 33323$^{T}$ | Type strain of *L. gasseri* | ATCC |
| *Lactobacillus fermentum* ATCC 9338$^{T}$ | Type strain of *L. fermentum* | ATCC |
| *Bacteroides fragilis* 638R | Clinical isolate | Privitera *et al*., (1979) |
| *Escherichia coli DH5α* | Gram-negative cloning host | Hanahan, (1983) |
| *Enterococcus faecalis* (P1.2) | MCB laboratory strain | MCB |
| *Clostridium perfringens* ATCC 13124$^{T}$ | Type strain of *C. perfringens* | ATCC |
| Plasmid pTZ57R/T | Linearized cloning vector with single 3'-ddT overhangs | Fermentas |

*__NCIMB:__ National Collection of Industrial, Food and Marine Bacteria, United Kingdom, __DSM:__ Deutsche Sammlung von Mikroorganismen, Germany, __ATCC:__ American Type Culture Collection, __MCB:__ Department of Molecular and Cell Biology, University of Cape Town

### 3.3.2 Genomic DNA extraction

Total bacterial DNA was extracted from faecal samples collected at T0 (section 2.3.2), using the ZR Faecal DNA Kit™ (ZYMO Research) (Yoshikawa *et al*., 2011). Approximately 200

mg of frozen faecal material was added directly to a ZR BashingBead$^{TM}$ lysis tube containing 750 µl lysis buffer (1.8% Tris; 8% NaCl; 18.6% EDTA and 4 M guanidine thiocyanate [Zymo Research MSDS]) and rapidly lysed for 5 minutes at maximum speed in a Mini-Beadbeater$^{TM}$ (Biospec). Following this, DNA extraction was performed according to the manufacturer's instructions.

DNA extraction from bacterial reference strains was performed using the Genomic DNA Purification Kit (Fermentas) for the isolation of nucleic acids from bacterial cultures, as described by the manufacturer with a slight modification. Bacterial cells, except *B. fragilis* and *E. coli*, were initially pre-treated with 200 µl lysis buffer (20 mM Tris-HCl pH 8.5, 2 mM EDTA pH 8, 1% (v/v) Triton X-100 and 20 mg/ml lysozyme) for 2 hours at 37$^{o}$C. Following this, DNA isolation was completed according to the manufacturer's instructions. The concentration of the extracted DNA was determined spectrophotometrically using a Nanodrop (NanoDrop® ND-1000).



### 3.3.3 PCR-DGGE analysis of selected faecal bacterial populations

*3.3.3.1 PCR amplification*

PCR amplification of the 16S rRNA gene, from faecal genomic DNA, was used for the detection of total bacteria as well as *Bifidobacterium* and *Lactobacillus* species. Optimal PCR products for DGGE analysis were obtained using two rounds of nested PCR. Primers used for this section of the work are shown in Table 3.2 and all PCR reactions were performed in a GeneAmp® PCR System 9700 (Applied Biosystems).

*First round PCR detection of Bifidobacterium species:*

PCR detection of *Bifidobacterium* species employed primers Bif164F and Bif662R. A PCR reaction mixture of 25 µl contained 100 ng of DNA template, 1X PCR reaction buffer, 1.5 mM $MgCl_2$, 0.5 µM of each primer, 200 µM of each dNTP (Fermentas), Bovine Serum Albumin [0.6 µg/µl] (Fermentas) and 0.25 units of Supertherm *Taq* polymerase (Southern Cross Biotechnology). The PCR cycling conditions used were performed according to the method described by Satokari *et al*., (2001) and Kullin, (2010). PCR amplification involved a denaturation step at 96$^{o}$C for 5 min, followed by 35 cycles of 96$^{o}$C for 30 sec, 63$^{o}$C for 20 sec and 68$^{o}$C for 40 sec, finishing off with a step at 62$^{o}$C for 20 sec and final extension at 68$^{o}$C for 7 min.

**Table 3.2** PCR primers employed in this study

| Primer | Primer Sequence (5'-3') | Reference |
|---|---|---|
| Bif164F | GGG TGG TAA TGC CGG ATG | Kok *et al*., (1996) |
| Bif662R | CCA CCG TTA CAC CGG GAA | Langendijk *et al*., (1995) |
| Univ341F | TCC TAC GGG AGG CAG CAG | Muyzer *et al*., (1993) |
| Lab0667R | CAC CGC TAC ACA TGG AG | Heilig *et al*., (2002) |
| Univ341F-GC | CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GTC CTA CGG GAG GCA GCA | Muyzer *et al*., (1993) |
| Univ515R | ATC GTA TTA CCG CGG CTG CTG GCA | Lane, (1991) |
| R2 | GGA CTA CCI GGG TAT CTA ATC C | Lee *et al*., (1993) |
| M13F | CGC CAG GGT TTT CCC AGT CAC GAC | Yanisch-Perron *et al.*, (1985) |
| M13R | GAG CGG ATA ACA ATT TCA CAC AGG | |

*First round PCR detection of the Lactobacillus group:*

PCR detection was performed using primers Univ341F and Lab0667R for the *Lactobacillus* group (*Lactobacillus*, *Leuconostoc*, *Pedicococcus* and *Weissella* genera). Reactions were carried out using the following PCR reaction mixture (25 µl): 100 ng of DNA template, 1X PCR reaction buffer, 1.5 mM $MgCl_2$, 0.5 µM of each primer, 200 µM of each dNTP (Fermentas), Bovine Serum Albumin [0.6 µg/µl] (Fermentas) and 0.25 units of Supertherm *Taq* polymerase (Southern Cross Biotechnology). PCR cycling conditions, previously optimized by Magwira, (2008), consisted of an initial step at 96$^{o}$C for 4 min, followed by 30 cycles of 96$^{o}$C for 40 sec, 55$^{o}$C for 30 sec and 72$^{o}$C for 45 sec, and a final extension step of 5 min at 72$^{o}$C.

*First round PCR detection of total bacteria:*

PCR detection of total bacteria was performed using primers Univ341F and R2 for total bacteria. The PCR reaction mixture used comprised (25 µl): 100 ng of DNA template, 1X PCR reaction buffer, 1.5 mM $MgCl_2$, 0.5 µM of each primer, 200 µM of each dNTP (Fermentas), Bovine Serum Albumin [0.6 µg/µl] (Fermentas) and 0.25 units of Supertherm *Taq* polymerase (Southern Cross Biotechnology). PCR cycling parameters consisted of an initial step at 96$^{o}$C for 4 min, followed by 30 cycles of 96$^{o}$C for 40 sec, 53.5$^{o}$C for 30 sec and 72$^{o}$C for 45 sec, and a final extension step of 5 min at 72$^{o}$C (Magwira, 2008).

*Second round PCR detection*

For the second round of amplification, to include the GC clamp and to obtain PCR products of similar length, 1 µl of the first round PCR product was used as template together with primers Univ341F-GC and Univ515R, producing PCR amplicons of approximately 216 bp in size. PCR conditions included initial denaturation at 96$^{o}$C for 4 min followed by 30 cycles of 96$^{o}$C for 40 sec, 55$^{o}$C for 30 sec and 72$^{o}$C for 40 sec with a final extension of 7 min at 72$^{o}$C (Magwira, 2008).

The detection of PCR products and their respective molecular sizes was determined using agarose gel electrophoresis. Approximately 5 µl of the PCR product was run on a 2% agarose (w/v) gel stained with ethidium bromide [10mg/ml] (Promega) and compared to a 100 bp molecular weight marker (Fermentas).

*3.3.3.2 Denaturing gradient gel electrophoresis (DGGE)*

PCR products generated using primers Univ341F-GC and Univ515R were used for DGGE analysis. Polyacrylamide gels consisted of 8% (v/v) polyacrylamide (37.5:1 acrylamide-bisacrylamide) and 50X TAE buffer (2 M Tris, 1 M Acetic acid, 50 mM EDTA, pH 8). PCR products were separated using deionized formamide and urea denaturing gradients of 45-65% (total bacteria); 45–55% (*Lactobacillus* group) and 55-65% (*Bifidobacterium*) respectively. A 100% denaturant is defined as a mixture of 7 M urea and 40% (v/v) deionized formamide. Electrophoresis was conducted using the DCode$^{TM}$ Universal Mutation Detection System (Bio-Rad Inc.) run at a constant voltage and temperature of 62 V and 60$^{o}$C for 17-18 h. Enhanced resolution of the DGGE banding pattern was achieved by pre-electrophoresis of the gel at 180V for 10 mins. A DGGE reference ladder made up of different bacterial species, specific to the group being investigated, was included in each run thereby allowing subsequent gel normalisation and inter-gel comparisons. Following electrophoresis, gels were stained with ethidium bromide (25 µl in 250 µl 1X TAE buffer [1 µg/µl]) for 15 mins and then de-stained in fresh 1X TAE buffer for 30 mins. DGGE banding patterns were visualized using the GelDoc XR$^{+}$ imaging system (Bio-Rad Inc.).

### 3.3.4 Statistical analyses of selected DGGE banding patterns

*3.3.4.1 Cluster analysis*

The relationship and similarities between the banding patterns generated by PCR-DGGE were analysed using the unweighted-pair group method (UPGMA) to construct dendrograms based on the Dice similarity coefficient between samples as previously described by van der Gucht *et al*., (2001). All DGGE banding profiles were digitally analysed in a multistep method using the FPQuest$^{TM}$ bioinformatics software version 4.5 (Bio-Rad Inc) (Vitali *et al*., 2010). Following gel normalisation, DNA bands present in each lane were automatically detected and matched based on their position relative to the standard reference ladder. Calculated similarity values were then visually represented in the form of a dendrogram (Vitali *et al*., 2010).

*3.3.4.2 Shannon-Wiener diversity index*

The Shannon-Wiener diversity index ($H'$) was used to determine the diversity of bacterial species present within the faecal samples of the two donor groups. The diversity index for each gel lane was evaluated using the Quantity One® software version 4.5.2 (Bio-Rad Inc), and calculated using the following formula (Gafan *et al*., 2005):

$$H' = -\sum_{i=1}^{s} (Pi)(\ln Pi)$$

where *s* is the number of bands present in each sample and *Pi* is the relative intensity of each band within the sample. Since the data was not uniformly distributed, a nonparametric analysis using the Mann-Whitney U Test (Mann and Whitney, 1947) was performed to compare the $H'$ values in the HIV-positive and HIV-negative groups and a $p$ value $\leq 0.05$ was interpreted as being statistically significant. All nonparametric statistical analyses, as well as box-and-whisker plots (expressed as medians with quartile ranges [QR]) were performed using Stata data analysis and statistical software version 11.1 (StataCorp LP, USA).

### 3.3.5 Sequence analysis of selected DGGE bands

*3.3.5.1 Excision of DNA fragments and cloning*

DGGE bands were excised as described by Omar and Ampe, (2000). Selected bands were excised under UV illumination (360 nm), using sterile surgical blades and re-suspended in 30

µl of nuclease-free water for 24 hours at 4°C. Samples were centrifuged for 1 min at maximum speed, and a 3 µl aliquot was re-amplified using primers Univ341F and Univ515R, as described in section 3.3.3.1. The resulting PCR products were purified using the BioSpin PCR Purification Kit (BioFlux), as per the manufacturer's instructions; ligated into the vector pTZ57R/T using the InsTAclone™ PCR Cloning Kit (Fermentas) and transformed into competent *E. coli* DH5α cells. Presence of the insert was confirmed through colony PCR (Dafa'alla *et al*., 2000; Zon *et al*., 1989) using primers M13F and M13R (Table 3.2). Plasmid DNA, containing the insert, was then isolated using the BioSpin Plasmid Extraction Kit (BioFlux) and sequenced (Macrogen Inc., Seoul, Korea) using M13F as the sequencing primer. Three independent clones were selected for each cloned fragment. Sequences were analysed using Chromas version 2.01 (Technelysium Ltd) and DNAMAN version 4.13 (Lynnon BioSoft), while the similarity of the sequence was compared with those available in the Genbank database, using the BLAST algorithm, (Zhang *et al*., 2000; Altschul *et al*., 1990) and the Ribosomal Database Project (Cole *et al*., 2009).

## 3.4 RESULTS AND DISCUSSION

### 3.4.1 PCR-DGGE optimisation

PCR-DGGE has become a valuable tool for monitoring the structure and dynamics of a complex microbial population, and has been successfully used in many studies focusing on the gastrointestinal microbiota of humans (Ariefdjohan *et al*., 2010; Wu *et al*., 2010), farm animals (Furet *et al*., 2009; Petersson *et al*., 2009) and mice (Bibiloni *et al*., 2005). To ensure that optimal DGGE conditions were used in this study several key components needed to be optimised prior to performing the experimental work. These components included: efficient genomic DNA extraction; primer selection; PCR conditions; the addition of PCR enhancers such as bovine serum albumin (BSA), as well as the denaturing gradient and electrophoresis run time to be used.

#### *3.4.1.1 Cell lysis and validation of genomic DNA extraction kit*

An accurate representation of the gastrointestinal microbial community is affected by factors such as the quality of the DNA isolated from the sample, as well as incomplete or preferential cell lysis. In this study, high quality, inhibitor-free DNA was isolated using the ZR Fecal DNA kit. The addition of the bead-beating step ensured that samples underwent efficient and rapid cell lysis, while any inhibitors released during the extraction procedure were removed

using the specially designed Zymo-Spin™ IV-HRC spin filter, to prevent any interference occurring during PCR amplification.

To validate the efficiency of the DNA extraction kit to extract inhibitor-free DNA, and to determine the optimum concentration of DNA to be used, PCR was performed on different concentrations of DNA (25 ng to 200 ng) using each primer set prior to PCR-DGGE. In most cases, PCR inhibition occurred when 150 ng or more of DNA template was used, whilst no amplification occurred when 25 ng DNA was used. Maximum PCR amplification was however, achieved when a DNA concentration of 100 ng was used, and confirmed that the ZR Fecal DNA kit had successfully removed any major inhibitors present within the sample (Figure 3.1). This kit was later also used for qPCR analysis (see Chapter 5). Different amounts of DNA were evaluated in the qPCR work, and no major inhibitors of the PCR reaction were detected. These results confirm the reports by Yoshikawa *et al.* (2011) that the ZR Fecal DNA kit effectively removes PCR inhibitors present in human faecal samples.

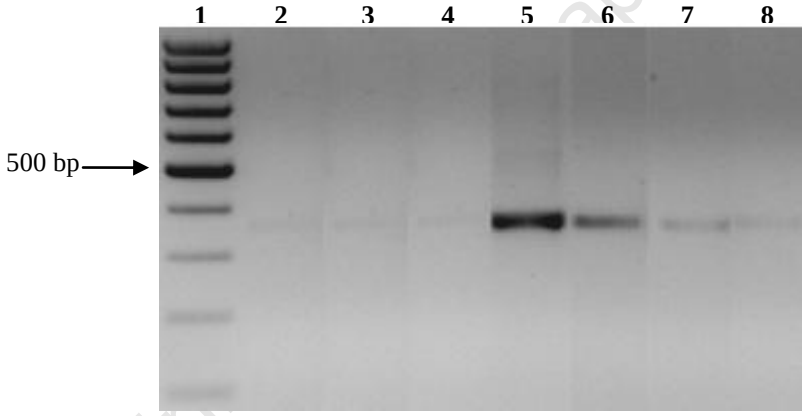



**Figure 3.1: PCR amplification of serial dilutions of faecal genomic DNA using optimised PCR conditions [Univ341F and Lab0667R].** Lane 1: 100 bp molecular weight marker (Fermentas); Lane 2: Negative control (no DNA); Lane 3: 200 ng; Lane 4: 150 ng; Lane 5: 100 ng; Lane 6: 75 ng; Lane 7: 50 ng and Lane 8: 25 ng.

*3.4.1.2 PCR Optimisation*

*3.4.1.2.1 Primer selection*

The sensitivity and specificity of PCR-DGGE does, in part, also rely on selecting the most efficient primer combinations. Primers Univ341F (Muyzer *et al.*, 1993) and R2 (Lee *et al.* 1993) are termed universal primers given that they will amplify all predominant bacterial species present within the sample.

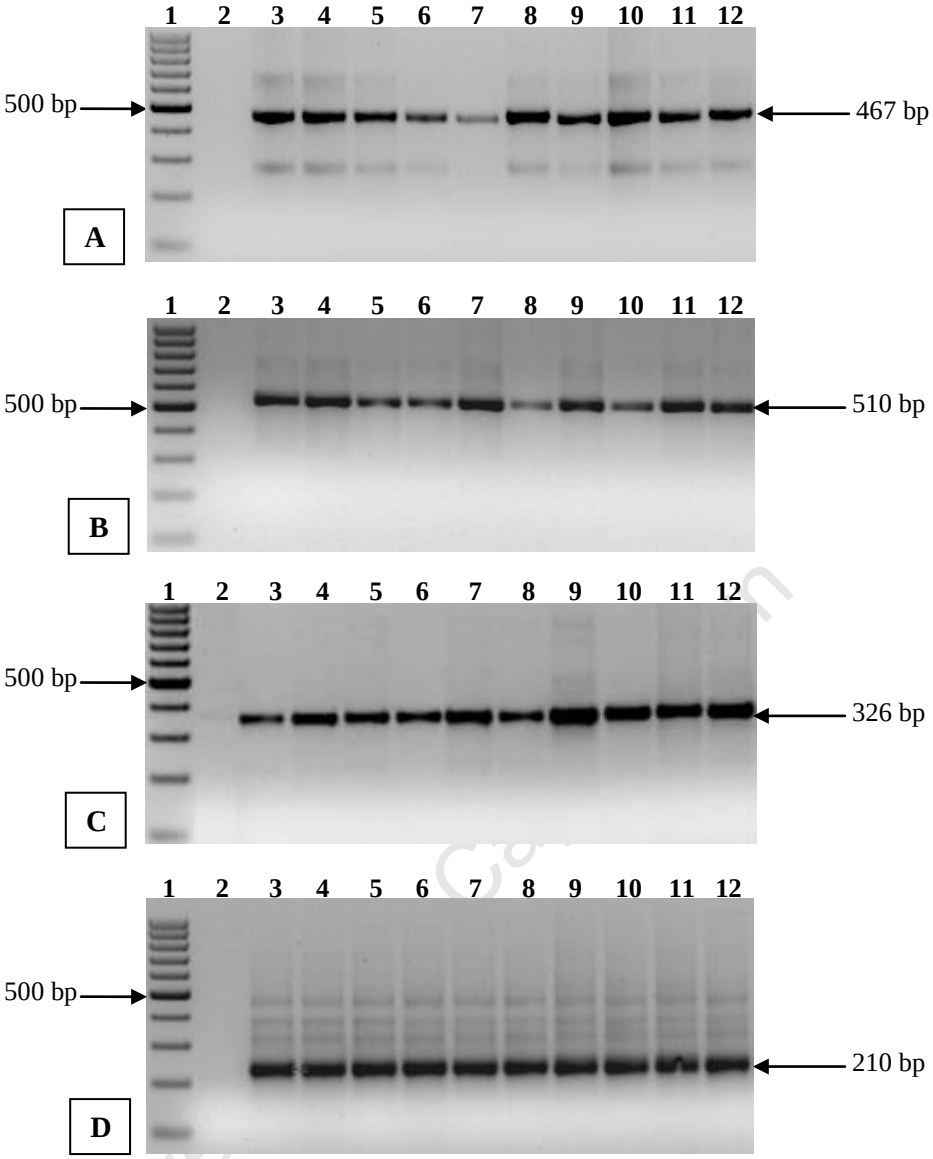



**Figure 3.2:** **First and second round PCR detection.** (A) Total bacteria employing primers Univ341F and R2. (B) *Bifidobacterium* species employing primers Bif164F and Bif662R. (C) *Lactobacillus* group employing primers Univ341F and Lab0667R. (D) Representative gel showing second round PCR detection employing primers Univ341F-GC and Univ515R [first round PCR products from panel A were used as templates]. Lane 1: 100 bp molecular weight marker (Fermentas); Lane 2: Negative control (no DNA); Lanes 3 – 7: HIV-positive donors (P1-P5) and Lanes 8 – 12: HIV-negative donors (H1-H5).

Samples tested using primers Univ341F and R2 all produced a distinct 467 bp DNA fragment after the first round of PCR amplification (Figure 3.2A). However, a major limitation of using universal primers is that individual shifts within specific smaller bacterial populations may not be seen. Thus the development of group-specific primers has meant that bacterial

species belonging to groups, present in much smaller numbers within the gastrointestinal tract, are able to be analysed, better revealing any subtle changes or differences within these communities.

In this study, several combinations of published PCR primers were tested (Heilig *et al*., 2002; Kok *et al*., 1996; Langendijk *et al*., 1995; Muyzer *et al*., 1993) and the following primers were selected as the most efficient. Group-specific primers Bif164F and Bif662R (*Bifidobacterium*) and Univ341F and Lab0667R (*Lactobacillus*) were used in the first round of PCR producing PCR amplicons of approximately 510 bp and 326 bp respectively (Figure 3.2B and Figure 3.2C). Lab0667R has been shown to be specific for lactic acid bacteria, and has been used for the detection of *Lactobacillus* species, while also simultaneously amplifying members belonging to the phylogenetically related *Leuconostoc*, *Pediococcus* and *Weisella* genera (Heilig *et al*., 2002).

Increased sensitivity and optimal fragment separation were achieved when a nested PCR was conducted. An advantage of using the nested PCR approach is that a standard length 16S rRNA gene fragment was generated prior to DGGE analysis. Figure 3.2D shows first round PCR products (from panel 3.2A) that were amplified using the second round primers. Similar results were found after using PCR products shown in Figures 3.2B and 3.2C as templates, and all produced equal length product sizes of 210 bp containing the GC-rich clamp essential for DGGE analysis. One of the most important functions of the GC clamp is to prevent the PCR product from completely dissociating during electrophoresis.

Faint shadow bands, associated with each PCR product, were seen when the universal primer sets were used (Figure 3.2A and 3.2D). Numerous attempts were made to eliminate this by modifying the PCR conditions, but these particular shadow bands were still evident even when relatively low DNA concentrations were used. Shadow bands have been previously reported by Qiu *et al*., (2001) who showed that the production of these PCR artefacts increased substantially when the diversity of species present within a sample increased. This may be the reason for the bands observed in this study. For the current study, therefore, PCR conditions showing the least amount of shadow bands were used (see section 3.3.3).

*3.4.1.2.2 PCR Conditions*

Optimisation of PCR cycling conditions was done using previously published methods as a basis for *Bifidobacterium* species (Satokari *et al*., 2001), as well as the *Lactobacillus* group and total bacteria (Magwira, 2008). To ensure that highly optimised PCR conditions were used for each primer set, the $MgCl_2$ concentration, annealing temperature, cycle number, and primer concentration were all evaluated and adjusted in various PCR test runs, prior to initiating this study. Several $MgCl_2$ titration curves, ranging from 1 mM to 2.5 mM were conducted in order to achieve maximum product production, as well as high specificity and sensitivity.

The addition of BSA has also been shown to increase PCR product production by stabilising the *Taq* polymerase, in addition to interacting with any remaining inhibitory substances present in the sample (Eilert and Foran, 2009; Al-Soud and Rådström, 2000; Nagai *et al*., 1998). Two related investigations conducted by Kullin, (2010) and Magwira, (2008) both showed that amplification of the 16S rRNA gene from human faecal material was enhanced when BSA was added directly to the PCR reaction mix. Therefore, to ascertain whether amplification of the 16S rRNA gene from faecal samples used in this study would be increased due to the presence of BSA, several PCR reactions were conducted in the presence or absence of BSA, and the resulting product examined using gel electrophoresis. A noticeable increase in PCR product production was seen for the reactions that had been supplemented with BSA (Figure 3.3) and as a result of this, BSA was included in all PCR reactions conducted in this study.



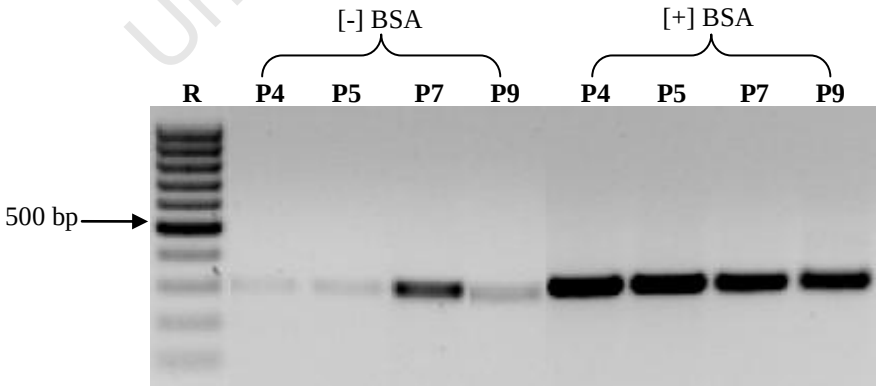



**Figure 3.3:** **The effect of BSA on PCR product production.** Duplicate PCR reactions, employing primers Univ341F and Lab0667R, in the presence [+] and absence [-] of BSA [0.6 µg/µl]. Lane R: 100 bp molecular weight marker (Fermentas).

*3.4.1.3 DGGE running conditions*

To achieve the best possible separation of bands, and in so doing make profiling of microbial communities easier, optimisation and modification of the denaturing gradient as well as the run time had to be performed. For the purpose of this study, a range of gradient concentrations were tested to ascertain the best band separation conditions (Temmerman *et al*., 2003a; Satokari *et al*., 2001). Gradient ranges of 55-65% and 45-55% were identified as suitable for the separation of the *Bifidobacterium* (Figure 3.4A) and *Lactobacillus* (Figure 3.4B) populations respectively, while a gradient of 45-65% was used for the analysis of the total bacteria profile (Figure 3.5).

Electrophoresis running conditions used for DGGE analysis is another contested issue, with some studies preferring a longer time period and low voltage (Boon *et al*., 2002; Gejman *et al*., 1998) while others have reported that shorter time periods at higher voltages (Sigler *et al*., 2004) result in efficient band separation. Optimisation of the electrophoresis run time carried out in this study confirmed the findings of Kullin, (2010) and Magwira, (2008) and demonstrated that a longer time period (17-18 hours) and a low constant voltage resulted in better separation of 16S rRNA fragments amplified from faecal material using the *Bifidobacterium* and *Lactobacillus* group-specific primers.

PCR products from all bacterial reference strains (Table 3.1) were amplified by means of the optimised methods, using either the universal or group-specific primers. Individual PCR products were pooled to create three standard DGGE reference ladders representative of *Bifidobacterium* spp. (Figure 3.4A), *Lactobacillus* spp. (Figure 3.4B) and total bacteria (Figure 3.5).

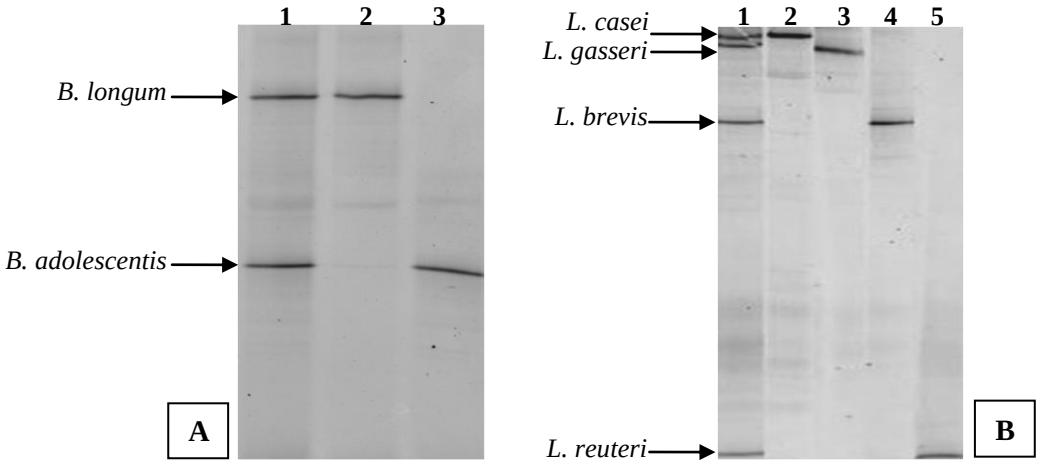



**Figure 3.4:** **(A) *Bifidobacterium* DGGE reference ladder.** Separation of PCR products from two *Bifidobacterium* species using a denaturing gradient of 55-65%. Lane 1: Pooled reference ladder; Lane 2: *B. longum* and Lane 3: *B. adolescentis*. **(B) *Lactobacillus* group DGGE reference ladder.** Separation of PCR products from four *Lactobacillus* species using a denaturing gradient of 45-55%. Lane 1: Pooled reference ladder; Lanes 2: *L. casei*; 3: *L. gasseri;* 4: *L. brevis* and 5: *L reuteri*.

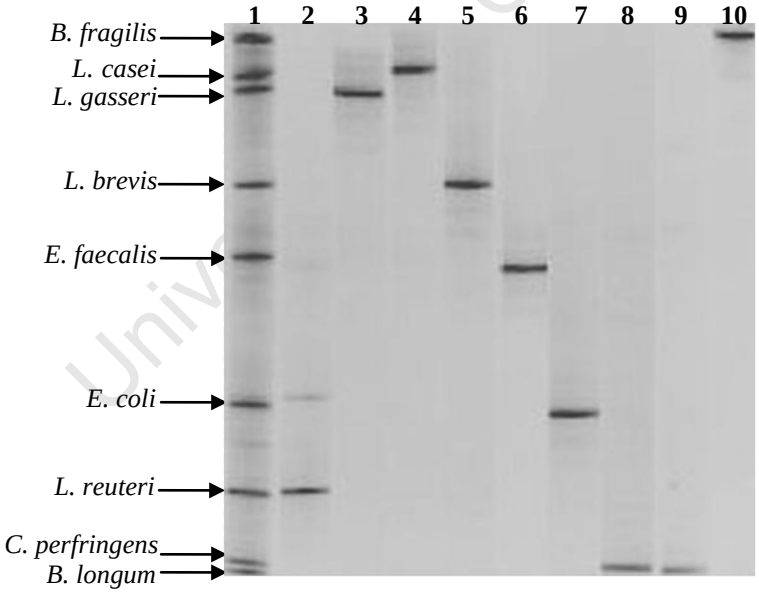



**Figure 3.5:** **Total bacteria DGGE reference ladder.** Separation of PCR products from nine reference strains using a denaturing gradient of 45-65%. Lane 1: Pooled reference ladder; Lanes 2: *L. reuteri*; 3: *L. gasseri*; 4: *L. casei*; 5: *L. brevis;* 6: *E. faecalis*; 7: *E. coli;* 8: *C. perfringens*; 9: *B. longum* and 10: *B. fragilis*.

### 3.4.2 DGGE profile and cluster analysis of different bacterial groups

DGGE was used in determining the diversity of the *Bifidobacterium* and *Lactobacillus* communities, as well as the major gut bacterial species, present in the faeces of HIV-positive and HIV-negative donors (Figure 3.6A, Figure 3.7A and Figure 3.8A). When examining the banding pattern generated for the *Bifidobacterium* population (Figure 3.6A), it was evident that 2 to 4 dominant bands occurred in most of the gel lanes. This corresponded with similar reported PCR-DGGE studies that also focused on *Bifidobacterium* species within the human intestinal tract (Vanhoutte *et al*., 2004; Nielsen *et al*., 2003; Satokari *et al*., 2001). The *Bifidobacterium* population appeared to be quite similar in both the HIV-positive and HIV-negative donors.

The faecal *Lactobacillus* population in contrast (making up less than 1% of the total intestinal community) showed very different DGGE profiles for each of the donors (Figure 3.7A). The banding patterns revealed a minimum of two and a maximum of six detectable bands in all the faecal samples screened suggestive of some variation in the diversity of the *Lactobacillus* community within the intestinal tracts of both HIV-positive and HIV-negative donors.

The universal DGGE profiles (Figure 3.8A) were considerably more complex than the *Bifidobacterium* and *Lactobacillus* profiles. DGGE banding patterns differed to some extent between donors both in the number of bands present, as well as the position and intensity of these bands within the gel. In most instances, the total bacteria profile was characterised by the presence of 8 to 18 different bands across the individual lanes indicating a higher diversity when compared to the *Bifidobacterium* and *Lactobacillus* groups. However, caution must be used when interpreting these results since DGGE provides only an indication of the microbial community's diversity rather than an exact representation (Eichner *et al*., 1999). The reason for this is that when the universal primers are employed to amplify a large mixture of 16S rRNA genes within a sample, species present in much lower abundances will not readily amplify, and the universal DGGE profile will be skewed to show only the predominant species present within that particular community (Muyzer *et al*., 1993).

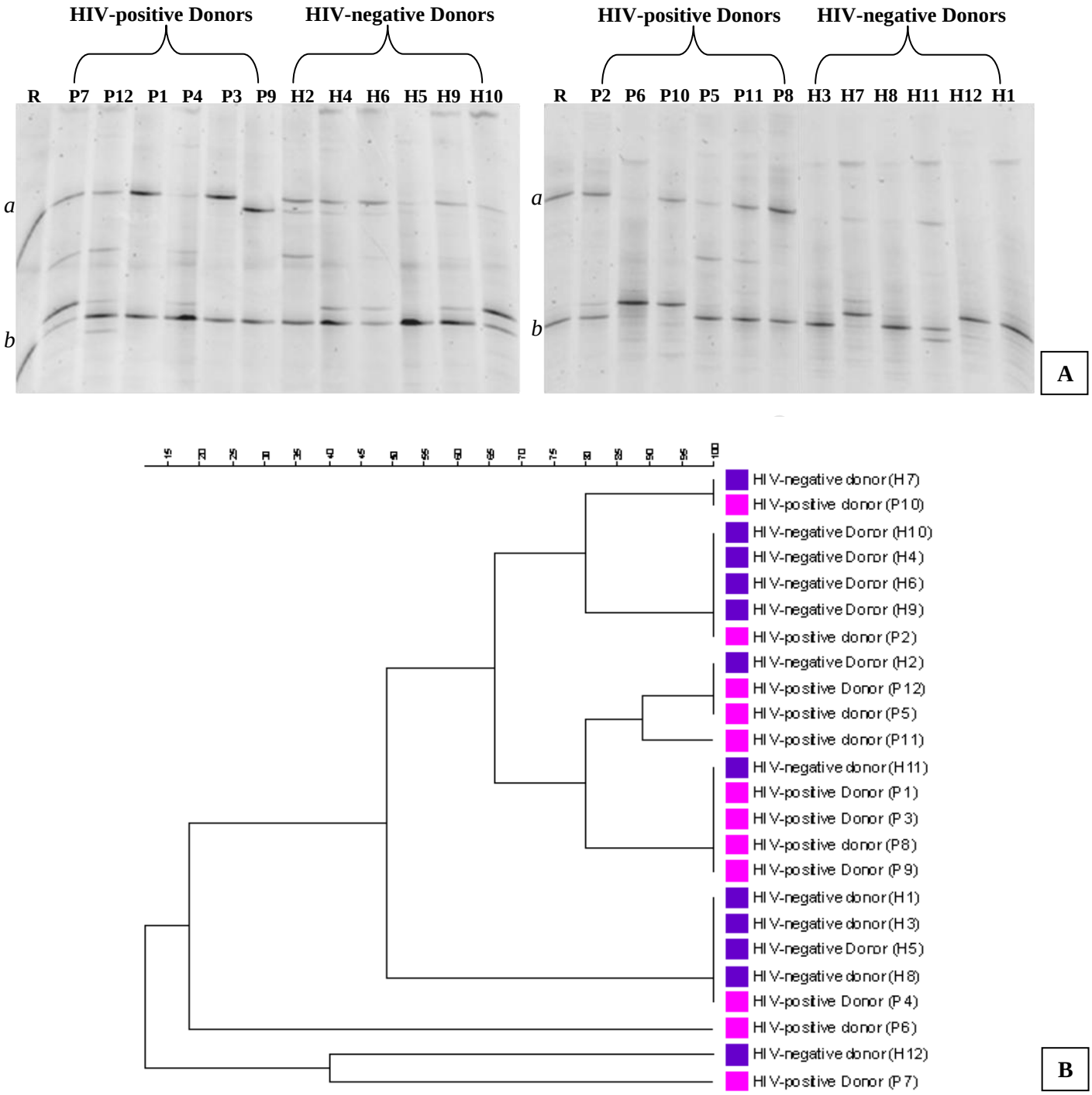



**Figure 3.6:** **DGGE and cluster analysis of bifidobacterial 16S rRNA genes amplified from the faecal material of 12 HIV-positive and 12 HIV-negative donors. (A)** Lane R: DGGE reference ladder [(*a*): *B. longum* and (*b*): *B. adolescentis*]. **(B)** The dendrogram was generated using the Dice Similarity coefficient and clustering algorithm UPGMA [(Pink): HIV-positive donor and (Purple): HIV-negative donor]. *Scale bar* indicates similarity (%).

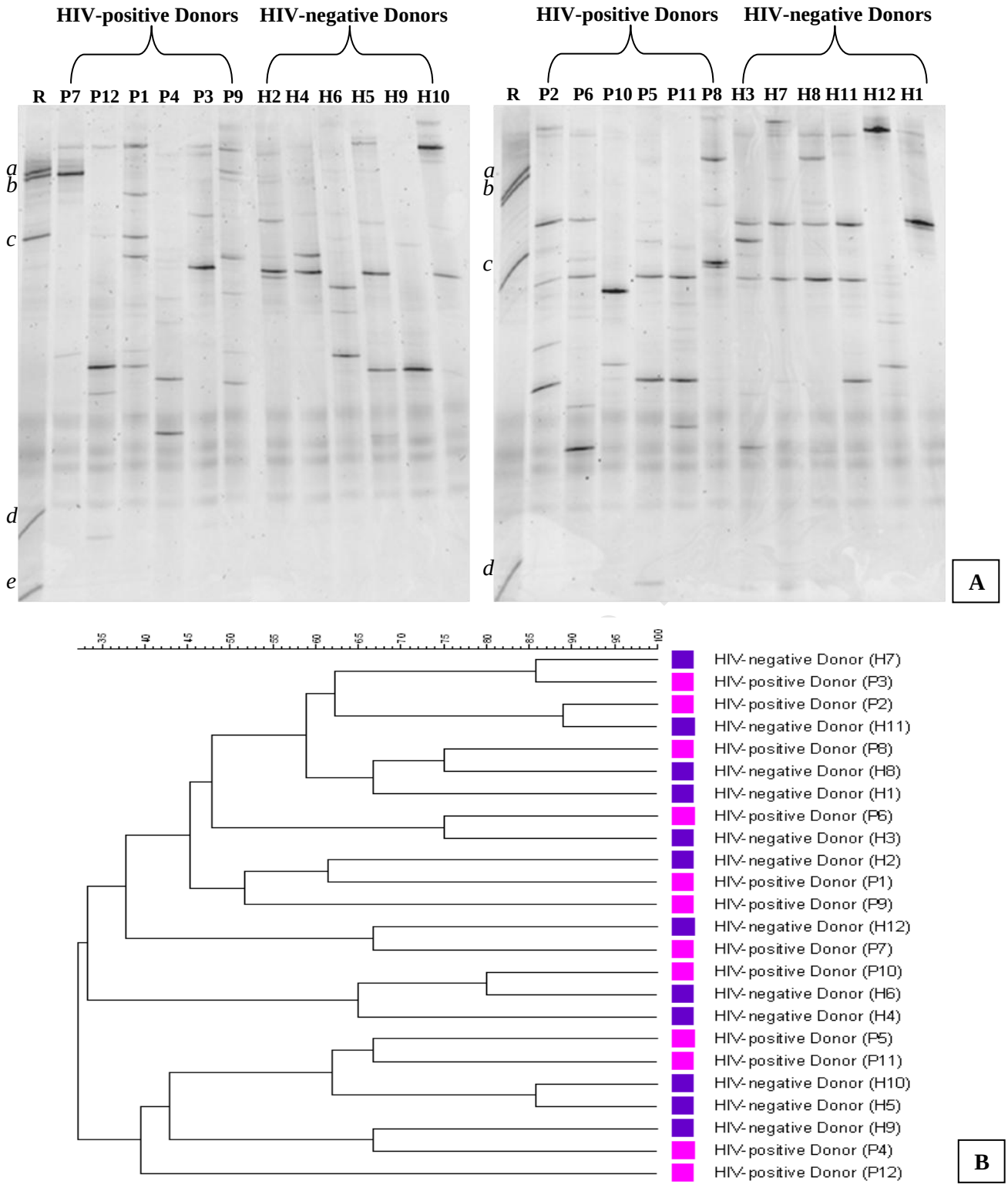



**Figure 3.7:** **DGGE and cluster analysis of the *Lactobacillus* group 16S rRNA genes amplified from the faecal material of 12 HIV-positive and 12 HIV-negative donors. (A)** Lane R: DGGE reference ladder [(*a*): *L. casei*; (*b*): *L. gasseri*; (*c*): *L. brevis*; (*d*): *L. reuteri* and (*e*): *L. fermentum*]. **(B)** The dendrogram was generated using the Dice Similarity coefficient and clustering algorithm UPGMA [(Pink): HIV-positive donor and (Purple): HIV-negative donor]. *Scale bar* indicates similarity (%).

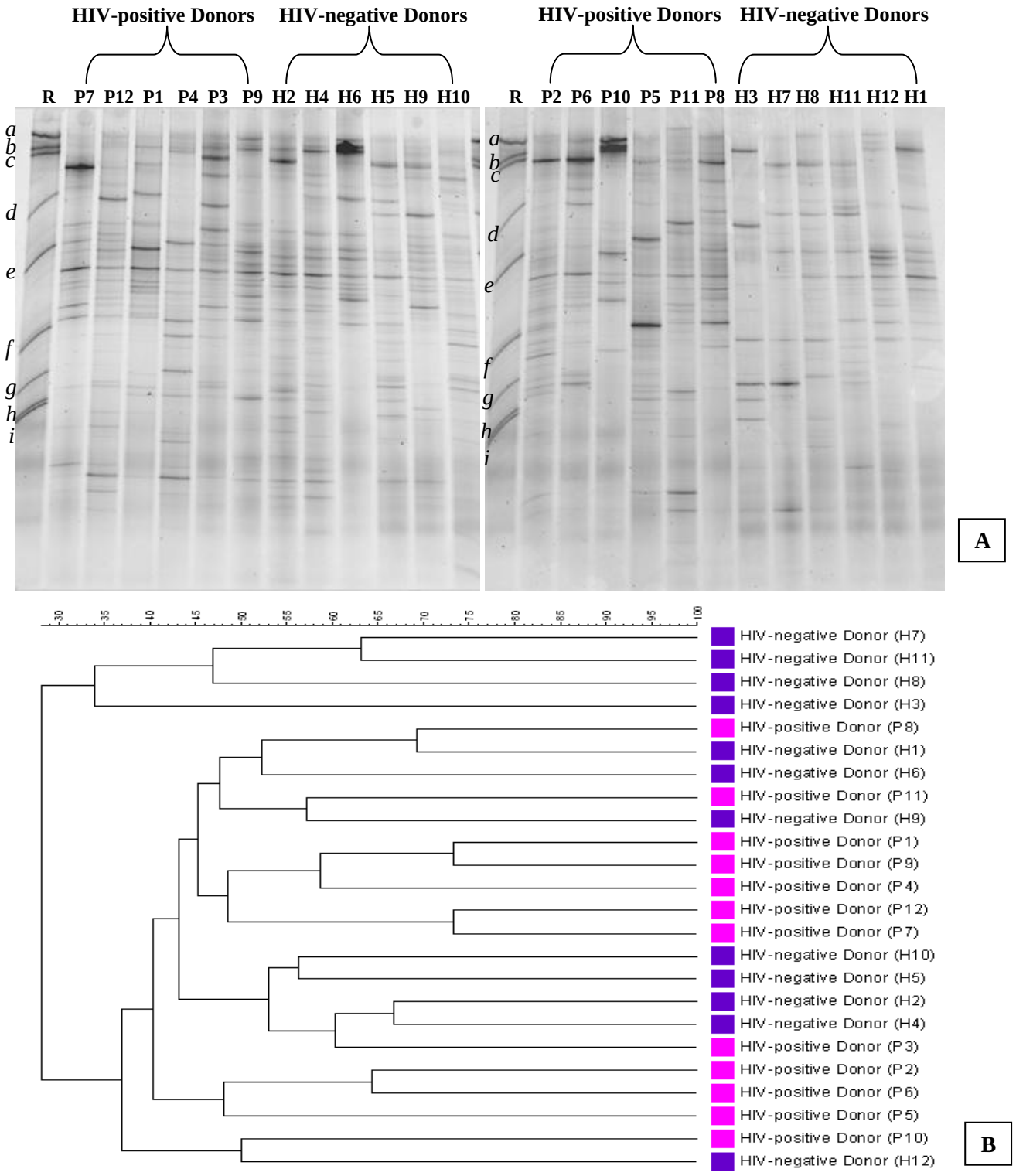



**Figure 3.8:** **Total bacteria DGGE and cluster analysis of 16S rRNA genes amplified from the faecal material of 12 HIV-positive and 12 HIV-negative donors. (A)** Lane R: DGGE reference ladder [(*a*): *B. fragilis*; (*b*): *L. casei*; (*c*): *L. gasseri*; (*d*): *L. brevis*; (*e*): *E. faecalis;* (*f*): *E. coli*; (*g*): *L. reuteri*; (*h*): *C. perfringens* and (*i*): *B. longum*]. **(B)** The dendrogram was generated using the Dice Similarity coefficient and clustering algorithm UPGMA [(Pink): HIV-positive donor and (Purple): HIV-negative donor]. *Scale bar* indicates similarity (%).

In both Figures 3.7A and 3.8A, a distinct common background region situated within the lower part of the gel was observed. The source of this background may be as a result of residual unbound ethidium bromide remaining within the gel after destaining (Vanhoutte *et al*., 2004). Ethidium bromide is capable of binding to both single and double stranded DNA (Waring, 1965), and unbound ethidium bromide has also been shown to have significant intrinsic fluorescence (Singer *et al*., 1999). Other nucleic acid gel stains such as SYBR Green I or SYBR Gold, produce very little background fluorescence when used in the detection of DNA (Vanhoutte *et al*., 2004). Extending destaining of the gel from 30 mins to 60 mins resulted in a reduction in this background region, but with a simultaneous decrease in the intensity of the DGGE bands. The background fluorescence was easily differentiated from the DGGE bands of interest due to its relatively low intensity and position within the gel, thus not influencing the analysis and interpretation of the data.

On the whole, DGGE profiles acquired for all three bacterial groups showed a strong tendency towards being host-specific even though some common bands (bands present at the same positions) were observed for different donors. This particular observation is consistent with what other studies have described (Satokari *et al*., 2001; Walter *et al*., 2001; Zoetendal *et al*., 1998).

The relationship of the DGGE banding profile between the HIV-positive and HIV-negative donors for the three bacterial groups was analysed and displayed as individual dendrograms (Figure 3.6B, Figure 3.7B and Figure 3.8B). The band variation and complex host specificity seen for both the *Lactobacillus* and total bacteria populations meant that no distinct clustering of the donors was seen. This general lack of clustering of donors for the *Lactobacillus* and total bacteria populations has also been reported in studies conducted by Maukonen *et al*., (2008) and Vanhoutte *et al*., (2004). In contrast, the *Bifidobacterium* population displayed strong similarities between the donors, thus enabling the clustering of certain donors. The clustering of donors seen for the *Bifidobacterium* group, however, was not related to the HIV-status of the donors.

### 3.4.3 Shannon-Wiener diversity index

The diversity of a specific population was calculated using the Shannon-Wiener diversity index, which took into account the species richness (number of bands present) as well as the relative intensity of the bands. When the diversity index ($H'$) was determined for the *Bifidobacterium* population, it initially appeared that the diversity was higher in the HIV-positive donor group (0.65) than the HIV-negative group (0.37) (Table 3.3). However, when the Mann-Whitney U Test was carried out to compare these $H'$ values statistically, the result showed that this difference was not statistically significant ($p = 0.10$).

**Table 3.3:** Shannon-Wiener diversity indices calculated for the three investigated bacterial groups

| Bacterial Group | Donor group | Mean ($H'$) | Range of ($H'$) | Statistical Significance |
|---|---|---|---|---|
| *Bifidobacterium* | HIV-positive | 0.65 | 0 – 0.93 | $p = 0.10$ |
| | HIV-negative | 0.37 | 0 – 1.00 | |
| *Lactobacillus* | HIV-positive | 1.06 | 0.36 – 1.71 | $p = 0.49$ |
| | HIV-negative | 1.12 | 0 – 1.73 | |
| Total bacteria | HIV-positive | 1.80 | 1.03 – 2.63 | $p = 0.009$ |
| | HIV-negative | 2.27 | 1.70 – 2.75 | |

The calculated diversity index ($H'$) values for the *Lactobacillus*-group (Table 3.3) showed that the HIV-negative group had a slightly higher mean diversity index (1.12) compared to the HIV-positive donors (1.06). This difference was also not statistically significant when a Mann-Whitney U test was performed ($p = 0.49$). However, when the total bacteria diversity for the two groups was determined (Table 3.3), the results showed that the HIV-positive group had a much lower $H'$ value (1.80) compared to that of the HIV-negative group (2.27). Comparison of these two groups showed that this difference was indeed statistically significant ($p = 0.009$), and that the diversity of the predominant microbiota within the HIV-positive donor group was reduced. This result could potentially indicate that the overall gastrointestinal microbiome of HIV-positive patients has been affected by the presence of the virus.

The box-and-whisker plot shown in Figure 3.9 was generated by taking the Shannon-Wiener diversity index values for all three groups and comparing them in terms of HIV status and gender.

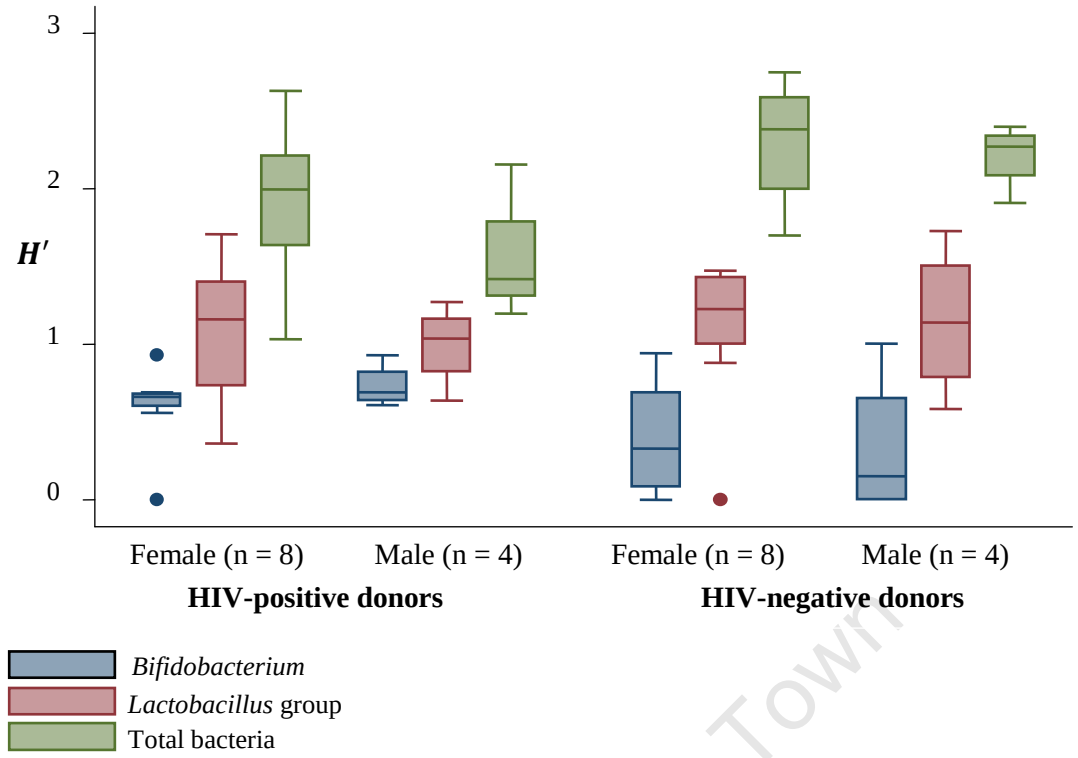



**Figure 3.9:** **Box-and-whisker plot showing the Shannon-Wiener diversity index (*H′*)** from the DGGE profiles of 12 HIV-positive and 12 HIV-negative donors.

Figure 3.9, shows that the overall diversity of total bacteria was higher in the HIV-negative donors, and mostly within the female donors with a median value of 2.30 (quartile ranges 1.70 - 2.75) compared to a median of 2.21 (quartile ranges 1.91 – 2.40) seen in the HIV-negative males. The diversity of the total bacterial population appeared to be reduced within the HIV-positive male donors recording a low median value of 1.55 (quartile ranges 1.19 – 2.16). However, this particular result should be interpreted cautiously due to the fact that the number of male donors participating in this study was extremely small and no statistical correlation could be inferred.

### 3.4.4 Sequence analysis

DGGE was employed to determine the diversity of selected species in the faecal material from both donor groups. The aims of sequencing particular DGGE bands were to evaluate the specificity of the group-specific primers, and to determine whether bands co-migrating with the standards were the expected species. In addition, bands migrating to other positions, within the gel, would also be identified. Bands from both donor groups were excised, cloned and sequenced. Selected DGGE bands found in the same position, but located within

different lanes were also excised to confirm their identity as well as the separation capability of DGGE in this study.

***Bifidobacterium* group:**

Figure 3.10 shows the bands, representing the *Bifidobacterium* population, which were excised and sequenced. Bands 1-7 were confirmed to be *Bifidobacterium* species thus validating the specificity of the genus-specific primers used in the first round of amplification. The presence or absence of these bands in all the donors was evaluated (Figure 3.6 and Table 3.5). Three predominant *Bifidobacterium* species were identified namely, *B. longum*, *B. pseudocatenulatum* and *B. adolescentis* in both the HIV-positive and HIV-negative donor groups (Table 3.4 and Table 3.5). These three *Bifidobacterium* species have previously been reported to be isolated from human faeces (Hopkins and Macfarlane, 2002).

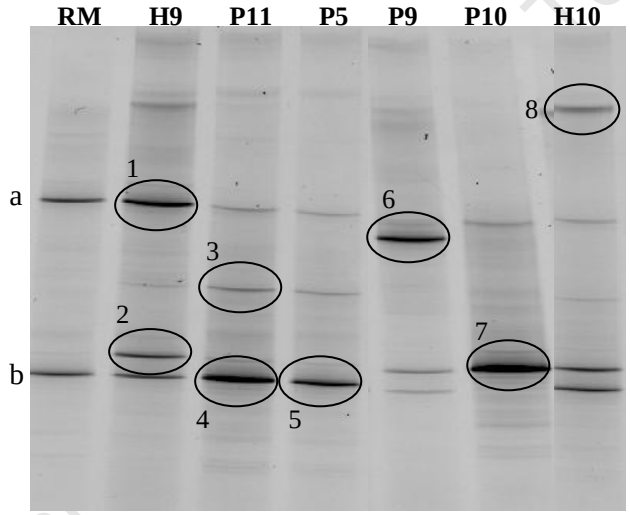



**Figure 3.10:** **Selected bifidobacterial DGGE bands excised for sequencing.** Bands 1 – 8 were excised, cloned and sequenced using the M13F primer. Lane RM: DGGE reference ladder [(a): *B. longum* and (b): *B. adolescentis*]. Composite figure generated using the FPQuest software.

Sequencing of both bands 3 and 4 identified them as *B. adolescentis*. Satokari *et al*., (2001) showed that *B. adolescentis* E-981074$^{T}$ (ATCC 15703$^{T}$) can produce two individual fragments within a DGGE gel on account of it having several 16S rRNA copies (5 copies) with specific sequence differences between these copies. Band 5, at the same position in P5, was similarly identified as *B. adolescentis*. The strong presence of *B. adolescentis* seen in this study, as well as other previously published investigations, shows that this particular species

is one of the most dominant and widely distributed *Bifidobacterium* species found within the intestinal tract of adults (Matsuki *et al*., 2004; Takada *et al*., 2004). *B. longum*, *B. breve* and *B. bifidum* represent the mucosa-adherent members of the *Bifidobacterium* genus, where despite being common faecal isolates, they are also found tightly associated with the human intestinal mucosa (Turroni *et al*., 2009). In this pilot study, *B. longum* appeared to be more predominant in the HIV-positive samples than in the HIV-negative donors (Table 3.5). However, DGGE is a qualitative technique and this observation should be followed up by a more quantitative approach.

**Table 3.4:** Sequence identities of cloned *Bifidobacterium* DGGE bands

| Donor ID | Excised Band | ID based on 16S rRNA gene sequence | % Similarity of sequence | Accession Number |
|---|---|---|---|---|
| **P5** | **5** | *Bifidobacterium adolescentis* ATCC 15703 | 100 | AP009256.1 |
| **P9** | **6** | *Bifidobacterium longum* subsp. *infantis* ATCC 15697 | 99 | CP001095.1 |
| **P10** | **7** | *Bifidobacterium pseudocatenulatum* JCM 1200 | 100 | D86187.1 |
| **P11** | **3** | *Bifidobacterium adolescentis* ATCC 15703 | 100 | AP009256.1 |
| **P11** | **4** | *Bifidobacterium adolescentis* ATCC 15703 | 100 | AP009256.1 |
| **H9** | **1** | *Bifidobacterium longum* NCC2705 | 99 | AE014295.3 |
| **H9** | **2** | *Bifidobacterium pseudocatenulatum* JCM 1200 | 100 | D86187.1 |
| **H10** | **8** | No identifiable sequence obtained | - | - |

A band found directly above the *B. longum* reference marker (band 8) was also sequenced (Figure 3.10). Sequencing of this fragment yielded either a mixed sequence result or an unreadable sequence, suggesting that this band was a heteroduplex complex formed during PCR (Qiu *et al*., 2001; Satokari *et al*., 2001).

**Table 3.5:** Presence of selected *Bifidobacterium* DGGE bands in the two donor groups

| Donor ID | *B. longum* | *B. pseudocatenulatum* | *B. adolescentis* | Donor ID | *B. longum* | *B. pseudocatenulatum* | *B. adolescentis* |
|---|---|---|---|---|---|---|---|
| **P1** | + | - | + | **H1** | - | - | + |
| **P2** | + | + | + | **H2** | + | - | + |
| **P3** | + | - | + | **H3** | - | - | + |
| **P4** | - | - | + | **H4** | + | + | + |
| **P5** | + | - | + | **H5** | - | - | + |
| **P6** | - | + | - | **H6** | + | + | + |
| **P7** | + | + | + | **H7** | + | + | - |
| **P8** | + | - | + | **H8** | - | - | + |
| **P9** | + | - | + | **H9** | + | + | + |
| **P10** | + | + | - | **H10** | + | + | + |
| **P11** | + | - | + | **H11** | + | - | + |
| **P12** | + | - | + | **H12** | - | + | - |

* [+]: Selected DGGE band is present

* [-]: Selected DGGE band is absent

* [P]: HIV-positive donors

* [H]: HIV-negative donors

***Lactobacillus group:***

Sequencing of DGGE bands excised from the *Lactobacillus* group (Figure 3.11) identified species that belonged mostly to the *Lactobacillus*, *Streptococcus* and *Weisella* genera (Table 3.6). In addition, bands 8, 10, 16 and 17, were all found to be closely related to *Eubacterium biforme*, a common constituent of the human gastrointestinal tract. As previously mentioned (section 3.4.1.2.1), the selected primer set used in this study was designed to specifically amplify species belonging to the *Lactobacillus* group. However, Heilig *et al*., (2002) also reported the unintended amplification of *E. biforme* owing to the fact that the 3' end of the Lab0667R primer is complementary to the 16S rRNA sequence of *E. biforme* and *Eubacterium cylindroides*. Analysis of the frequency of occurrence of the various bands in all the donors is complicated by the fact that the primers are not specific for the *Lactobacillus* genus, and bands migrating to the same position are not necessarily the same species (Table 3.6).

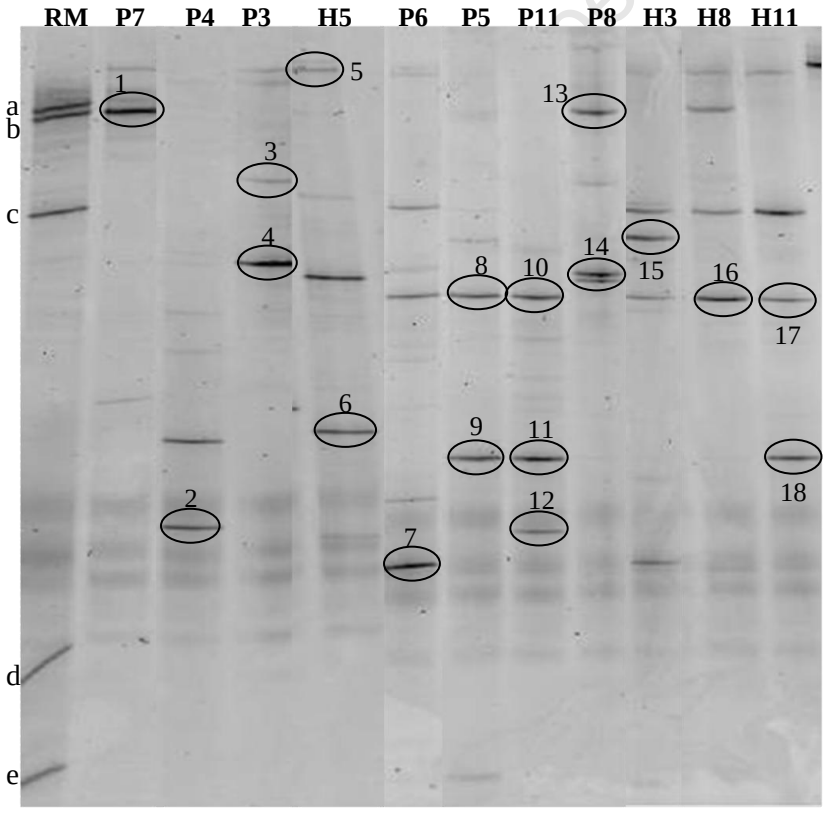



**Figure 3.11:** **Selected *Lactobacillus* group DGGE bands excised for sequencing.** Bands 1 – 18 were excised, cloned and sequenced using the M13F primer. Lane RM: DGGE reference ladder [(a): *L. casei*; (b): *L. gasseri*; (c): *L. brevis*; (d): *L. reuteri* and (e): *L. fermentum*]. Composite figure generated using the FPQuest software.

Identification of the dominant DGGE bands in both donor groups showed identities most similar to *L. ruminis*, *L. reuteri*, *L. jensenii*, *L. rossiae* and *L. crispatus* (Table 3.6). Furthermore, three of the bands sequenced were identified as *Streptococcus thermophilus* (Bands 2 and 12), a member of the Lactic Acid bacteria group found mainly in yogurt and other dairy products (Vodnar *et al*., 2010) and *Weissella confusa* (Band 5), a bacterial species commonly found in food products and to a lesser extent faecal material (Fusco *et al*., 2011). Both *S. thermophilus* and *W. confusa* are associated with beneficial properties such as, inducing the production of different cytokines (Aattouri and Lemonnier, 1997) and reducing the infectivity and persistence of pathogens such as *Helicobacter pylori* (Fusco *et al*., 2011) respectively. Only one of the cloned DGGE bands sequenced (Band 6) was identified as an uncultured bacterium clone originating from a human colon biopsy sample (Table 3.6).

**Table 3.6:** Sequence identities of cloned *Lactobacillus* group DGGE bands

| Donor ID | Excised Band | ID based on 16S rRNA gene sequence | % Similarity of sequence | Accession Number |
|---|---|---|---|---|
| **P3** | **3** | *Lactobacillus mucosae* strain TB-H32 | 100 | AB425938.1 |
| **P3** | **4** | *Lactobacillus rossiae* strain DSM 15814 | 99 | AB370880.1 |
| **P4** | **2** | *Streptococcus thermophilus* ND03 | 99 | CP002340.1 |
| **P5** | **8** | *Eubacterium biforme* strain EBA11-8 | 99 | JF298897.1 |
| **P5** | **9** | *Lactobacillus ruminis* strain SL1090 | 100 | HQ022863.1 |
| **P6** | **7** | *Lactobacillus reuteri* SD2112 | 98 | CP002844.1 |
| **P7** | **1** | *Lactobacillus johnsonii* strain ATCC 33200 | 100 | NR_025273.1 |
| **P8** | **13** | *Lactobacillus sakei* strain SM7 | 100 | HM568887.1 |
| **P8** | **14** | *Lactobacillus jensenii* strain KC36b | 100 | AF243159.1 |
| **P11** | **10** | *Eubacterium biforme* strain EBA11-8 | 98 | JF298897.1 |
| **P11** | **11** | *Lactobacillus ruminis* strain JCM 1152 | 99 | AB289286.1 |
| **P11** | **12** | *Streptococcus thermophilus* ND03 | 99 | CP002340.1 |
| **H3** | **15** | *Lactobacillus crispatus* strain K2-4-3 | 98 | HQ716720.1 |
| **H5** | **5** | *Weissella confusa* partial 16S rRNA clone 3EV3 | 99 | AM117131.1 |
| **H5** | **6** | Uncultured bacterium clone KU74 from human colon biopsy | 98 | AY916140.1 |
| **H8** | **16** | *Eubacterium biforme* strain EBA11-8 | 98 | JF298897.1 |
| **H11** | **17** | *Eubacterium biforme* strain EBA11-8 | 98 | JF298897.1 |
| **H11** | **18** | *Lactobacillus ruminis* strain SL1090 | 100 | HQ022863.1 |

The occurrence of bands at the same positions as those shown in Table 3.6 was evaluated in all donor samples (Figure 3.7), and the results are shown in Table 3.7. However, due to the lack of specificity of the primers, each of these bands would need to be sequenced to confirm their identity. An interesting finding was the identification of a characteristically food-associated *Lactobacillus* species, namely *Lactobacillus sakei* (Table 3.6 and 3.7). Several studies have suggested that the presence of these food-related species in faecal material

demonstrates that certain members of the *Lactobacillus* population are transient in nature, acquired through food eaten by the host, and that only a few *Lactobacillus* species, such as *L. ruminis, L. reuteri, L. gasseri* and *L. salivarius* are considered truly endogenous (Reuter, 2001; Walter *et al*., 2001; Tannock *et al*., 2000).

A band representing *Lactobacillus mucosae* was isolated and sequenced from two of the HIV-positive donors (Table 3.6 and 3.7). Bands in the same position were detected in other donors from both groups, but these should be independently sequenced to confirm their identity. This particular *Lactobacillus* species is of interest since it has been reported to represent approximately 3% of the total lactic acid bacteria population within the colonic mucin, and has been more frequently isolated from gastrointestinal biopsies of healthy adult donors, where it has been shown to have a tight association with the intestinal epithelium (Kinoshita *et al*., 2006). However, the strong presence of *L. mucosae* in the faecal material of patients who have Short Bowel Syndrome (SBS) has been reported by Joly *et al*., (2010). SBS is a condition, characterised by diarrhoea and malabsorption of vitamins and nutrients that can develop in patients suffering from illnesses such as Crohn's Disease, who have undergone a process known as gut re-sectioning. This particular procedure is known to severely modify the lumen of the intestinal tract, and Joly *et al*., (2010) showed that *L. mucosae* was detected only in the samples obtained from the SBS patients and not the healthy control group. It would be interesting to explore the specific occurrence of this bacterium in both the mucosa and faeces of HIV-positive patients as a future extension of this study.

Overall, the sequencing of DGGE bands in conjunction with the cluster analysis (section 3.4.2) and the calculated Shannon-Wiener diversity index (section 3.4.3), revealed that the diversity of the *Lactobacillus* population appeared to be fairly similar in both the HIV-positive and HIV-negative donor groups.

**Table 3.7:** Presence of selected *Lactobacillus* DGGE bands in the two donor groups

| Donor ID | *L. johnsonii* | *S. thermophilus* | *L. reuteri* | *L. rossiae* | *W. confusa* | *L. mucosae* | *E. biforme* | *L. ruminis* | *L. sakei* | *L. jensenii* | *L. crispatus* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **P1** | - | - | - | - | + | - | - | - | - | + | + |
| **P2** | - | - | - | - | + | + | + | + | - | - | - |
| **P3** | - | - | - | + | + | + | - | - | - | - | - |
| **P4** | - | + | + | - | - | - | - | - | - | - | - |
| **P5** | - | - | - | - | - | - | + | + | - | - | + |
| **P6** | - | - | + | - | + | - | + | - | - | + | - |
| **P7** | + | - | - | - | + | - | - | - | - | - | - |
| **P8** | - | - | - | + | - | - | - | - | + | + | - |
| **P9** | + | - | - | - | - | - | - | - | - | + | - |
| **P10** | - | - | - | - | - | - | + | - | - | - | - |
| **P11** | - | + | - | - | - | - | + | + | - | - | - |
| **P12** | - | - | - | - | + | - | - | + | - | - | - |
| **H1** | - | - | - | - | + | - | - | - | - | - | - |
| **H2** | - | - | - | - | - | - | + | - | + | + | - |
| **H3** | - | - | + | - | + | - | + | - | - | - | + |
| **H4** | - | - | - | - | - | - | - | - | - | + | - |
| **H5** | - | - | - | - | + | - | - | - | - | - | - |
| **H6** | - | - | - | - | - | - | - | - | - | - | - |
| **H7** | - | - | - | - | + | - | + | - | - | - | - |
| **H8** | - | - | - | - | + | - | + | - | + | - | - |
| **H9** | - | - | - | - | - | - | - | - | - | - | + |
| **H10** | - | - | - | - | - | - | + | - | - | - | - |
| **H11** | - | - | - | - | + | - | + | + | - | - | - |
| **H12** | - | - | - | - | - | - | - | - | - | - | - |

* [+] Selected DGGE band is present; [-] Selected DGGE band is absent

* [P]: HIV-positive donors; [H]: HIV-negative donors

***Total bacteria:***

A selection of strong bands, representative of co-migrating bands, was made to get an indication of the major species present. A total of 25 different DGGE bands were excised and sequenced from the total bacteria gels (Figure 3.12). However, several of the HIV-negative and positive donors displayed a region comprised of several bands in close proximity to each other, with many of these bands being too close together to be accurately excised. 16S rRNA clonal libraries of this region in H2 and H6 were therefore made to identify the species present (CL1 and CL2 in Figure 3.12). A similar partial 16S rRNA clonal library was not created for any of the HIV-positive donors, but individual bands from these donors, falling within the same region, were amongst the 25 bands excised and sequenced (Figure 3.12). A total of 96 individual clones from the partial clonal libraries were randomly selected and sequenced (Figure 3.13).

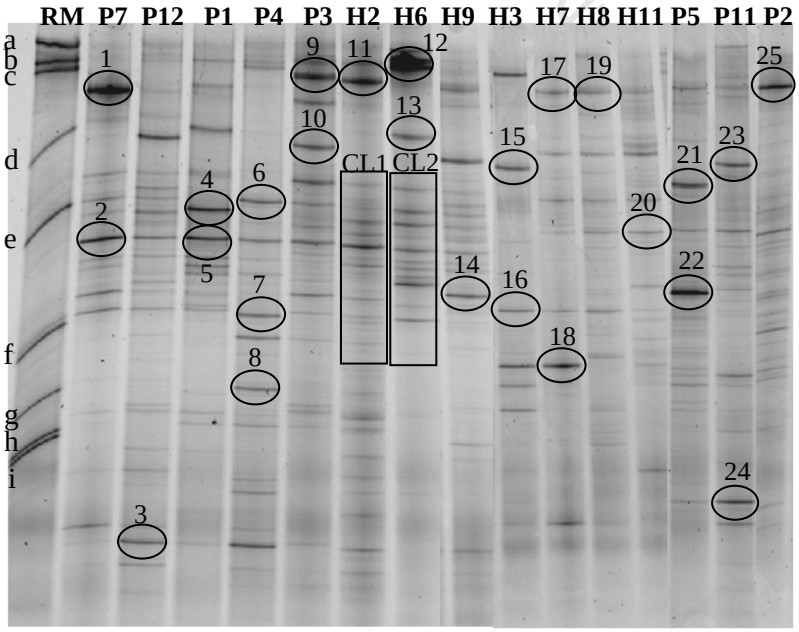



**Figure 3.12:** **Selected total bacteria DGGE bands excised for sequencing.** Bands 1 – 25 were excised, cloned and sequenced using the M13F primer. Lane RM: DGGE reference ladder [(a): *B. fragilis*; (b): *L. casei*; (c): *L. gasseri*; (d): *L. brevis*; (e): *E. faecalis;* (f): *E. coli*; (g): *L. reuteri*; (h): *C. perfringens* and (i): *B. longum*]. Composite figure generated using the FPQuest software. [(CL): Clone library].

A number of bands were identified as species belonging mostly to the *Bacteroides/Prevotella* genera, the *Clostridium coccoides* cluster and the *Clostridium leptum* group (Table 3.8), representing some of the most abundant bacterial groups found within the human intestinal tract. Bands 3, 7, 8, 16 and 20 were identified as *Prevotella copri*, *Alistipes shahii*, *Prevotella stercorea*, *Flavonifractor plautii* and *Clostridium orbiscindens* respectively (Table 3.8).

Sequences derived from unidentified species of the *Clostridium* genus were also detected for a few of the fragments sequenced. *Akkermansia mucinphila*, (band 6) identified in HIV-positive donor P4, is a common inhabitant of the human intestinal tract and plays an important role in the degradation of mucin (Derrien *et al*., 2008; Collado *et al*., 2007; Derrien *et al*., 2004). Bands 23 and 24 present in HIV-positive donor P11 were also excised, cloned and sequenced. However, after a number of sequencing attempts, no identifiable sequence information could be obtained for these bands (Table 3.8).



**Table 3.8:** Sequence identities of cloned total bacteria DGGE bands

| Donor ID | Excised Band | ID based on 16S rRNA gene sequence | % Similarity of sequence | Accession Number |
|---|---|---|---|---|
| **P1** | **4** | *Odoribacter splanchnicus* DSM 20712 | 98 | CP002544.1 |
| **P1** | **5** | *Faecalibacterium prausnitzii* strain ATCC 27768 | 98 | NR_028961.1 |
| **P2** | **25** | *Clostridium sp.* YIT 12069 gene for 16S rRNA | 93 | AB491207.1 |
| **P3** | **9** | *Clostridium sp.* YIT 12069 gene for 16S rRNA | 94 | AB491207.1 |
| **P3** | **10** | *Oscillibacter sp.* G2 16S ribosomal RNA gene | 96 | HM626173.1 |
| **P4** | **6** | *Akkermansia muciniphila* strain ATCC BAA-835 | 98 | CP001071.1 |
| **P4** | **7** | *Alistipes shahii* strain EBA11-1 | 98 | JF298871.1 |
| **P4** | **8** | *Prevotella stercorea* strain CB35 | 100 | AB244774.1 |
| **P5** | **21** | *Ruminococcus* sp. ID1 | 98 | AY960569.1 |
| **P5** | **22** | *Butyrivibrio crossotus* strain DSM2876 | 100 | FR733670.1 |
| **P7** | **1** | *Parasutterella excrementihominis* strain DSM 21040 | 97 | AB370250.1 |
| **P7** | **2** | *Ruminococcus bromii* strain ATCC 27255 | 96 | NR_025930.1 |
| **P11** | **23** | No identifiable sequence obtained | - | - |
| **P11** | **24** | No identifiable sequence obtained | - | - |
| **P12** | **3** | *Prevotella copri* strain CB7 | 96 | AB064923.2 |
| **H2** | **11** | *Clostridium clostridioforme* strain 136069/2010 | 98 | HM008264.1 |
| **H3** | **15** | Uncultured bacterium clone VDRD42cont3 | 98 | JN021854.1 |
| **H3** | **16** | *Flavonifractor plautii* strain aK2 | 96 | HQ455040.1 |
| **H6** | **12** | *Parabacteroides distasonis* ATCC 8503 | 98 | CP000140.1 |
| **H6** | **13** | *Bacteroides vulgatus* ATCC 8482 | 98 | CP000139.1 |
| **H7** | **17** | *Clostridium sp.* AbxANB1 | 98 | JF813180.1 |
| **H7** | **18** | *Succinivibrio dextrinosolvens* strain 0554 | 98 | NR_026476.1 |
| **H8** | **19** | *Clostridium sp.* YIT 12069 gene for 16S rRNA | 93 | AB491207.1 |
| **H9** | **14** | *Eubacterium rectale* ATCC 33656 | 97 | CP001107.1 |
| **H11** | **20** | *Clostridium orbiscindens* strain AIP028.07 | 97 | EU541437.1 |

Upon further examination of the sequencing data (Table 3.8), it was evident that bands 2 and 5; 7 and 16; and 1 and 25, all ran at the same position in the gel, but had different sequence identities. As previously mentioned, this is a frequent problem encountered with the use of PCR-DGGE, particularly when using universal primers; PCR fragments aligning at the same positions do not necessarily prove they are identical or derived from the same species. Depending on the location of sequence differences, it is possible for 16S rRNA gene fragments arising from different strains to migrate to the same position on the gel (Vallaeys *et al*., 1997). In order to do an in-depth analysis of the species present, additional DGGE bands would have to be excised and sequenced to confirm their identity.

The most commonly identified species from the clonal libraries of the CL1 and CL2 regions from HIV-negative donors H2 and H6 are shown in Figure 3.13. The majority of the clones sequenced showed a high level of DNA similarity (96-100%) to *Bacteroides uniformis, Faecalibacterium prausnitzii*, *Roseburia intestinalis*, *Clostridium orbiscindens* and *Eubacterium rectale,* with *Bacteroides* spp. making up 43% of the total isolates. The following species were found in both the partial clonal libraries and the individual HIV-positive bands: *F. prausnitzii*, *Alistipes* spp. and *Clostridium* spp. However, the predominant occurrence of *Bacteroides* spp. found in the HIV-negative donors was not evident in the HIV-positive donors. The following species were not found in the HIV-negative clonal libraries but were seen as individual DGGE bands in the HIV-positive patients in the corresponding region: *Ruminococcus* spp., *Odoribacter splanchnicus*, *Akkermansia muciniphila* and *Butyrivibrio crossotus.*

Many of the species identified in the partial clonal libraries analysed in this study using PCR-DGGE and sequencing were also reported by Qin *et al*., (2010) who used a metagenomic approach. This latter technique would also be useful for future, more extensive studies of HIV-positive and HIV-negative donors. If a clonal library approach is used, full libraries from more of the HIV-negative, as well as the HIV-positive donors should be constructed to obtain a more complete data set.

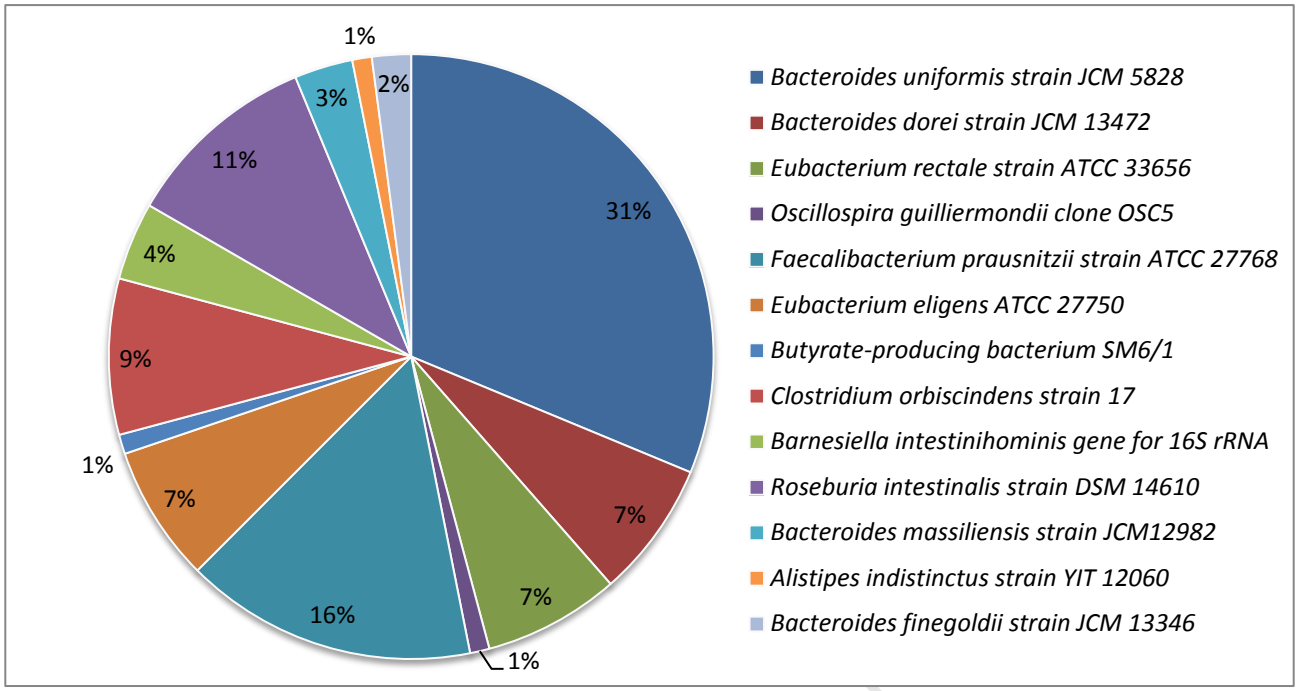



**Figure 3.13:** **Predominant species identified in the partial clonal libraries from the CL1 and CL2 regions from donors H2 and H6**. Percentage occurrence within 96 clones sequenced is shown.



## 3.5 CONCLUSION

Despite the fact that knowledge regarding the gastrointestinal tract microbiota is continually increasing and previously unidentified species are being detected, very little is known about the endogenous microbiota within the gastrointestinal tract of HIV-positive patients. The main objective of the work presented in this chapter was to determine the diversity of the total endogenous microbiota and to evaluate whether there were any noticeable differences between the HIV-positive and HIV-negative donor groups with respect to the diversity of *Lactobacillus* and *Bifidobacterium* spp.

The results from the DGGE analyses clearly suggested that the HIV-positive donors had a significantly lower diversity in the total bacteria present, but there were no significant differences with respect to the diversity of the *Bifidobacterium* and *Lactobacillus* groups. This was an interesting finding since it showed that these latter groups were not disrupted in any major way in HIV-positive patients, and that the prescribing of these bacteria (commonly found in commercial probiotic preparations) as probiotics would not necessarily aid HIV recovery. The findings also pointed the way toward the investigation of other protective gut

species (e.g. *Bacteroides*). This was done later in this study using qPCR since PCR-DGGE is not a quantitative technique (Chapter 5).

As a scientific approach, the use of DGGE, together with cluster analysis and the Shannon-Wiener diversity index provided useful data in analysing the relationships and differences that exist within complex microbial communities. The DGGE profiles generated showed that although some common bands were shared between the two donor groups, a unique microbial profile in terms of composition and diversity was displayed for each individual.

However, the usefulness of the PCR-DGGE technique can be limited by several technical factors (see section 3.2), and the results obtained may not necessarily give an accurate representation of the diversity of a particular species present within the gastrointestinal tract. In this study, several of the main sources of technical bias were eliminated. Careful optimisation of the cell lysis procedure was done through the introduction of a bead-beating step, and a commercial DNA purification kit was used for extraction of the genomic DNA from faecal samples, which has been independently evaluated and shown to be 94% efficient in extracting DNA from all faecal bacteria (Yoshikawa *et al*., 2011). The PCR conditions used were also optimised.

The development of group-specific primers for use in DGGE has led to easier detection and identification of bacterial species present in much smaller numbers within the intestinal tract. However, as was shown in this and several other studies, published *Lactobacillus*-specific primers are not entirely genus-specific, and primer Lab0067R has been found to amplify up not only *Lactobacillus* species, but species belonging to other phylogenetically related genera. The presence of multiple copies of the 16S rRNA gene can also affect the DGGE result obtained for a specific population, as was the case in this study for *B. adolescentis*, which produced more than one DGGE band. These sources of error have been considered when interpreting the data in the current pilot study. It is clear that alternative approaches should be used in future studies to test the validity of the results, and take this research forward.

One such approach would be to use a gene target other than the 16S rRNA gene. Requena *et al*., (2002) and Rantsiou *et al*., (2004) both showed that alternative gene targets, such as the transaldolase gene and the *rpo*B gene could be successfully used in the PCR-DGGE identification of both *Bifidobacterium* and *Lactobacillus* species respectively. Alternatively,

the use of RNA as opposed to DNA targets, has also been suggested. This has the added advantage of increasing the sensitivity and specificity of the reaction, while also providing a more accurate representation of the targeted bacterial community (Matsuda *et al*., 2009).

Another approach of achieving a more accurate result would be to use a more quantitative technique, such as FISH, qPCR, high-throughput sequencing or metagenomics. Over the past several years, the use of metagenomics has grown and has been applied to the study of microbial populations in different ecosystems, such as the gastrointestinal tract. This has resulted in the characterisation and identification of new previously unidentified species. It is also becoming an affordable research option, where it was formerly too expensive (Caporaso *et al*., 2011). In the context of the current study reported here, these methods would serve as the next step in acquiring a more robust profile of the intestinal microbiota of HIV-positive and HIV-negative donors.

The strong association of specific bacterial species with the intestinal epithelial lining suggests a significant interaction between the host and bacteria. As an extension of this current research, future studies could compare the composition of bacterial species found attached to the lining of the intestinal tract of HIV-positive patients, as a means of determining the actual state of the mucosal lining. To conduct such a study, biopsy samples would be required. However, the challenges faced in following this course of invasive action for this type of project are significant as was previously discussed in Chapter 2.

The administration of HAART to HIV-positive patients results in significant reductions in the viral load, with a simultaneous increase in the individual's $CD_4^+$ T cell count. However, a question that needs to be addressed is whether the initiation of HAART itself exerts any effect on the gut microbiota. The condition of the gastrointestinal microbiota prior to the initiation of HAART has been described in Chapter 3. This provides a basis on which the diversity and dynamics of the *Bifidobacterium* and *Lactobacillus* populations, both important probiotic groups, within HIV-positive donors receiving HAART could be determined. This qualitative longitudinal investigation monitored the diversity of these two bacterial groups in eight HIV-positive donors over a 6-month period using PCR-DGGE analysis. This study is described in Chapter 4.

# CHAPTER FOUR

## PCR-DGGE analysis of *Bifidobacterium* and *Lactobacillus* species in South African HIV-positive donors prior to and during HAART

### Contents

## 4.1 SUMMARY

Recent virological investigations have shown that HIV replication and $CD_4^+$ T cell depletion continues to take place within the GALT of HIV-positive patients during HAART. These observations suggest that the gastrointestinal tract might remain in a compromised state. It is not known whether the endogenous microbiota are disrupted during HAART, and whether *Bifidobacterium* and *Lactobacillus* species are still present to provide a protective function to the host. Therefore, the diversity of the *Bifidobacterium* and *Lactobacillus* populations present in the faecal material of eight South African HIV-positive donors and one HIV-negative donor was characterised, using PCR-DGGE during the first 6 months of HAART. Analysis of the PCR-DGGE results revealed that the *Bifidobacterium* population remained fairly stable within individual donors over time with no significant inter-donor variation throughout the longitudinal study. There was a strong predominance of *B. adolescentis* and *B. longum* throughout the course of the study. The *Lactobacillus* population showed greater variation of species within individual donors, as well as between donors, but this was not significantly linked to HAART. One of the recruited HIV-positive donors (P5) received multiple antibiotics during this study, resulting in a marked reduction in the diversity of the *Bifidobacterium* and *Lactobacillus* groups present. The fact that no significant changes were observed in these bacterial probiotic groups over the 6 month treatment period investigated indicates that neither the presence of HIV nor the use of HAART adversely affected their diversity.

## 4.2 INTRODUCTION

The efficacy of HAART is clinically determined by monitoring two important factors, namely, suppression of viral load, and an increase in $CD_4^+$ T cells in the peripheral blood (Wood *et al*., 2005). Even though the gastrointestinal tract is one of the earliest sites of HIV replication, knowledge regarding viral suppression, restoration of the mucosal immune system and the overall microbial conditions found within the intestinal tract during HAART administration is limited.

A longitudinal study conducted by Guadalupe *et al*., (2003) attempted to determine and evaluate the dynamics of viral suppression, $CD_4^+$ T cell restoration, and regulation of gene expression in both blood and biopsy samples obtained from HIV-positive patients who had initiated HAART during either a primary (4 weeks after exposure) or chronic infection (8 months to 1 year after exposure). The results of this particular study provided evidence of ongoing viral replication, accompanied by slow and incomplete restoration of $CD_4^+$ T cells within the GALT of HIV-positive patients, possibly contributing to a continual loss of $CD_4^+$ T cells from the mucosal tissue. Immunologic analysis revealed that the majority of repopulating $CD_4^+$ T cells, detected within the gastrointestinal tract, all appeared to express HIV co-receptors CCR5 and CXCR4, which ultimately could result in them again being targets for infection by HIV. Furthermore, after one year of HAART, restoration of $CD_4^+$ T cells within the GALT remained slow, while elevated $CD_8^+$ T cell levels were recorded suggesting that T-cell homeostasis had not yet been normalised. Guadalupe *et al*., (2003) concluded that, even after 24 months of HAART, only a minimal $CD_4^+$ T cell restoration had occurred, and that poor $CD_4^+$ T cell restoration in patients was associated with an increased expression of genes responsible for inflammation, cell injury and apoptosis.

Consequently, in light of the above research, the continuous replication of HIV together with the delayed restoration of $CD_4^+$ T cells within the GALT results in conflicting reports between conditions in the gastrointestinal tract and the peripheral blood. It has thus been proposed that, for treatment purposes, as well as for monitoring the efficiency of HAART, both blood and conditions within the gastrointestinal tract need to be monitored, in order to achieve a more accurate overview of disease progression and mucosal immune restoration. It was also suggested by Guadalupe *et al*., (2003) that access of HAART to the GALT may not be as efficient as in the blood or other lymphoid tissue, which could also be contributing towards the incomplete suppression of viral replication during HAART.

Extensive temporal based studies have been conducted to determine the effect of external factors on the endogenous microbiota of the gut, such as treatment with broad spectrum antibiotics (Dethlefsen *et al*., 2008; De La Cochetière *et al*., 2005), intestinal disorders (Mättö *et al*., 2005), and nutritional and dietary influences (Spor *et al*., 2011). Stability of the gastrointestinal microbiota has been examined over several weeks, months or even a year by means of culture-based techniques (Finegold *et al*., 1983), PCR-DGGE (Vanhoutte *et al*., 2004; Zoetendal *et al*., 2001; Zoetendal *et al*., 1998) or FISH (Franks *et al*., 1998). Temporal based studies that have been previously conducted have shown that both the faecal *Bifidobacterium* and *Bacteroides* populations are relatively stable (Maukonen *et al*., 2008; Vanhoutte *et al*., 2004), while the *Lactobacillus* community tends to be more unstable with substantial variations (Maukonen *et al*., 2008; Vanhoutte *et al*., 2004).

Current research studies strongly point towards a significant delay in the improvement of conditions within the gastrointestinal tract, even when HAART has been initiated. This particular outcome tends to suggest that the endogenous intestinal microbiota may continuously be subjected to disruption, which possibly negatively affects the beneficial bacterial groups, resulting in them no longer being able to provide a beneficial function to the host.

In Chapter 3 of this study, it was established that, in spite of the chronic and advanced nature of the infections in the HIV-positive patients, there was no significant difference in the diversity of their *Bifidobacterium* and *Lactobacillus* populations as compared to the HIV-negative control donors prior to the initiation of HAART. There was, however, a statistically significant lower diversity of the total bacteria in the HIV-positive donors. The research described in this chapter aimed at establishing whether the existing *Bifidobacterium* and *Lactobacillus* populations in the HIV-positive patients were affected in any way during six months of HAART administration. These two groups were targeted (rather than the general total bacteria) because of their well-established protective and probiotic potential. It was decided that the nature of the observed changes in the total bacterial population would best be addressed using qPCR (Chapter 5) in order to specifically investigate the numbers of a larger range of specific bacterial groups in the gut microbiota of HIV-positive patients.

## 4.3 MATERIALS AND METHODS

Faecal samples were provided by 8 HIV-positive donors at specific time points, for the duration of the 6 month longitudinal study, resulting in a total of 40 samples being collected. Sampling time points for donors (donors P2, P3, P4, P5, P7, P8, P10 and P11) were: prior to HAART (T0), and then 2 weeks (T1), 6 weeks (T2), 3 months (T3) and 6 months (T4) after HAART initiation. Donor P 11, however, could not be sampled at T3 due to non-attendance at the HIV clinic. In some instances, an additional 12 month (T5) faecal sample was provided by four of the participating longitudinal donors (P2, P5, P8 and P10). In addition, five faecal samples were collected at the same time points from one HIV-negative donor (donor H3) over the 6 month longitudinal study. Total bacterial DNA was extracted from all faecal samples using the ZR Faecal DNA Kit™ (ZYMO Research), and all materials and methods (PCR-DGGE, statistical and sequence analyses) employed were identical to those described in Chapter 3.

## 4.4 RESULTS AND DISCUSSION

### 4.4.1 Longitudinal DGGE analysis of *Bifidobacterium* species

The 6 month longitudinal DGGE profiles of the *Bifidobacterium* population from 8 HIV-positive donors (Figure 4.1A – H) and one HIV-negative donor (H3) (Figure 4.1I) were included in this longitudinal study, to establish the temporal stability of the *Bifidobacterium* and *Lactobacillus* populations during HAART.

The *Bifidobacterium* population detected using primers Bif164F and Bif662R (Chapter 3), appeared relatively stable throughout the longitudinal study, indicative of the presence of a core, host specific *Bifidobacterium* community in each of the 8 HIV-positive donors (Figure 4.1A - H). In most cases (7 out of 8 donors), 2 to 3 dominant DGGE bands were detected and identified as *B. longum, B. adolescentis* and *B. pseudocatenulatum* (Table 4.1). HIV-positive donors P3 (Figure 4.1B), P7 (Figure 4.1E), P8 (Figure 4.1F) and P11 (Figure 4.1H) all showed a strong predominance of both *B. adolescentis* and *B. longum* throughout the 6 month study (Table 4.2). HIV-positive donor P11 missed the 3 month sampling appointment (Chapter 2), which resulted in no faecal sample, $CD_4^+$ T cell count or clinical data being collected at this point. HIV-positive donor P10 (Figure 4.1G) was unique when compared to the other donors, in that a strong and stable *B. pseudocatenulatum* population was observed, with an absence of *B. adolescentis*. *B. longum* and *B. adolescentis* are both major species

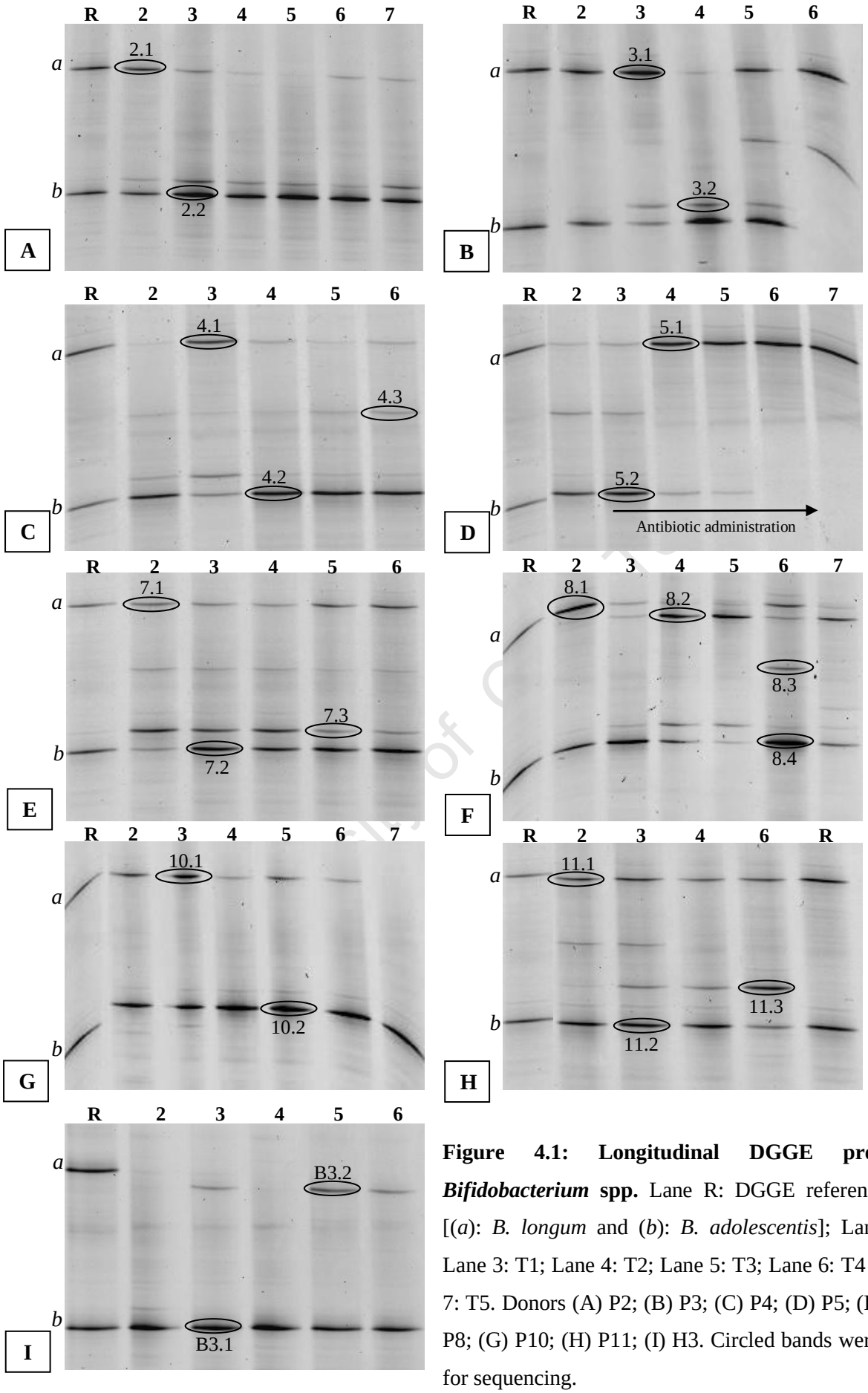



**Figure 4.1: Longitudinal DGGE profile of *Bifidobacterium* spp.** Lane R: DGGE reference ladder [(*a*): *B. longum* and (*b*): *B. adolescentis*]; Lane 2: T0; Lane 3: T1; Lane 4: T2; Lane 5: T3; Lane 6: T4 and Lane 7: T5. Donors (A) P2; (B) P3; (C) P4; (D) P5; (E) P7; (F) P8; (G) P10; (H) P11; (I) H3. Circled bands were excised for sequencing.

associated with the mucosal surfaces of the intestinal tract (Turroni *et al*., 2009; Kleessen *et al*., 2003). The presence of these two species within the faecal material could be as a result of the disruption caused to the gastrointestinal mucosa due to continued HIV replication. *Bifidobacterium* species may be detected more frequently in HIV-positive donors if they are unable to adhere to the disrupted gastrointestinal tract, but continue to multiply in the gut lumen and are shed in the faeces. This hypothesis would require testing by investigating intestinal epithelial biopsy samples from HIV-positive patients for the presence or absence of *Bifidobacterium* species.

**Table 4.1:** Sequence identities of cloned *Bifidobacterium* DGGE bands from longitudinal study

| Donor ID | Excised Band | ID based on 16S rRNA gene sequence | % Similarity of sequence | Accession Number |
|---|---|---|---|---|
| **P2** | **2.1** | *Bifidobacterium longum* NCC2705 | 99 | AE014295.3 |
| **P2** | **2.2** | *Bifidobacterium adolescentis* ATCC 15703 | 100 | AP009256.1 |
| **P3** | **3.1** | *Bifidobacterium longum* NCC2705 | 99 | AE014295.3 |
| **P3** | **3.2** | *Bifidobacterium pseudocatenulatum* JCM 1200 | 100 | D86187.1 |
| **P4** | **4.1** | *Bifidobacterium longum* NCC2705 | 99 | AE014295.3 |
| **P4** | **4.2** | *Bifidobacterium adolescentis* ATCC 15703 | 100 | AP009256.1 |
| **P4** | **4.3** | *Bifidobacterium adolescentis* ATCC 15703 | 100 | AP009256.1 |
| **P5** | **5.1** | *Bifidobacterium longum* NCC2705 | 99 | AE014295.3 |
| **P5** | **5.2** | *Bifidobacterium adolescentis* ATCC 15703 | 100 | AP009256.1 |
| **P7** | **7.1** | *Bifidobacterium longum* NCC2705 | 99 | AE014295.3 |
| **P7** | **7.2** | *Bifidobacterium adolescentis* ATCC 15703 | 100 | AP009256.1 |
| **P7** | **7.3** | *Bifidobacterium pseudocatenulatum* JCM 1200 | 100 | D86187.1 |
| **P8** | **8.1** | *Bifidobacterium longum* NCC2705 | 99 | AE014295.3 |
| **P8** | **8.2** | *Bifidobacterium longum* subsp. *infantis* ATCC 15697 | 99 | CP001095.1 |
| **P8** | **8.3** | *Bifidobacterium adolescentis* ATCC 15703 | 100 | AP009256.1 |
| **P8** | **8.4** | *Bifidobacterium adolescentis* ATCC 15703 | 100 | AP009256.1 |
| **P10** | **10.1** | *Bifidobacterium longum* NCC2705 | 99 | AE014295.3 |
| **P10** | **10.2** | *Bifidobacterium pseudocatenulatum* JCM 1200 | 100 | D86187.1 |
| **P11** | **11.1** | *Bifidobacterium longum* NCC2705 | 99 | AE014295.3 |
| **P11** | **11.2** | *Bifidobacterium adolescentis* ATCC 15703 | 100 | AP009256.1 |
| **P11** | **11.3** | *Bifidobacterium adolescentis* ATCC 15703 | 100 | AP009256.1 |
| **H3** | **B3.1** | *Bifidobacterium adolescentis* ATCC 15703 | 100 | AP009256.1 |
| **H3** | **B3.2** | *Bifidobacterium longum* subsp. *infantis* ATCC 15697 | 99 | CP001095.1 |

HIV-positive donor P2 (Figure 4.1A) showed a stable *Bifidobacterium* population, with no major changes being observed, even though this donor had fallen pregnant between sampling time points T4 and T5. The substantial decrease in the $CD_4^+$ T cell count shown in Figure 4.2A was thought to be as a result of the pregnancy and not an increase in HIV replication within the blood (discussed in Chapter 2).

**Table 4.2:** Presence of selected *Bifidobacterium* DGGE bands in donors P2-P11 and H3 throughout the 6 month longitudinal study

| Donor ID | *Time Point* | *B. longum* | *B. pseudocatenulatum* | *B. adolescentis* |
|---|---|---|---|---|
| **P2** | T0 | + | + | + |
| | T1 | + | + | + |
| | T2 | + | + | + |
| | T3 | - | + | + |
| | T4 | + | + | + |
| | T5 | + | + | + |
| **P3** | T0 | + | - | + |
| | T1 | + | + | + |
| | T2 | + | + | + |
| | T3 | + | + | + |
| | T4 | + | - | - |
| **P4** | T0 | + | + | + |
| | T1 | + | + | + |
| | T2 | + | - | + |
| | T3 | + | - | + |
| | T4 | + | - | + |
| **P5** | T0 | + | - | + |
| | T1 | + | - | + |
| | T2 | + | - | + |
| | T3 | + | - | + |
| | T4 | + | - | - |
| | T5 | + | - | - |
| **P7** | T0 | + | + | + |
| | T1 | + | + | + |
| | T2 | + | + | + |
| | T3 | + | + | + |
| | T4 | + | + | + |
| **P8** | T0 | + | - | + |
| | T1 | + | + | + |
| | T2 | + | + | + |
| | T3 | + | + | + |
| | T4 | + | + | + |
| | T5 | + | + | + |
| **P10** | T0 | + | + | - |
| | T1 | + | + | - |
| | T2 | + | + | - |
| | T3 | + | + | - |
| | T4 | + | + | - |
| | T5 | - | + | - |
| **P11** | T0 | + | - | + |
| | T1 | + | - | + |
| | T2 | + | - | + |
| | T4 | + | - | + |
| **H3** | T0 | - | - | + |
| | T1 | + | - | + |
| | T2 | - | - | + |
| | T3 | + | - | + |
| | T4 | + | - | + |

* [+]: Selected DGGE band is present; [-]: Selected DGGE band is absent

* [P]: HIV-positive donors; [H]: HIV-negative donors

The longitudinal DGGE profile obtained for HIV-positive donor P5 (Figure 4.1D) showed a reduction in *B. adolescentis* and a subsequent increase in the abundance of *B. longum*, following HAART initiation and treatment with multiple antibiotics. The multiple antibiotics administered to donor P5, consisted of ciprofloxacin (250 mg) at sampling time point T1, cefixime (400 mg) and doxycycline (100 mg) at T2 and T3 and cefixime (400 mg), doxycycline (100 mg) and metronidazole (400 mg) at T4 (discussed in Chapter 2). Recent investigations have shown that, the *Bifidobacterium* population is generally susceptible to a number of antibiotics including cephalosporins, penicillins, macrolides (Mättö *et al*., 2008), and doxycycline (Saarela *et al*., 2007). However, Moubareck *et al*., (2005) reported an increase in the resistance of *B. longum* to cefoxitin (a second generation cephalosporin), tetracycline and metronidazole. This particular trend of increasing resistance shown in *B. longum* species should be noted, especially in HIV-positive patients where treatment with multiple antibiotics is sometimes required.

In a study conducted by Dethlefsen and Relman, (2010), various different measures of biological diversity, acquired during a longitudinal investigation, were plotted and compared to each other for individual donors. In order to assess whether HAART had any impact on microbial diversity over time, the Shannon-Wiener diversity index was determined for each of the sampling time points [blue lines], and was plotted together with the $CD_4^+$ T cell counts [red squares] provided by the clinic (Chapter 2) for each of the donors (Figure 4.2A-H). When assessing the Shannon-Wiener diversity indices for all eight HIV-positive donors, no distinctive common trend was observed that could be attributed to HAART administration. For the most part, donors showed some variations in the diversity of the *Bifidobacterium* group over time, but these were specific for each donor and were not statistically significant. HIV-positive donor P5 ($H'$ = 0.72 at T0 relative to $H'$ = 0 at T5) received multiple antibiotics which eliminated *B. adolescentis* (Figure 4.2D). It is interesting to note that donor P10 (Figure 4.2.G) ($H'$ = 0.61 at T0 relative to $H'$ = 0 at T5) had less *B. adolescentis* and *B. longum* after HAART, with *B. pseudocatenulatum* becoming the dominant species. The exact reasons for this are not known. Throughout the 6 month study period, a gradual increase in $CD_4^+$ T cell numbers, relative to the individual starting values, was seen following the initiation of HAART in all of the HIV-positive donors (Figure 4.2A–H). The only variation in this trend was seen in donor P2, who became pregnant at T3, resulting in a decrease in the $CD_4^+$ T cell numbers. Even so, the $CD_4^+$ T cell level was still higher than before HAART. The increase in $CD_4^+$ T cell numbers, along with a significant reduction in the HIV viral load within the blood, indicates that the ARV

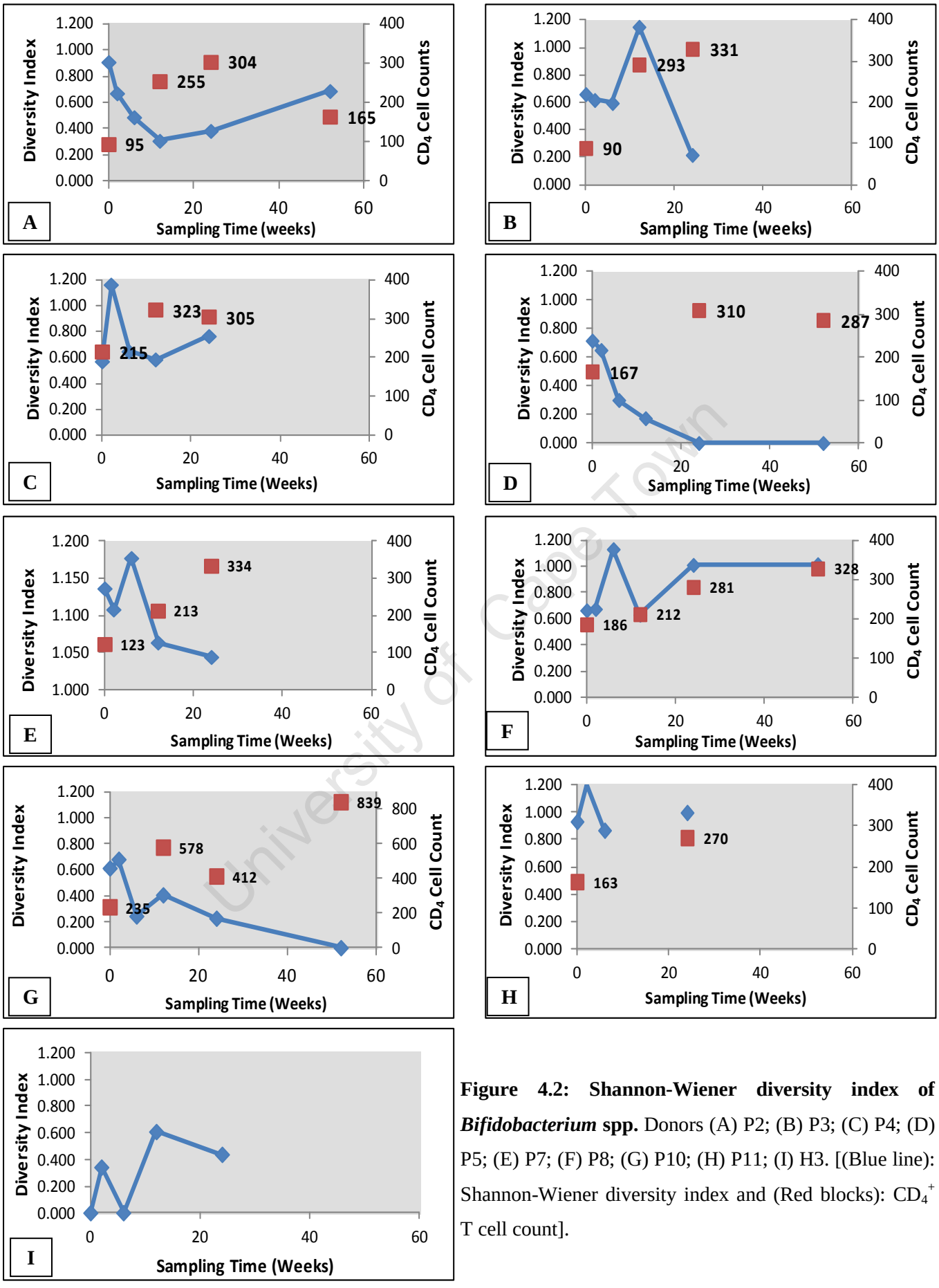



**Figure 4.2: Shannon-Wiener diversity index of *Bifidobacterium* spp.** Donors (A) P2; (B) P3; (C) P4; (D) P5; (E) P7; (F) P8; (G) P10; (H) P11; (I) H3. [(Blue line): Shannon-Wiener diversity index and (Red blocks): $CD_4^+$ T cell count].

regime prescribed for each donor is effective. Nevertheless, it is important to remember that this particular result is an indication of conditions within the blood only, and not the gastrointestinal tract (Guadalupe *et al*., 2006).

The *Bifidobacterium* DGGE profile obtained for the single longitudinal HIV-negative donor H3 was generally stable, with no significant changes in the diversity occurring during the 6 month period (Figure 4.1I). *B. adolescentis* was detected throughout the 6 months, whilst *B. longum* was only detected at sampling points T1, T3 and T4 (Table 4.1 and 4.2). The absence of *B. longum* could be due to the fact that *B. longum* is adhering to the healthy, intact gastric mucosa of donor H3. He *et al*., (2001), reported that the adhesion of *B. longum* within the gastrointestinal tract is strain-specific, in that not all *B. longum* species will adhere strongly to the mucosa, and adhesion is also influenced by factors such as substrate availability (mucins or fructans). Kleessen *et al*., (2003) suggested that changes in the mucin composition of the gastrointestinal tract, caused by intestinal diseases, could prevent *Bifidobacterium* species from adhering, thereby affecting the composition and diversity of this group. Therefore, in light of the fact that the replication of HIV within the gastrointestinal tract creates a compromised mucosa, it may be possible that this disruption could be affecting the adherence of *Bifidobacterium* species to the gastrointestinal tract. This would result in species transiting through the intestinal tract, and higher numbers being detected within the faecal material of HIV-positive donors. However, this conclusion should be viewed with caution due to the fact that only one healthy control was available for comparison in this study, and additional healthy, HIV-negative controls would be needed to validate this conclusion, together with analysis of gut epithelial biopsies.



### 4.4.2 Longitudinal DGGE analysis of *Lactobacillus* species

Figure 4.3(A-H) shows the longitudinal DGGE profiles of the *Lactobacillus* population during 6 months of HAART, along with the Shannon-Wiener Diversity Index and $CD_4^+$ T cell counts plots (Figure 4.4A-H). Longitudinal PCR-DGGE analysis of the *Lactobacillus* population revealed a strong host specificity in all 8 HIV-positive donors. All of the eight investigated HIV-positive donors exhibited their own distinctive *Lactobacillus* population with one or two species remaining dominant throughout the 6 months (Figure 4.3A-H and Table 4.3). Most of these patients showed variation in the *Lactobacillus* population over time, with P2, P4 and P7 maintaining a more stable profile (see Figure 4.6A, 4.6C and 4.6E). The temporal changes seen

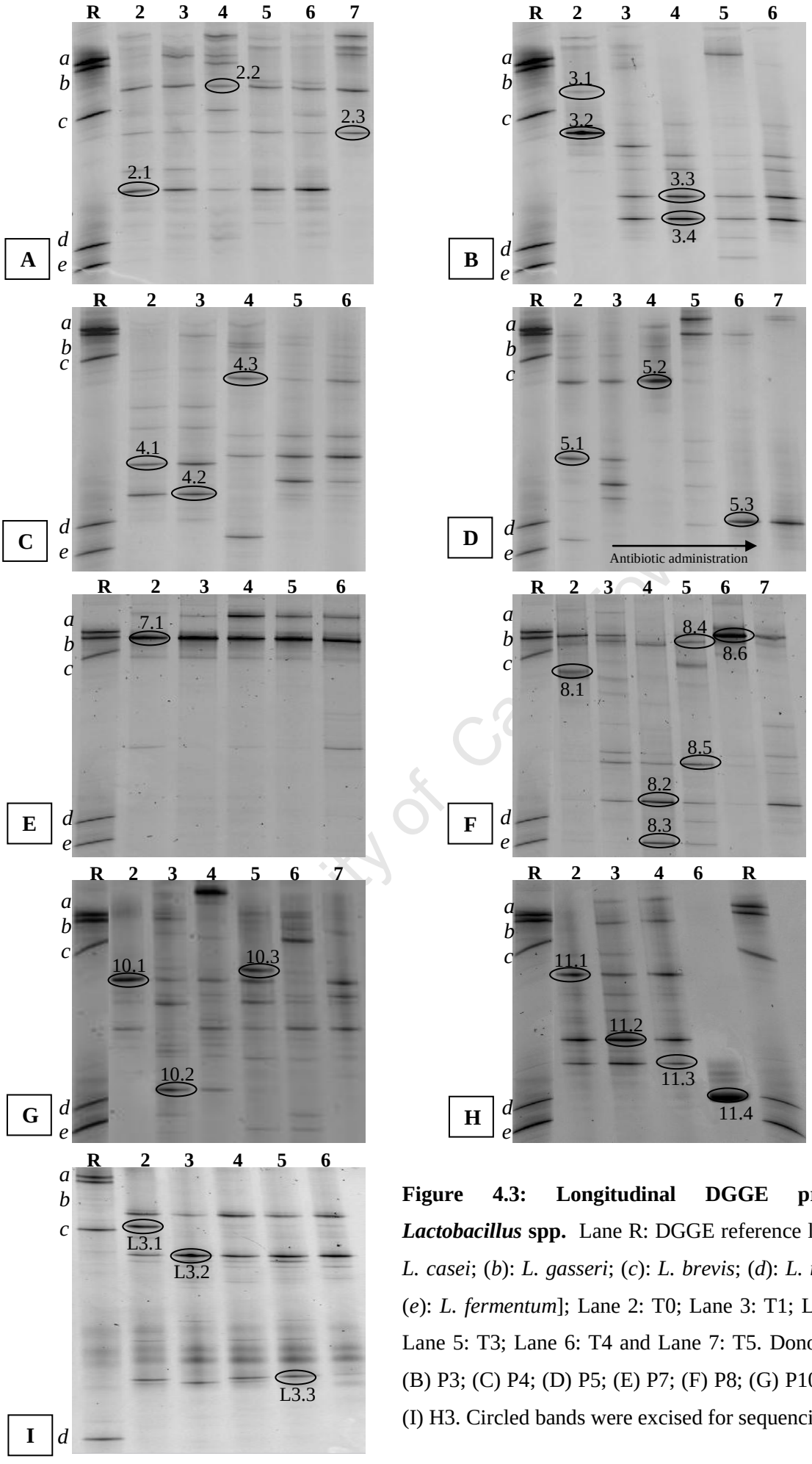



**Figure 4.3: Longitudinal DGGE profile of *Lactobacillus* spp.** Lane R: DGGE reference ladder [(*a*): *L. casei*; (*b*): *L. gasseri*; (*c*): *L. brevis*; (*d*): *L. reuteri* and (*e*): *L. fermentum*]; Lane 2: T0; Lane 3: T1; Lane 4: T2; Lane 5: T3; Lane 6: T4 and Lane 7: T5. Donors (A) P2; (B) P3; (C) P4; (D) P5; (E) P7; (F) P8; (G) P10; (H) P11; (I) H3. Circled bands were excised for sequencing.

for the *Lactobacillus* group is consistent with the findings from a previous longitudinal PCR-DGGE study conducted by Vanhoutte *et al*., (2004) on four healthy volunteers. Employing primers Univ341F and Lab0677R, as discussed in Chapter 3, resulted in the identification of not only *Lactobacillus* species, but also *S. thermophilus* and *E. biforme* (Table 4.3). Several food-associated *Lactobacillus* species were detected in some donors, and included species such as *L. sakei*, *L. delbrueckii* subsp. *bulgaricus*, *L. fermentum*, and *L. brevis*.

A relatively diverse but stable DGGE profile was obtained for the *Lactobacillus* group present in HIV-positive donor P2 (Figure 4.3A). Interestingly, donor P2 was the only donor that exhibited a strong *L. mucosae* presence throughout the longitudinal study, including the 12 month sampling time point, although it was transiently seen in P3 (Table 4.4A). As previously mentioned in Chapter 3, *L. mucosae* has been characterised as a mucosa-associated species, frequently identified in biopsy samples obtained from healthy adult volunteers (Joly *et al*., 2010). The presence of this bacterium in the faecal samples from two of the HIV-positive donors may possibly indicate that conditions within the gastrointestinal tract are in a disrupted state. Carroll *et al*., (2010), conducted a study in which they investigated the luminal and mucosal-associated intestinal microbiota in patients with diarrhoea-predominant irritable bowel syndrome (D-IBS). Their findings demonstrated a significant increase in the levels of *Lactobacillus* species in faecal samples from D-IBS patients compared to the healthy controls. However, in the context of the current study reported in this thesis, the conclusion stated above is based on the results for a limited number of HIV-positive donors. Validation of this hypothesis would require examination of faecal and biopsy samples from more HIV-positive donors. Donor P2 became pregnant during the study, but no significant diversity changes between T4 and T5 were observed, indicating that pregnancy did not influence the *Lactobacillus* composition of this patient.

Another interesting result was recorded for the longitudinal DGGE profile of HIV-positive donor P3 (Figure 4.3B). This particular donor showed a completely different *Lactobacillus* profile after the initiation of HAART, which seemed to remain stable throughout the remainder of the sampling period, accompanied by a fairly consistent increase in $CD_4^+$ T cells.

The effect of multiple antibiotics, namely, ciprofloxacin, cefixme, doxycycline and metronidazole, on the diversity and composition of the *Lactobacillus* population was evident in HIV-positive donor P5 (Figure 4.3D). Common gastrointestinal species *L. ruminis* and *E.*

*biforme* were both completely reduced by T2 while, at sampling time points T4 and T5, a strong *L. reuteri* presence was identified and maintained. A study conducted by Temmerman *et al*., (2003b) reported that certain probiotic *Lactobacillus* strains showed resistance to tetracycline (29.5%), chloramphenicol (8.5%) and erythromycin (12%). Interestingly, HIV-positive donor P5 received 100 mg of doxycycline, a member of the tetracycline antibiotic group, from T2 up until T4. Temmerman *et al*., (2003b) evaluated six *L. reuteri* strains for their susceptibility to tetracycline and all six strains were found to be highly resistant. This particular finding might explain the emergence of *L. reuteri* as the dominant species in P5.

**Table 4.3:** Sequence identities of cloned *Lactobacillus* group DGGE bands from longitudinal study

| Donor ID | Excised Band | ID based on 16S rRNA gene sequence | % Similarity of sequence | Accession Number |
|---|---|---|---|---|
| **P2** | **2.1** | *Lactobacillus ruminis* strain SL1090 | 100 | HQ022863.1 |
| **P2** | **2.2** | *Lactobacillus mucosae* strain CCUG 43179 | 99 | NR_024994.1 |
| **P2** | **2.3** | *Eubacterium biforme* strain EBA11-8 | 98 | JF298897.1 |
| **P3** | **3.1** | *Lactobacillus mucosae* strain CCUG 43179 | 99 | NR_024994.1 |
| **P3** | **3.2** | *Lactobacillus rossiae* strain DSM 15814 | 99 | AB370880.1 |
| **P3** | **3.3** | *Lactobacillus* sp. KLDS 1.0716 | 100 | EU600921.1 |
| **P3** | **3.4** | *Streptococcus thermophilus* ND03 | 99 | CP002340.1 |
| **P4** | **4.1** | *Lactobacillus reuteri* JCM 1112 | 96 | AP007281.1 |
| **P4** | **4.2** | *Streptococcus thermophilus* ND03 | 99 | CP002340.1 |
| **P4** | **4.3** | *Eubacterium biforme* strain EBA11-8 | 98 | JF298897.1 |
| **P5** | **5.1** | *Lactobacillus ruminis* strain SL1090 | 100 | HQ022863.1 |
| **P5** | **5.2** | *Eubacterium biforme* strain EBA11-8 | 99 | JF298897.1 |
| **P5** | **5.3** | *Lactobacillus reuteri* JCM 1112 | 97 | AP007281.1 |
| **P7** | **7.1** | *Lactobacillus johnsonii* strain ATCC 33200 | 100 | NR_025273.1 |
| **P8** | **8.1** | *Lactobacillus jensenii* strain KC36b | 100 | AF243159.1 |
| **P8** | **8.2** | *Lactobacillus ruminis* ATCC 27782 | 99 | CP003032.1 |
| **P8** | **8.3** | *Lactobacillus fermentum* strain 4353A2 | 98 | HM173677.1 |
| **P8** | **8.4** | *Lactobacillus brevis* strain k-0004 | 98 | JF520563.1 |
| **P8** | **8.5** | *Lactobacillus gallinarum* strain ATCC 33199 | 99 | NR_042111.1 |
| **P8** | **8.6** | *Lactobacillus sakei* strain SM7 | 100 | HM568887.1 |
| **P10** | **10.1** | *Eubacterium biforme* strain EBA11-8 | 98 | JF298897.1 |
| **P10** | **10.2** | *Lactobacillus paracasei* strain T2-2 | 100 | AB368899.1 |
| **P10** | **10.3** | *Lactobacillus delbrueckii* subsp. *bulgaricus* ND02 | 100 | CP002341.1 |
| **P11** | **11.1** | *Eubacterium biforme* strain EBA11-8 | 98 | JF298897.1 |
| **P11** | **11.2** | Bacterium La3 16S ribosomal RNA gene | 99 | AY766419.1 |
| **P11** | **11.3** | *Lactobacillus ruminis* strain SL1090 | 99 | HQ022863.1 |
| **P11** | **11.4** | *Lactobacillus paracasei* strain p-027 | 100 | JF520605.1 |
| **H3** | **L3.1** | *Lactobacillus crispatus* strain K2-4-3 | 98 | HQ716720.1 |
| **H3** | **L3.2** | *Eubacterium biforme* strain EBA11-8 | 98 | JF298897.1 |
| **H3** | **L3.3** | *Lactobacillus reuteri* SD2112 | 98 | CP002844.1 |

**Table 4.4A:** Presence of selected *Lactobacillus* DGGE bands in donors P2-P5 throughout the 6 month longitudinal study

| Donor ID | Time Point | *L. john.* | *S. ther.* | *L. reut.* | *L. ros.* | *L. par.* | *L. muc.* | *E. bif.* | *L. rum.* | *L. sak.* | *L. jen.* | *L. cris.* | *L. ferm.* | *L. delb.* | *L. brev.* | *L. gall.* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **P2** | T0 | - | - | - | - | - | + | + | + | - | - | - | - | - | - | - |
| | T1 | - | - | - | - | - | + | + | + | - | - | - | - | - | - | - |
| | T2 | - | - | - | - | - | + | + | + | - | - | - | - | - | - | - |
| | T3 | - | - | - | - | - | + | + | + | - | - | - | - | - | - | - |
| | T4 | - | - | - | - | - | + | + | + | - | - | - | - | - | - | - |
| | T5 | - | - | - | - | - | + | + | - | - | - | - | - | - | - | - |
| **P3** | T0 | - | - | - | + | - | + | - | - | - | - | - | - | - | - | - |
| | T1 | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | T2 | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | T3 | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | T4 | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **P4** | T0 | - | + | + | - | - | - | - | - | - | - | - | - | - | - | - |
| | T1 | - | + | + | - | - | - | - | - | - | - | - | - | - | - | - |
| | T2 | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - |
| | T3 | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - |
| | T4 | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - |
| **P5** | T0 | - | - | - | - | - | - | + | + | - | - | - | - | - | - | - |
| | T1 | - | - | - | - | - | - | + | + | - | - | - | - | - | - | - |
| | T2 | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - |
| | T3 | - | - | + | - | - | - | - | + | - | - | - | - | - | - | - |
| | T4 | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - |
| | T5 | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - |

* [+] Selected DGGE band is present; [-] Selected DGGE band is absent and * [P]: HIV-positive donors; [H]: HIV-negative donors

* Strain Abbreviations: [ *L. john]: L. johnsonii;[ S. ther]: Streptococcus thermophilus;[ L. reut]: L. reuteri;[ L. par]: L. paracasei;[ L. muc]: L. mucosae; [E. bif]: E. biforme; [L. rum]: L. ruminis; [L. sak]: L. sakei; [L. jen]: L. jensenii; [L. cris]: L. crispatus; [L. ferm]: L. fermentum; [L. delb]: L. delbrueckii; [L. brev]: L. brevis and [L. gall]: L. gallinarum.*

**Table 4.4B:** Presence of selected *Lactobacillus* DGGE bands in donors P7-P11 and H3 throughout the 6 month longitudinal study

| Donor ID | Time Point | *L. john.* | *S. ther.* | *L. reut.* | *L. ros.* | *L. par.* | *L. muc.* | *E. bif.* | *L. rum.* | *L. sak.* | *L. jen.* | *L. cris.* | *L. ferm.* | *L. delb.* | *L. brev.* | *L. gall.* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **P7** | T0 | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | T1 | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | T2 | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | T3 | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | T4 | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **P8** | T0 | - | - | - | - | - | - | - | - | + | + | - | - | - | - | - |
| | T1 | - | - | - | - | - | - | - | + | + | - | - | - | - | + | + |
| | T2 | - | - | - | - | - | - | - | + | - | - | - | + | - | + | + |
| | T3 | - | - | - | - | - | - | - | + | - | - | - | + | - | + | + |
| | T4 | - | - | - | - | - | - | - | + | + | - | - | - | - | - | - |
| | T5 | - | - | - | - | - | - | - | + | + | - | - | - | - | - | - |
| **P10** | T0 | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - |
| | T1 | - | - | - | - | + | - | + | - | - | - | - | - | - | - | - |
| | T2 | - | - | - | - | + | - | + | - | - | - | - | - | - | - | - |
| | T3 | - | - | - | - | - | - | + | - | - | - | - | - | + | - | - |
| | T4 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | T5 | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - |
| **P11** | T0 | - | - | - | - | - | - | + | + | - | - | - | - | - | - | - |
| | T1 | - | - | - | - | - | - | + | + | - | - | - | - | - | - | - |
| | T2 | - | - | - | - | - | - | + | + | - | - | - | - | - | - | - |
| | T4 | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - |
| **H3** | T0 | - | - | + | - | - | - | + | - | - | - | + | - | - | - | - |
| | T1 | - | - | + | - | - | - | + | - | - | - | - | - | - | - | - |
| | T2 | - | - | + | - | - | - | + | - | - | - | - | - | - | - | - |
| | T3 | - | - | + | - | - | - | + | - | - | - | - | - | - | - | - |
| | T4 | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - |

* [+] Selected DGGE band is present; [-] Selected DGGE band is absent and * [P]: HIV-positive donors; [H]: HIV-negative donors

* Strain Abbreviations: [ *L. john]: L. johnsonii;[ S. ther]: Streptococcus thermophilus;[ L. reut]: L. reuteri;[ L. par]: L. paracasei;[ L. muc]: L. mucosae; [E. bif]: E. biforme; [L. rum]: L. ruminis; [L. sak]: L. sakei; [L. jen]: L. jensenii; [L. cris]: L. crispatus; [L. ferm]: L. fermentum; [L. delb]: L. delbrueckii; [L. brev]: L. brevis and [L. gall]: L. gallinarum.*

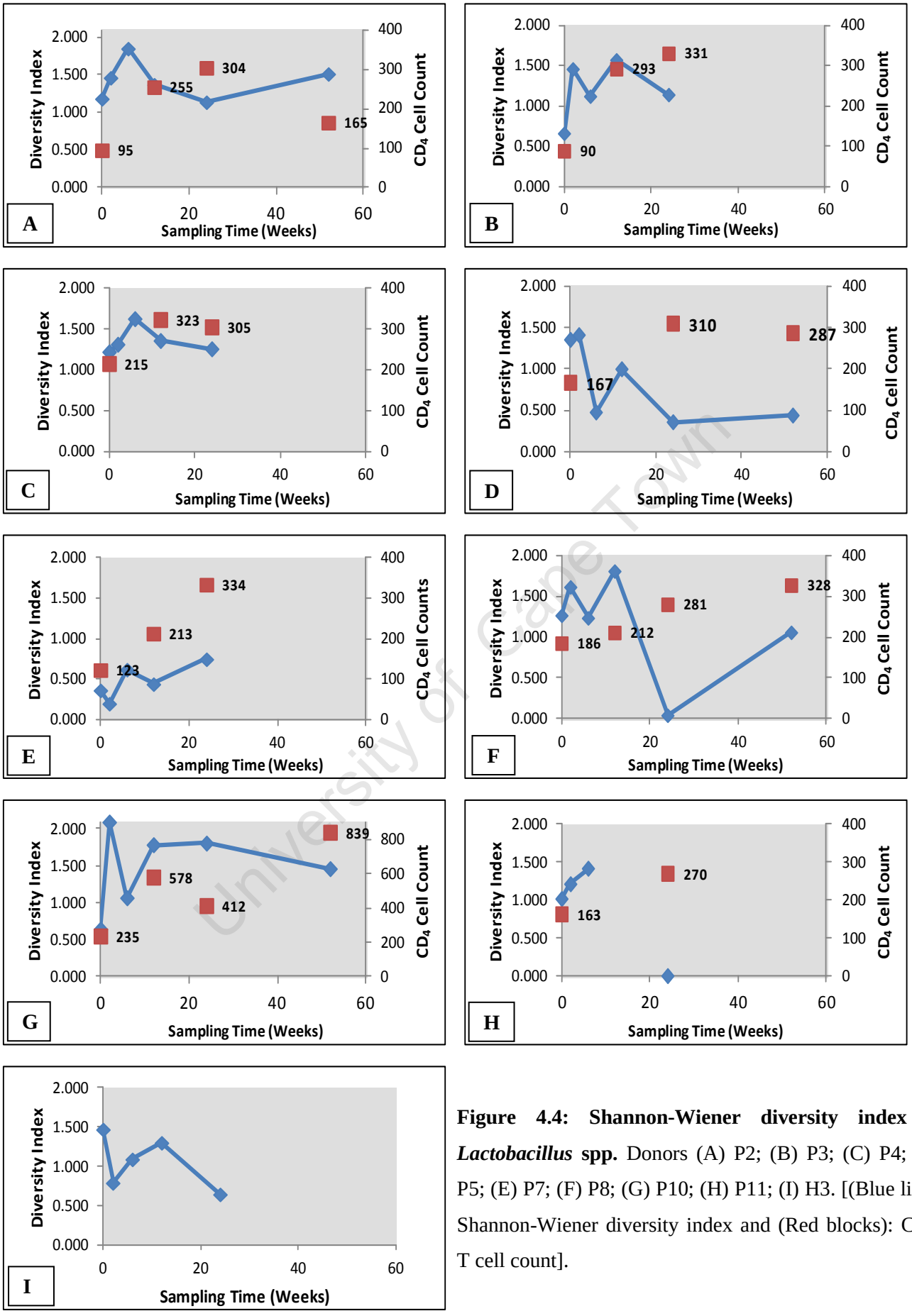



**Figure 4.4: Shannon-Wiener diversity index of *Lactobacillus* spp.** Donors (A) P2; (B) P3; (C) P4; (D) P5; (E) P7; (F) P8; (G) P10; (H) P11; (I) H3. [(Blue line): Shannon-Wiener diversity index and (Red blocks): $CD_4^+$ T cell count].

HIV-positive donor P7 (Figure 4.3E) exhibited a simple *Lactobacillus* population in comparison to the more complex DGGE profiles shown for donors P8 (Figure 4.3F), P10 (Figure 4.3G) and P11 (Figure 4.3H). When referring to the donor demographic data received from the Groote Schuur clinic (Chapter 2), HIV-positive donor P7 was the youngest female donor to participate in this study (aged 21), and the only apparent similarity between donors P8, P10 and P11, was the fact that they were all males. A noticeable alteration to the *Lactobacillus* population of donor P11 (Figure 4.3H) occurred between sampling time point T2 and T4. However, because the T3 sample was not provided, and no clinical data was available for donor P11 at T3, no reliable conclusion regarding the cause of this alteration could be made.

The *Lactobacillus* profile for H3 (Figure 4.3I) showed, at T0, a unique and donor specific profile, but differed slightly from the profiles seen for the HIV-positive donors in that the *Lactobacillus* group appeared to be less complex with fewer variations being evident over the 6 month period (Figure 4.4I). Two out of the four bands detected were present throughout the 6 month period and showed a high level of similarity to *L. reuteri* (band L3.3) and *E. biforme* (band L3.2) (Table 4.3). The findings reported in this chapter showed that, despite certain *Lactobacillus* strains persisting throughout the longitudinal sampling period, there was some variability in the diversity of the *Lactobacillus* group over the 6 months (Figure 4.4A-H). This was, however, not linked to HAART administration.



### 4.4.3 Longitudinal cluster analysis of *Bifidobacterium* and *Lactobacillus* species

The *Bifidobacterium* population in all 8 HIV-positive donors (Figure 4.5A and B) tended to show a very stable and established community throughout the 6 month longitudinal study. This was not affected in a temporal way by the administration of HAART. In contrast, the diversity of the *Lactobacillus* community (Figure 4.6A and B) throughout the 6 months of HAART showed some variation, which is an accurate reflection of the fluctuating and transient nature of *Lactobacillus* species within the intestinal tract, of healthy adult subjects (Vanhoutte *et al*., 2004). The variation, however, did not reflect a correlation with the administration of HAART. In order for a more objective conclusion regarding stability to be made, this study needs to be enlarged further thereby allowing more statistical and cluster analyses to be performed, and reducing any experimental scatter or inter-subject variation from the analysis.

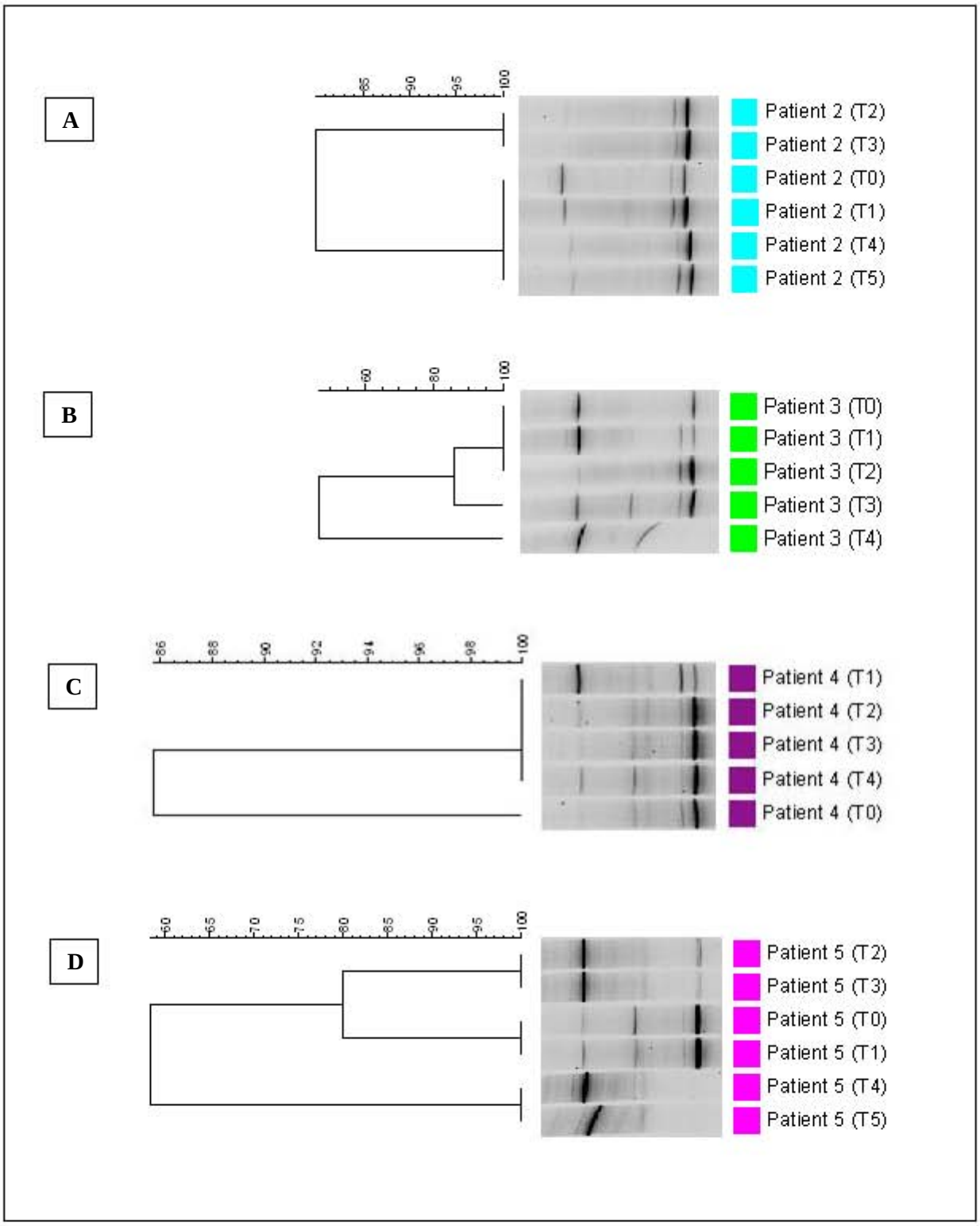



**Figure 4.5A:** **DGGE and cluster analysis of bifidobacterial 16S rRNA genes amplified from the faecal material of 4 HIV-positive donors over a 6-12 month longitudinal study.** The dendrogram was generated using the Dice Similarity coefficient and clustering algorithim UPGMA. Donors: (A) P2; (B) P3; (C) P4; (D) P5.

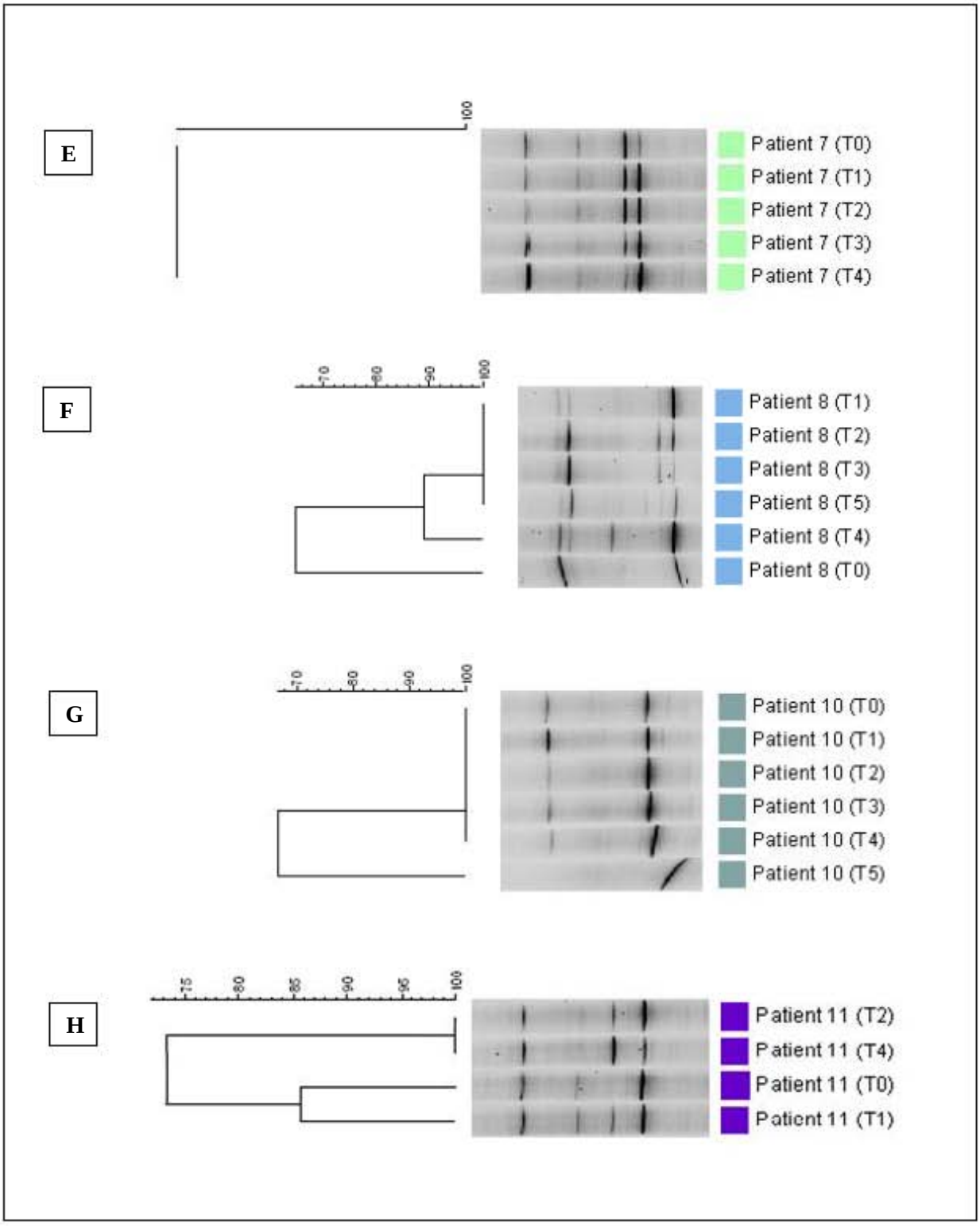



**Figure 4.5B:** **DGGE and cluster analysis of bifidobacterial 16S rRNA genes amplified from the faecal material of 4 HIV-positive donors over a 6-12 month longitudinal study.** The dendrogram was generated using the Dice Similarity coefficient and clustering algorithim UPGMA. Donors: (E) P7; (F) P8; (G) P10; (H) P11.

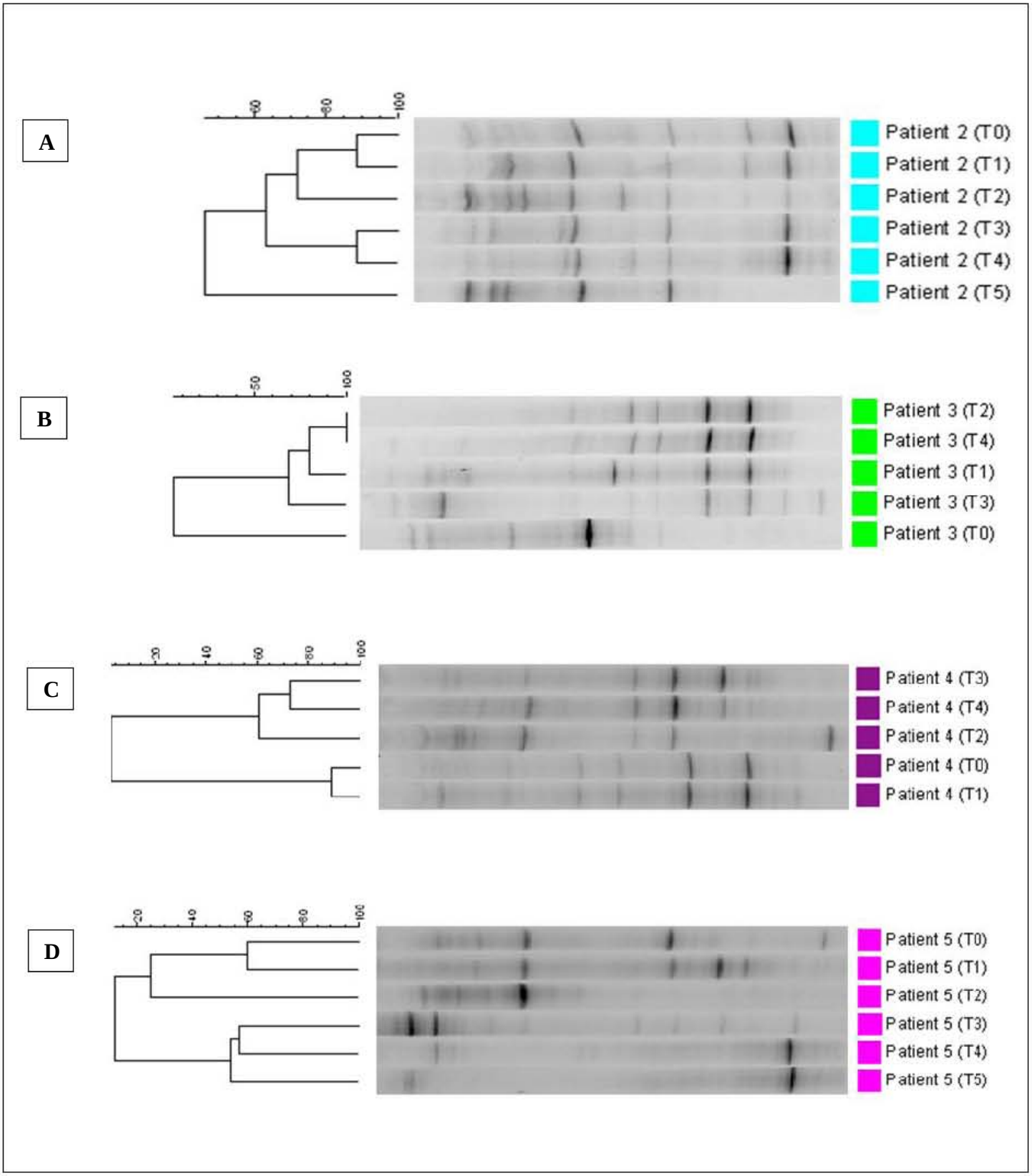



**Figure 4.6A:** **DGGE and cluster analysis of the *Lactobacillus* group 16S rRNA genes amplified from the faecal material of 4 HIV-positive donors over a 6-12 month longitudinal study.** The dendrogram was generated using the Dice Similarity coefficient and clustering algorithim UPGMA. Donors: (A) P2; (B) P3; (C) P4; (D) P5.

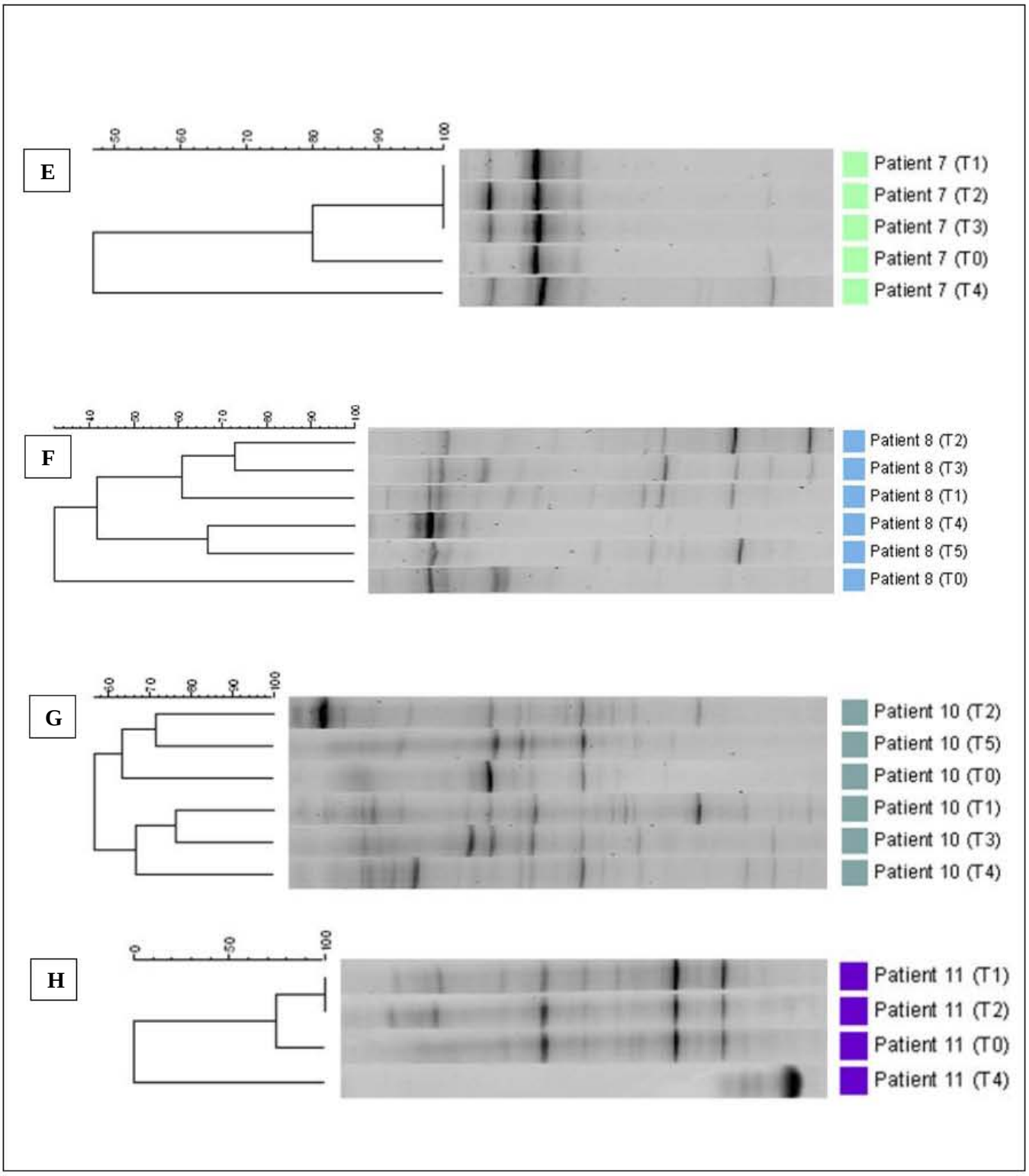



**Figure 4.6B: DGGE and cluster analysis of the *Lactobacillus* group 16S rRNA genes amplified from the faecal material of 4 HIV-positive donors over a 6-12 month longitudinal study.** The dendrogram was generated using the Dice Similarity coefficient and clustering algorithim UPGMA. Donors: (E) P7; (F) P8; (G) P10; (H) P11.

## 4.5 CONCLUSION

The overall objective of this chapter was to ascertain whether the diversity of the known beneficial bacterial groups, *Bifidobacterium* and *Lactobacillus* was affected during 6 months of HAART. The findings of this chapter showed that no significant changes occurred in either of these populations during HAART, and that any alterations seen were specific for that particular donor. In general, however, the *Bifidobacterium* population tended to be less diverse, but temporally more stable than the *Lactobacillus* population.

A direct comparison between HIV-positive donors receiving the same ARV regime and *Bifidobacterium* and *Lactobacillus* species present was not conducted. Carrying out this particular comparison would have been complicated in that, although several HIV-positive donors were on identical ARV treatments, they all had unique and very different characteristics including, age, gender, race and previous medical conditions, all of which could play a role in influencing the endogenous gastrointestinal microbiota.

The administration of multiple antibiotics over a 22 week period in HIV-positive donor P5, led to a reduction in the *Lactobacillus* population, with a persistence in *L. reuteri*, which may possibly be resistant to these antibiotics. *B. longum* also appeared to be less affected by the antibiotics than *B. adolescentis*. However, since only one donor received multiple antibiotics, general conclusions regarding the responses of these bacteria to antibiotics, in HIV-positive donors, requires a more extensive study since the observed responses may be host specific and not linked to medication.

Clinical data summarised in Chapter 2 and graphically represented in this chapter, showed a general improvement of conditions within the peripheral blood namely, a clinically significant reduction in the viral load and an increase in $CD_4^+$ T cell count, except for donor P2 (who became pregnant during the study). All of the HIV-positive donors were classified as having a chronic HIV infection when HAART was initiated, which means that, according to the study conducted by Guadalupe *et al*., (2006) a prolonged period of time would need to elapse before conditions within the gastrointestinal tract began to improve.

Even though the *Bifidobacterium* and *Lactobacillus* population diversity did not seem to be affected by the persistence of HIV within the gastrointestinal tract, these conditions or the HAART itself could possibly be affecting other dominant intestinal bacterial groups. The

results reported in Chapter 3 showed a significant reduction in the total bacterial diversity before the initiation of HAART relative to the healthy donors. It would have been interesting to also investigate possible changes in the diversity of the total bacteria longitudinally over the 6 month HAART period. This, however, could not be done in the course of this study due to time and equipment constraints, but future work should include this analysis.

As an alternative research strategy (given the available resources), it was decided that the abundance and dynamics of other key bacterial groups in the gastrointestinal tract should be investigated using qPCR. The groups selected were the four dominant (*Bacteroides/Prevotella*, *Clostridium leptum* group, *Clostridium coccoides* cluster and *Bifidobacterium*) and three sub-dominant (*Lactobacillus*, *Escherichia coli* and *Enterococcus*) gastrointestinal groups. These would be determined in the faecal samples of 12 HIV-positive and 12 HIV-negative donors, using qPCR. Samples would be analysed prior to HAART, as well as at 6 months post HAART initiation (in the 8 longitudinal patients), in an attempt to establish whether the numbers of endogenous microbiota were affected by the conditions found within the intestinal tract during HAART. Furthermore, the prevalence of three intestinal pathogens within HIV-positive donors would also be evaluated. The findings and conclusions of this work are described and discussed in Chapter 5.

# CHAPTER FIVE

## Quantitative real time PCR analysis of selected gastrointestinal bacterial groups and pathogens in South African HIV-positive and HIV-negative donors

### Contents

## 5.1 SUMMARY

Real-time PCR has been successfully employed in the quantification of bacterial DNA from various sources such as faeces (Malinen *et al*., 2003; Requena *et al*., 2002), colonic tissue (Fujita *et al*., 2002) and gastric tissue (He *et al*., 2002). The final section of this study aimed to establish whether there were any significant changes in the abundance of four dominant and three sub-dominant gastrointestinal bacterial groups, present in the faeces of HIV-positive donors prior to, as well as 6 months after HAART initiation. The dominant groups were *Bacteroides/Prevotella*, *Clostridium leptum* group, *Clostridium coccoides* cluster and *Bifidobacterium* and the subdominant were *Lactobacillus*, *Escherichia coli* and *Enterococcus*. The findings of this chapter showed that prior to the initiation of HAART, the abundance of the *C. leptum* group was significantly reduced in the HIV-positive donors relative to the HIV-negative donor group ($p = 0.02$). The *Bacteroides* group was also reduced in the HIV-positive patients, but this did not attain statistical significance ($p = 0.08$). There was no significant difference between the two donor groups with respect to any of the other groups tested. No detectable levels of *Enterococcus* spp. were found in either the HIV-positive or HIV-negative donor groups. After 6 months of HAART, significant reductions in the abundance of the *C. leptum* ($p = 0.05$) and *Bacteroides* ($p = 0.004$) groups as well as the total bacteria ($p = 0.03$) were seen relative to the HIV-negative donor group. This suggests that the microbial composition within the HIV-positive gastrointestinal tract was different to the HIV-negative state, and that during 6 months of HAART these potentially protective groups decreased further. The possible presence of three intestinal pathogens was also determined utilising *Taq*Man probes. No detectable levels of *Campylobacter jejuni* were found in either of the two donor groups, while *Clostridium* cluster XI species were found in both groups of donors. *Salmonella* species were detected more frequently within the HIV-negative donors ($p = 0.02$). These results showed that qPCR enabled the identification of specific changes in the intestinal microbiota, following chronic HIV infection and during a 6 month period during which HAART was administered.

## 5.2 INTRODUCTION

The findings discussed in Chapter 3 and Chapter 4 of this study showed that PCR-DGGE could be successfully used in determining the composition and diversity of complex microbial communities within the gastrointestinal tract. However, the abundance of strains in these bacterial communities could not be accurately measured as DGGE is not a truly quantitative technique due to several limitations which were addressed in Chapter 3. Therefore, in order to establish any alterations in the abundance of certain species under specific disease conditions, a more sensitive, quantitative method, such as real-time PCR, is required (Ahn *et al*., 2009).

Real-time PCR is an accurate and sensitive technique that has been used in various quantitative nucleic acid analyses, such as gene expression and pathogen quantification (Schmittgen *et al*., 2008). Current detection methods employed include either SYBR Green I or *Taq*Man (Kubista *et al*., 2006). SYBR Green I is an asymmetric, cyanine intercalating dye, that has been shown to fluoresce 50 to 100 times more brightly when bound to double-stranded DNA, and is the most commonly used dye in quantitative PCR (Kubista *et al*., 2006; Inglis and Kalischuk, 2004; Malinen *et al*., 2003). The *Taq*Man assay, also referred to as the 5'-nuclease PCR assay, exploits the 5' to 3' exonuclease activity of *Taq* polymerase and measures the increase in fluorescence when a hydrolysis probe, specifically designed to bind inside the amplified region is cleaved (Heid *et al*., 1996). An increase in fluorescence will occur only if the target sequence is amplified, thus preventing the detection of non-specific products and increasing the overall specificity and sensitivity. *Taq*Man has been reported to be a hundred times more sensitive than conventional PCR (Haarman and Knol, 2005). Unlike the *Taq*Man assay, SYBR Green has a tendency for non-specific binding to any double-stranded nucleic acid or primer dimers, which may significantly reduce the specificity of the PCR assay (Inglis and Kalischuk, 2004; Malinen *et al*., 2003). However, SYBR Green is associated with several advantages, including low running costs and its suitability for use in most PCR amplification reactions, and thorough experimental controls can minimize the formation of primer dimers and non-specific binding (Inglis and Kalischuk, 2004; Malinen *et al*., 2003).

Baseline comparisons established between HIV-positive and HIV-negative donors, in Chapter 3, showed that the diversity of the total bacterial community had been significantly affected in the former group. The development of primers specific for the major and minor bacterial groups found in the gut means that an accurate representation of the abundance of these groups within the gastrointestinal tract can be determined using qPCR.

Several scientific reports have indicated that patients living with a more advanced and progressive HIV infection ($CD_4^+$ < 200 cells/$mm^3$), are at higher risk of acquiring and developing diarrhoea attributable to a bacterial pathogen (Sanchez *et al*., 2005; Obi and Bessong, 2002; Navin *et al*., 1999). Enteric pathogens identified as causative agents of bacterial diarrhoea in HIV-positive patients, include *Clostridium*, *Campylobacter* and *Salmonella* species. These accounted for 53.6%, 13.8% and 7.4% of diarrhoea cases, respectively, reported during 1992-2002 (Sanchez *et al*., 2005). As recorded in Chapter 2, none of the recruited HIV-positive donors developed diarrhoea during the course of this study. However, the absence of diarrhoea within these donors should be interpreted with caution, since potential intestinal pathogens may not be completely absent, but could be present in considerably lower numbers, suggesting that HIV-positive patients may still be carriers of these pathogens.

The focus of the research reported in this chapter was to evaluate quantitatively whether any significant changes in the abundance of four dominant (*Bacteroides/Prevotella*, *Clostridium leptum* group, *Clostridium coccoides* cluster and *Bifidobacterium*) and three sub-dominant (*Lactobacillus*, *Escherichia coli* and *Enterococcus*) gastrointestinal groups had occurred. SYBR Green was used for the detection of these groups within the faecal material of both HIV-positive donors (prior to [T0] and 6 months [T4] after HAART initiation), and in the control HIV-negative group. Furthermore, the abundance of three bacterial intestinal pathogens, namely *S. enterica*, *C. jejuni* and *C. difficile*, within both the HIV-positive and HIV-negative donor groups was determined using the *Taq*Man method.

## 5.3 MATERIALS AND METHODS

### 5.3.1 Bacterial strains and plasmids

Bacterial strains used as positive controls for each of the bacterial groups investigated in this chapter, are listed in Table 5.1. All reference strains, with the exception of *C. leptum* and *C. coccoides*, were cultured as previously described in section 3.3.1. DNA extracted from *C. leptum* DSM 753 and *C. coccoides* DSM 935, was purchased from the DSM bacterial culture collection (Deutsche Sammlung von Mikroorganismen, Germany).

**Table 5.1** Bacterial strains and cloning plasmid used in this study

| Bacterial strains and plasmid | Characteristics | Reference* |
|---|---|---|
| *Bifidobacterium longum* NCIMB 702259$^T$ | Type strain of *B. longum* | NCIMB |
| *Lactobacillus gasseri* ATCC 33323$^T$ | Type strain of *L. gasseri* | ATCC |
| *Bacteroides fragilis* 638R | Clinical isolate | Privitera *et al*., (1979) |
| *Escherichia coli DH5α* | Gram-negative cloning host | Hanahan, (1983) |
| *Enterococcus faecalis* (P1.2) | MCB laboratory strain | MCB |
| *Clostridium leptum* DSM 753$^T$ | Type strain of *C. leptum* | DSM |
| *Clostridium coccoides* DSM 935$^T$ | Type strain of *C. coccoides* | DSM |
| pTZ57R/T | Linearized cloning vector with single 3'-ddT overhangs | Fermentas |

*__NCIMB:__ National Collection of Industrial, Food and Marine Bacteria, United Kingdom, __DSM:__ Deutsche Sammlung von Mikroorganismen, Germany, __ATCC:__ American Type Culture Collection, __MCB:__ Department of Molecular and Cell Biology, University of Cape Town

The three bacterial pathogens used in this study are described in Table 5.2. *Salmonella enterica* was grown aerobically at 37$^o$C in LB broth or agar plates, whilst *C. difficile* was cultured anaerobically in both a supplemented brain heart infusion medium and a *Clostridium* basal growth medium as mentioned in section 3.3.1. A pure, clinical isolate of *Campylobacter jejuni*, grown on Tryptose blood agar under microaerophilic conditions at 33.5$^o$C, was acquired from the National Health Laboratory Services (NHLS) in Cape Town.

**Table 5.2** Bacterial pathogens used in this study

| Bacterial pathogen | Characteristics | Reference* |
|---|---|---|
| *Salmonella enterica* ATCC 14028$^T$ | Type strain of *S. enterica* | BD Diagnostics |
| *Campylobacter jejuni* type I | Clinical isolate | NHLS |
| *Clostridium difficile* ATCC 9689$^T$ | Type strain of *C. difficile* | BD Diagnostics |

*__BD:__ Becton, Dickinson and Company, __NHLS:__ National Health Laboratory Services

### 5.3.2 Genomic DNA Extraction

Total bacterial DNA was extracted from faecal samples collected at T0 and T4, using the ZR Faecal DNA Kit™ (ZYMO Research), as previously described in Chapter 3 (section 3.3.2). Bacterial gDNA was isolated from the reference strains (Table 5.1) and the three pathogens (Table 5.2) using the Genomic DNA Purification Kit (Fermentas) which was also described in Chapter 3 (section 3.3.2).

### 5.3.3 Preparation of plasmid DNA controls

PCR products generated from the different primer combinations (Tables 5.3 and 5.4) were purified, using the BioSpin PCR Purification Kit (BioFlux).

**Table 5.3** Target bacterial groups and qPCR primers used in this study

| Target Group (amplicon size) | Primer | Sequence (5'-3') | Reference |
|---|---|---|---|
| *Total bacteria* | F_Bact 1369 | CGG TGA ATA CGT TCC CGG | Suzuki *et al*. |
| (123 bp) | R_Prok1492 | TAC GGC TAC CTT GTT ACG ACT T | (2000) |
| *C. leptum* | F_Clept 09 | CCT TCC GTG CCG SAG TTA | Furet *et al*., |
| (115 bp) | R_Clept 08 | GAA TTA AAC CAC ATA CTC CAC TGC TT | (2009) |
| *Bifidobacterium* | F_Bifid 09c | CGG GTG AGT AAT GCG TGA CC | Furet *et al*., |
| (137 bp) | R_Bifid 06 | TGA TAG GAC GCG ACC CCA | (2009) |
| *C. coccoides* | F_Ccoc 07 | GAC GCC GCG TGA AGG A | Furet *et al*., |
| (198 bp) | R_Ccoc 14 | AGC CCC AGC CTT TCA CAT C | (2009) |
| *Bacteroides* | F_Bacter 11 | CCT WCG ATG GAT AGG GGT T | Furet *et al*., |
| (130 bp) | R_Bacter 08 | CAC GCT ACT TGG CTG GTT CAG | (2009) |
| *E. coli* | *E. coli* F | CAT GCC GCG TGT ATG AAG AA | Huijsdens *et* |
| (100 bp) | *E. coli* R | CGG GTA ACG TCA ATG AGC AAA | *al*., (2002) |
| *Lactobacillus* | F_Lacto 05 | AGC AGT AGG GAA TCT TCC A | Furet *et al*., |
| (352 bp) | R_Lacto 04 | CGC CAC TGG TGT TCY TCC ATA TA | (2009) |
| *E. faecalis* | F_Entero | CCC TTA TTG TTA GTT GCC ATC ATT | Rinttilä *et al*., |
| (143 bp) | R_Entero | ACT CGT TGT ACT TCC CAT TGT | (2004) |

**Table 5.4** Real-time primers and probes employed in this study

| Target Group | Primer/Probe | Sequence (5'-3') | Reference |
|---|---|---|---|
| *S. enterica* | ttr-6(F) | CTC ACC AGG AGA TTA CAA CAT GG | Malorny *et al*., (2004) |
| | ttr-4(R) | AGC TCA GAC CAA AAG TGA CCA TC | |
| | **ttr-5 (P)** | **FAM-CAC CGA CGG CGA GAC CGA CTT T** | |
| *C. jejuni* | *yph*C-F | CAT TCT CAA ATA GCA CTT TTG GTT TT | Iijima *et al*., (2004) |
| | *yph*C-R | GCC CAG CAA TGC GTT CA | |
| | ***yph*C-P** | **TET-TGG CGC ATG AGG GCT TTA ACG AGC** | |
| *Clostridium* cluster XI *(C. difficile)* | CXI-F1 | ACG CTA CTT GAG GAG GA | Song *et al*., (2004) |
| | CXI-R2 | GAG CCG TAG CCT TTC ACT | |
| | **Probe I** | **FAM-GTG CCA GCA GCC GCG GTA ATA CG** | |

**Probe sequences and names are in bold.**

The PCR fragments were ligated into the pTZ57R/T vector (Fermentas), and transformed into competent *E. coli* DH5α cells by means of heat shock (Chapter 3, section 3.3.5.1). The presence of the insert was confirmed through PCR amplification, using group or pathogen specific primers (Table 5.3 and Table 5.4). Plasmid DNA was extracted using the BioSpin Plasmid Extraction Kit (BioFlux) and the concentration determined using the Nanodrop (NanoDrop® ND-1000).

### 5.3.4 Quantitative real-time PCR amplification

*5.3.4.1 SYBR Green Method*

Quantitative PCR detection of the eight selected bacterial groups was carried out in triplicate, using optical-quality 96-well PCR plates (0.2 ml volume/well) and sealing film (Lasec SA). Assays were performed using the Bio-Rad MyIQ$^{TM}$ Real-Time PCR detection system (Bio-Rad, Inc) and a reaction mixture (12.5 µl) composed of 1X SensiMix$^{TM}$ SYBR No-ROX Master Mix (BioLine), 0.2 µM of each specific primer (Table 5.3) and 1 µl (10 ng) of DNA template. Cycling parameters consisted of one cycle at 95$^{o}$C for 10 min, followed by 40 cycles of 95$^{o}$C for 10 sec, 60$^{o}$C for 15 sec, and 72$^{o}$C for 20 sec.

A detailed melt curve analysis was performed after the last cycle of amplification to confirm PCR specificity. The melt curve data was acquired by heating the PCR reaction from 72$^{o}$C to 95$^{o}$C at a ramp rate of 0.1$^{o}$C/sec. Measurements were then recorded at intervals of 1$^{o}$C. Following this, the plasmid copy number/µl was determined by dividing the plasmid DNA

concentration with its corresponding plasmid mass, approximately 3.3 x $10^{-18}$ g (Pèlissier *et al*., 2010). All cloned group and pathogen specific inserts were serially diluted (10-fold dilutions) (Table 5.5), and were subsequently used in the optimisation of each qPCR run.

Negative and positive controls, in addition to a standard curve, were included on every plate for all qPCR runs. The MyIQ system was calibrated, as prescribed by the manufacturer, with the iCycler iQ external well factor solution (Bio-Rad, Inc) which improved the quality of the fluorescent data recorded.

*5.3.4.2 Taqman (5'- nuclease PCR assay) Method*

Pathogen specific primers and probes used in this study are summarized in Table 5.4. PCR amplification and detection of pathogen DNA was performed in a Rotor-Gene™ 6000 system (Corbett Life Science) with optical-quality PCR tubes (0.1 ml) and caps (Gene target solutions). Real-time PCR reactions were performed in triplicate, in a total volume of 12.5 µl, which consisted of 1X SensiMix™ II Probe Master Mix (BioLine), 0.4 µM (*S. enterica*) and 0.3 µM (*C. jejuni* and *Clostridium* cluster XI) of each primer, 0.25 µM (*S. enterica* and *C. jejuni*) and 0.2 µM (*Clostridium* cluster XI) of the hydrolysis probe and 1 µl of DNA template. All *Taq*man probes used were labeled with a 5'-reporter dye, either 6-carboxyfluorescein (FAM) or 6-carboxy-4,7,2',7'-tetrachloro-fluorescein (TET) and the 3'-quencher dye dimethylaminoazosulfonic acid (DABSYL). The reaction conditions used for amplification were 95$^{o}$C for 10 min, followed by 45 cycles of 95$^{o}$C for 15 sec, and annealing/elongation for 30 sec at either 61$^{o}$C (*S. enterica*), 60$^{o}$C (*C. jejuni*) or 58$^{o}$C (*Clostridium* cluster XI). Product formation was detected by measuring the intensity of the reporter dye, which is cleaved off from the probe by the 5'- 3' exonuclease activity of *Taq* polymerase.

**5.3.5 Normalisation of data and statistical analysis**

PCR quantification and melt curve analysis was performed using the iQ5™ Optical System software version 2.0 (Bio-Rad, Inc) and the Rotor-Gene 6000 software version 1.7 (Corbett Life Sciences). For each of the investigated bacterial groups, the number of 16S rRNA gene copies/mg of faecal material was converted into the log of 16S rRNA gene copies/g of faecal material.

A Mann-Whitney U Test was performed, to compare differences in the abundance of the bacterial groups between the HIV-positive and HIV-negative donors, and a $p$ value $\leq 0.05$ was considered to be statistically significant. The qPCR results were also graphically represented as box-and-whisker plots, constructed using Stata data analysis and statistical software version 11.1 (StataCorp LP, USA) and expressed as median values with quartile ranges (QR).

## 5.4 RESULTS AND DISCUSSION

### 5.4.1 Optimisation of the SYBR Green I-based real-time PCR assays

All qPCR optimisation experiments were performed according to the relevant internationally recognized MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments (Bustin *et al*., 2009). All qPCR assays performed in this study, were run in triplicate, together with two independent negative qPCR controls on each PCR plate. In addition, genomic DNA was also extracted twice from individual faecal samples.

#### *5.4.1.1 qPCR primer selection*

Previously published primers, described in Table 5.3, were used in the detection of eight specific gastrointestinal bacterial groups. The specificity of each primer set was validated by means of conventional PCR, using genomic DNA isolated from the bacterial reference strains summarised in Table 5.1. Each individual primer set was also tested against all eight representative species from each bacterial group to confirm specificity. When the total bacterial primer set (F_Bact 1369 and R_Prok 1492) was evaluated, a PCR product of approximately 123 bp was produced in all eight species (Figure 5.1A). The remaining bacterial group qPCR primer sets were highly specific for each group and produced a positive result for only the corresponding target species, with no cross reactions or non-specific binding occurred with any of the other target groups (Figure 5.1B – H).

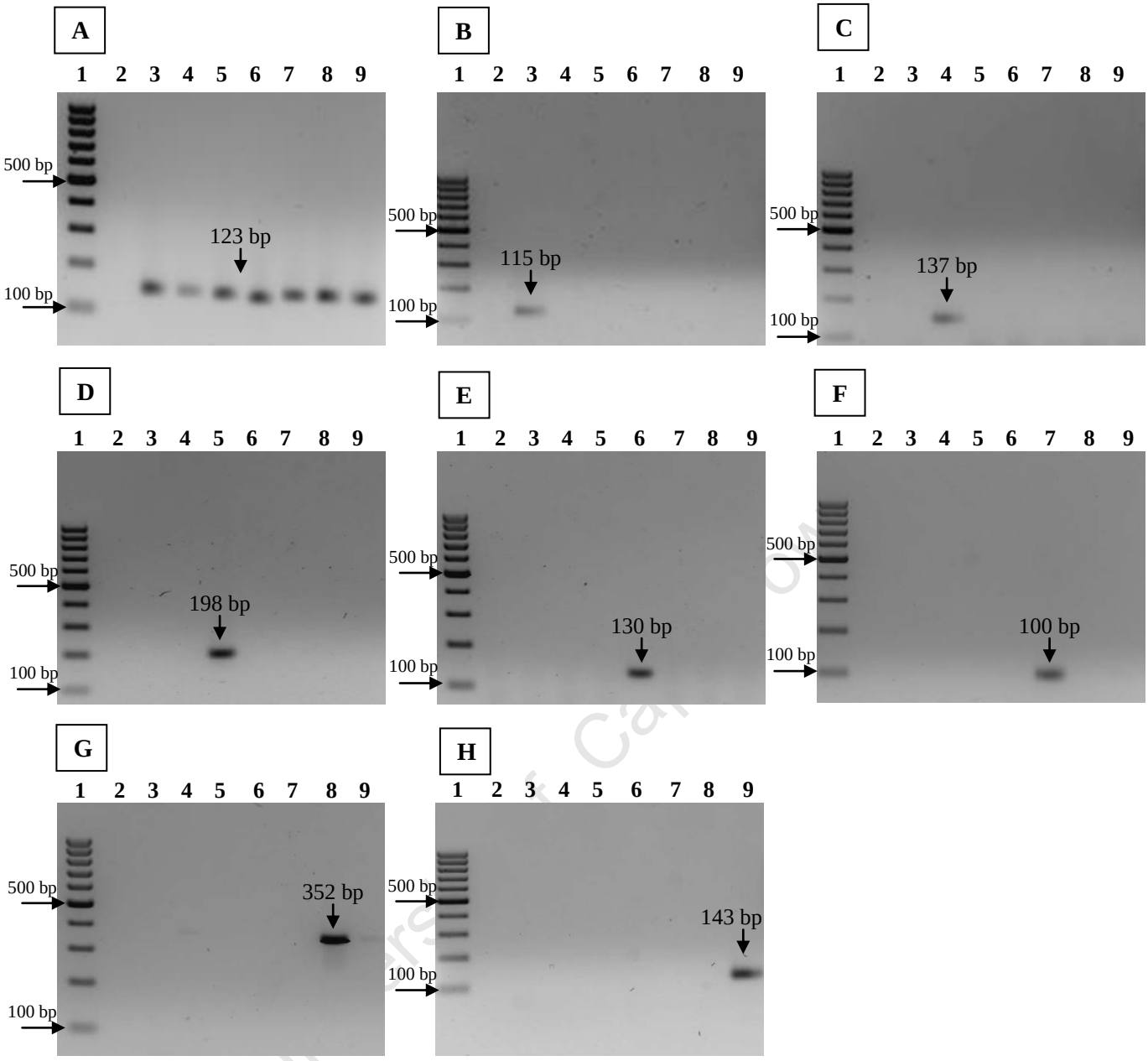



**Figure 5.1:** **Specificity of the qPCR primers employed in the SYBR Green Method. (A)** Total bacteria [F_Bact 1369 and R_Prok 1492]; **(B)** *C. leptum* group [F_Clept 09 and R_Clept 08]; **(C)** *Bifidobacterium* group [F_Bifid 09c and R_Bifid 06]; **(D)** *C. coccoides* group [F_Ccoc 07 and R_Ccoc 14]; **(E)** *Bacteroides* group [F_Bacter 11 and R_Bacter 08]; **(F)** *E. coli* [E. coli F and E. coli R]; **(G)** *Lactobacillus* group [F_Lacto 05 and R_Lacto 04] and **(H)** *Enterococcus* group [F_Entero and R_Entero]. Lane 1: 100 bp molecular weight marker (Fermentas); Lane 2: Negative control (no DNA); Lane 3: *C. leptum*; Lane 4: *B. longum*; Lane 5: *C. coccoides*; Lane 6: *B. fragilis*; Lane 7: *E. coli*; Lane 8: *L. gasseri* and Lane 9: *E. faecalis*. Arrows indicate sizes of PCR products in bp.

In the study published by Furet *et al*., (2009), a final primer concentration of 0.2 µM was used for all of the evaluated primer sets. To some extent lower qPCR primer concentrations (0.2-0.4 µM) are usually favoured when employing SYBR Green I in an attempt to reduce non-specific amplification (Sigma Aldrich, 2008). Therefore, the optimal qPCR primer concentration for this study was determined by testing each primer set, using qPCR, at the following concentrations: 0.1 µM, 0.2 µM, 0.3 µM, and 0.4 µM. Optimal qPCR conditions were achieved when a primer concentration of 0.2 µM was used.

To determine the purity of the extracted gDNA, as well as the concentration which would result in the greatest amount of amplification, a standard DNA dilution series was established using varying amounts of gDNA (1 ng/µl to 100 ng/µl). A ten-fold increase in DNA should lead to a decrease in the threshold cycle (Ct) value by approximately 3.32, provided the PCR efficiency is acceptable. Relatively high concentrations of DNA could result in the inhibition of the qPCR reaction, whereas too little DNA may be undetectable (Edwards, 2004). To determine the reproducibility of the qPCR assays, two separate DNA extracts (derived from the same faecal sample) were evaluated under optimised conditions. Briefly, 10 ng of each gDNA extract was run in triplicate, under the same reaction conditions, with strongly reproducible results being achieved for both samples.



*5.4.1.2 qPCR reaction conditions*

PCR cycling conditions, previously optimised by Furet *et al*., (2009), were also evaluated for use in the current study. A very important variable in ensuring that optimal conditions and acceptable qPCR efficiencies are achieved is the annealing temperature. Prior to the initiation of qPCR, the annealing temperature for all eight primer sets were tested and evaluated. After performing numerous qPCR runs at four different annealing temperatures (58 $^{o}$C, 59 $^{o}$C, 60 $^{o}$C, 61 $^{o}$C), the optimum annealing temperature, for all eight primer sets, was determined to be 60$^{o}$C. This result was in agreement with the conditions recorded and employed by Furet *et al*., (2009). Another key variable in the optimisation of qPCR is the magnesium chloride ($MgCl_2$) concentration. High $MgCl_2$ concentrations may result in low product formation and a significant increase in the production of non-specific PCR products, such as primer dimers (Edwards, 2004). Several $MgCl_2$ titration curves, ranging from 1 mM to 5 mM were conducted, and the optimal $MgCl_2$ concentration determined for this research study was 3 mM.

*5.4.1.3 qPCR validation: specificity, sensitivity and efficiency (using SYBR Green)*

The presence of primer dimers or non-specific amplification can affect the overall PCR reaction efficiency and limit the detection range (Bustin *et al*., 2009). Therefore, performing a melt curve analysis is necessary when using SYBR Green I, on account of its relatively high affinity for non-specific binding. As the temperature is increased during the melt curve analysis, double stranded DNA begins to denature, causing SYBR Green I to dissociate from this complex and results in a decrease in the fluorescence. Non-specific PCR products or primer dimers formed during PCR, have different melting properties compared to the product of interest (primer dimers usually melt at lower temperatures than specific PCR products), and would therefore been seen as additional peaks (van der Velden *et al*., 2003). The melt curve analyses carried out in this study were observed from 72$^{o}$C to 95$^{o}$C. A single product was obtained for all of the primer sets (Figure 5.2). Furthermore, when analysing each of these products using agarose gel electrophoresis and ethidium bromide staining, the gels also confirmed that no non-specific products were produced at 72$^{o}$C.

The reaction efficiency, sensitivity and detection limits of the SYBR Green qPCR assays were determined, by creating 10-fold serial dilutions of the plasmids containing each of the cloned bacterial group inserts (Table 5.5). Standard curves were generated by plotting the value of the threshold cycle (Ct) against the log copy number of the corresponding known standard (Figure 5.3A - C), with standard curve correlation coefficient values ($R^2$) ranging between 0.994 and 1.000 being recorded.

**Table 5.5** Standard curve ranges used for each target group

| Target Group | Standard curve range (copies/µl) | Target Group | Standard curve range (copies/µl) |
|---|---|---|---|
| *Total bacteria* | $3.03x10^3 – 3.03x10^8$ | *S. enterica* | $2.08x10^3 – 2.08x10^9$ |
| *C. leptum* | $3.04x10^3 – 3.04x10^8$ | *C. jejuni* | $3.07x10^4 – 3.04x10^9$ |
| *Bifidobacterium* | $3.02x10^3 – 3.02x10^8$ | *Clostridium cluster XI* | $3.02x10^4 – 3.02x10^9$ |
| *C. coccoides* | $2.96x10^3 – 2.96x10^8$ | | |
| *Bacteroides* | $3.03x10^3 – 3.03x10^8$ | | |
| *E. coli* | $3.06x10^3 – 3.06x10^8$ | | |

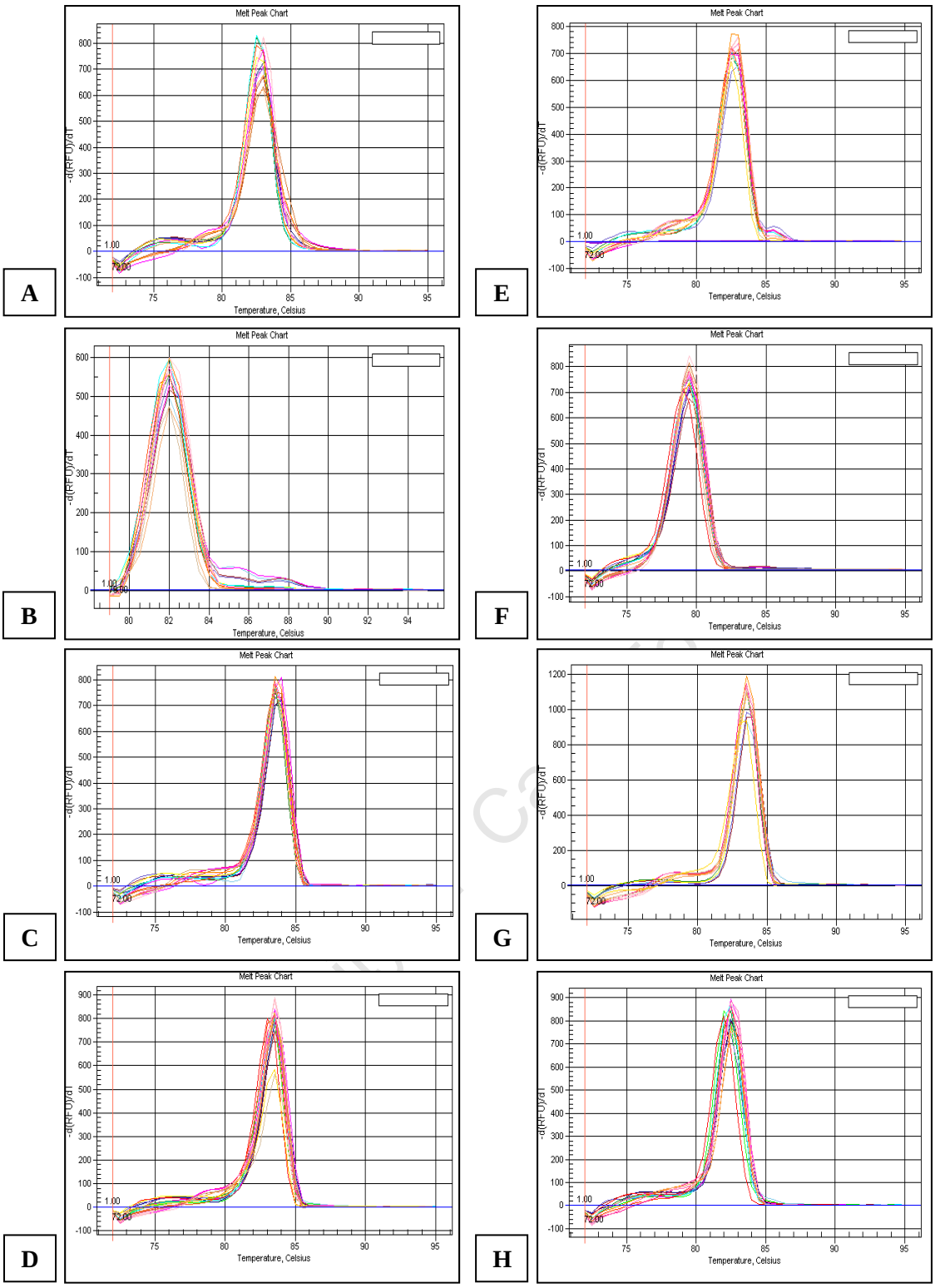



**Figure 5.2:** **Melt curve analysis of bacterial group specific PCR products.** (A): Total bacteria [F_Bact 1369 and R_Prok 1492]; (B): *C. leptum* [F_Clept 09 and R_Clept 08]; (C): *Bifidobacterium* [F_Bifid 09c and R_Bifid 06]; (D): *C. coccoides* [F_Ccoc 07 and R_Ccoc 14]; (E): *Bacteroides* [F_Bacter 11 and R_Bacter 08]; (F): *E. coli* [E. coli F and E. coli R]; (G): *Lactobacillus* [F_Lacto 05 and R_Lacto 04] and (H): *Enterococcus* [F_Entero and R_Entero]. Specific primers used are shown in the square brackets and in Table 5.3. [Experiments A-H were performed in triplicate].

Overall, both the primer sensitivity and reaction efficiencies were good, in that a template range of between $10^3$ to $10^8$ copies was easily detected using SYBR Green I, and the lowest and highest efficiencies, calculated using the formula $E = [10^{(-1/slope)} - 1]$ (Penders *et al.*, 2005), were 86% and 107.7% respectively (Figure 5.3A – C). A slope between -3.9 and -3.0 (80-110% efficiency) is considered, in the literature, to be acceptable (Stapleton *et al.*, 2009; Sigma Aldrich, 2008). Numerous attempts were made to try to bring the PCR efficiencies recorded in this study closer to 100%. However, changing the annealing temperature and primer concentrations did not improve the efficiencies shown in Figure 5.3 significantly.

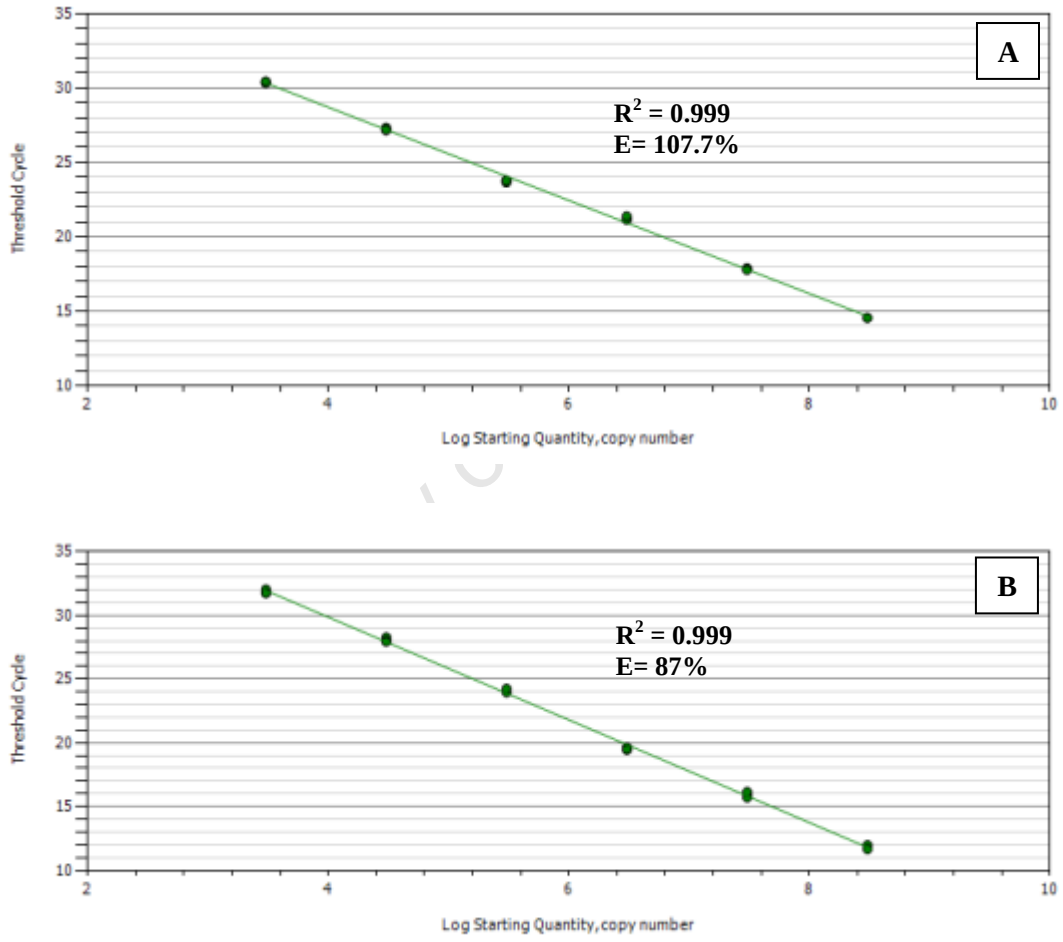



**Figure 5.3A: Standard curves of bacterial group specific PCR products generated using the SYBR Green method.** (A): Total bacteria [F_Bact 1369 and R_Prok 1492] and (B): *C. leptum* [F_Clept 09 and R_Clept 08]. Specific primers used are shown in the square brackets and summarized in Table 5.3. [All standard curve dilutions were assayed in triplicate for each experiment].

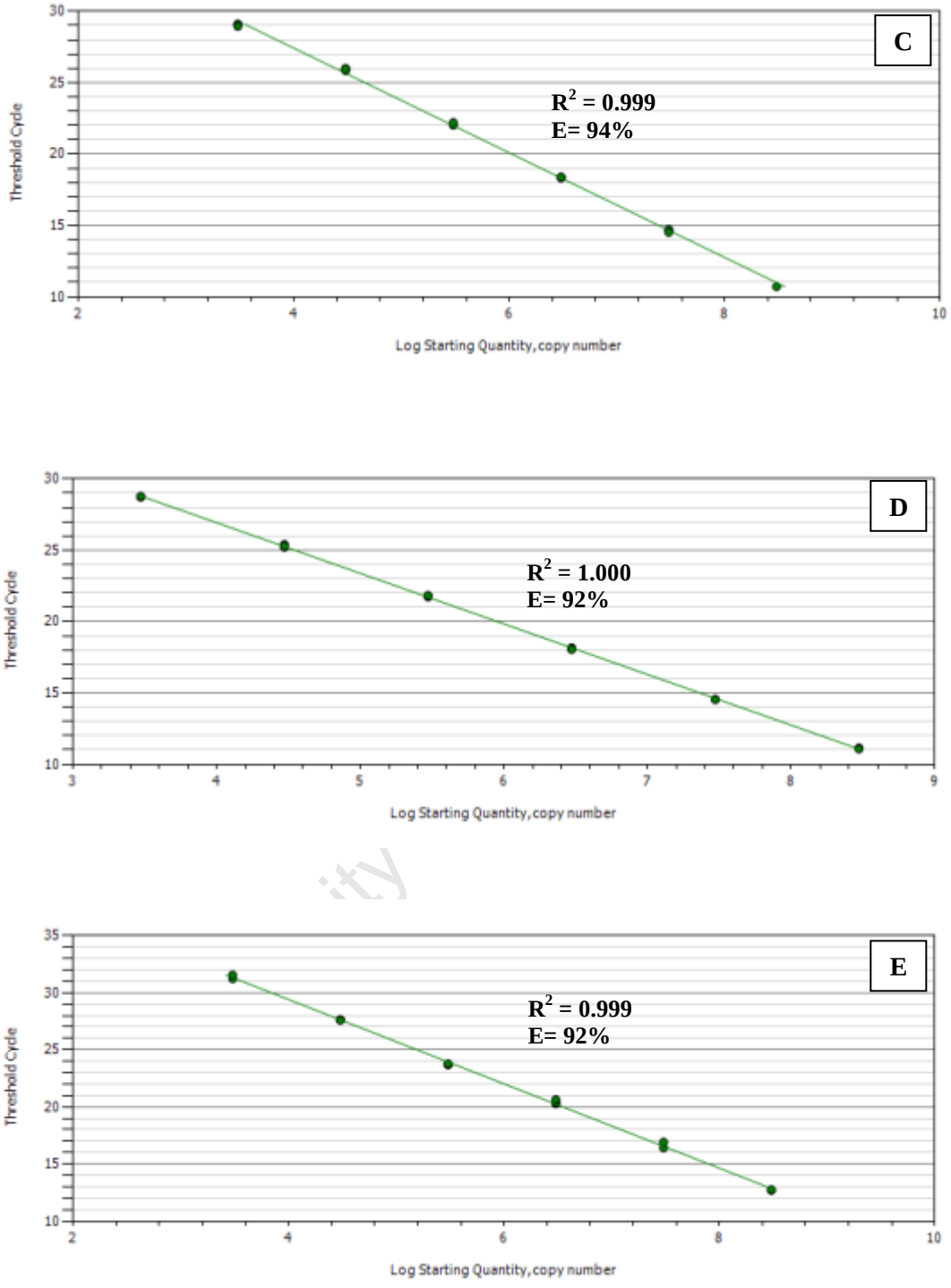



**Figure 5.3B:** **Standard curves of bacterial group specific PCR products generated using the SYBR Green method.** (C): *Bifidobacterium* [F_Bifid 09c and R_Bifid 06]; (D): *C. coccoides* [F_Ccoc 07 and R_Ccoc 14]; (E): *Bacteroides* [F_Bacter 11 and R_Bacter 08]. Specific primers used are shown in the square brackets and summarized in Table 5.3. [All standard curve dilutions were assayed in triplicate for each experiment].

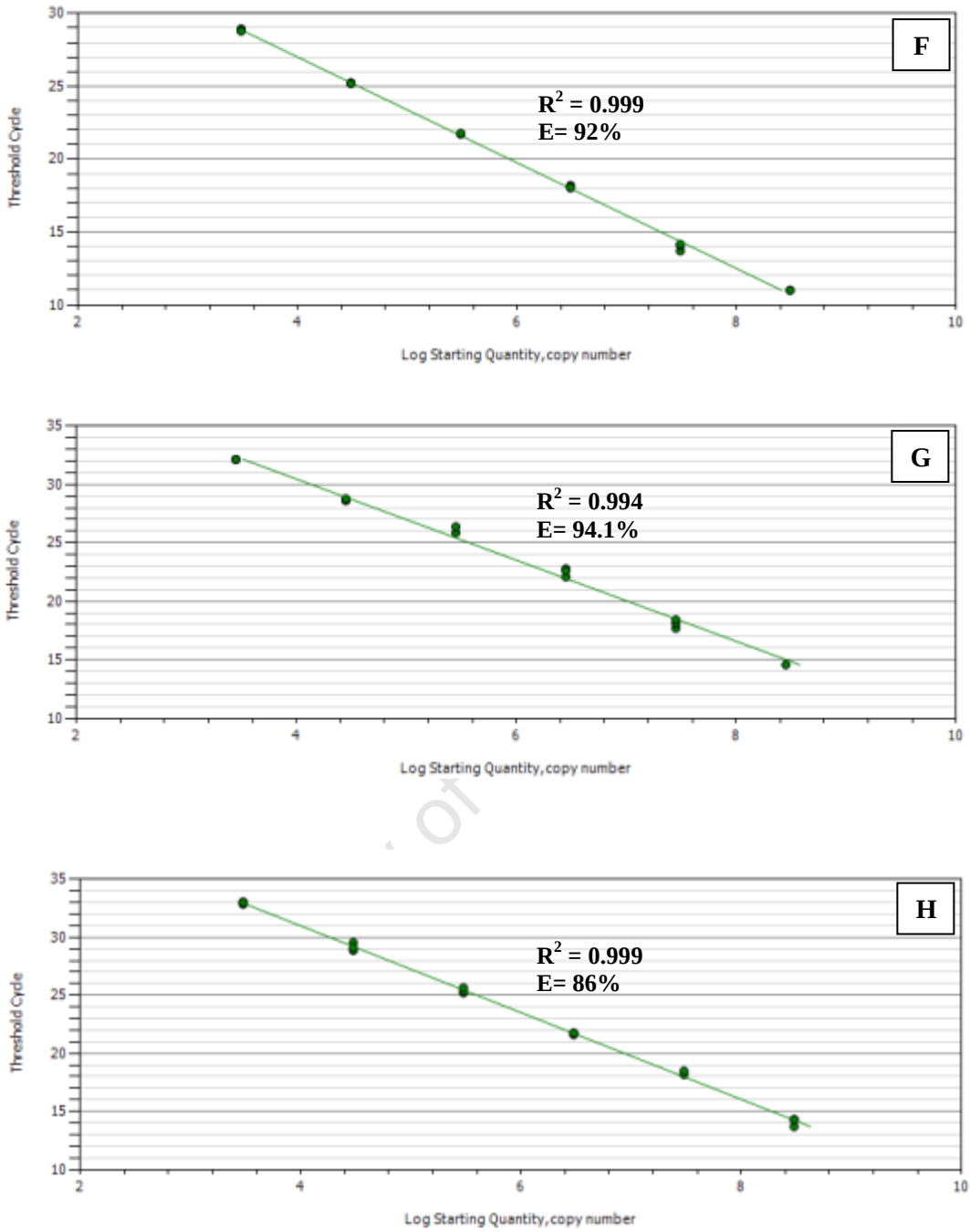



**Figure 5.3C: Standard curves of bacterial group specific PCR products generated using the SYBR Green method.** (F): *E. coli* [E. coli F and E. coli R]; (G): *Lactobacillus* [F_Lacto 05 and R_Lacto 04] and (H): *Enterococcus* [F_Entero and R_Entero]. Specific primers used are shown in the square brackets and summarized in Table 5.3. [All standard curve dilutions were assayed in triplicate for each experiment].

### 5.4.2 Sensitivity of the qPCR primers and probes used for the bacterial pathogens

The DNA target region for each pathogen was cloned into pTZ57R/T and the presence of the insert was validated, using the pathogen specific primers. Three clones, randomly selected, all had the correct insert present in each case (Figure 5.4). The sensitivity of the *Taq*Man primer and probe combinations, as well as the reaction efficiencies were both determined in the same manner as the bacterial group primers discussed above. The detection limits for all three primer and probe sets were approximately $10^3$ to $10^9$ copies, with reaction efficiencies of 96%, 104% and 96% obtained for *S. enterica*, *C. jejuni* and *C. difficile* respectively.

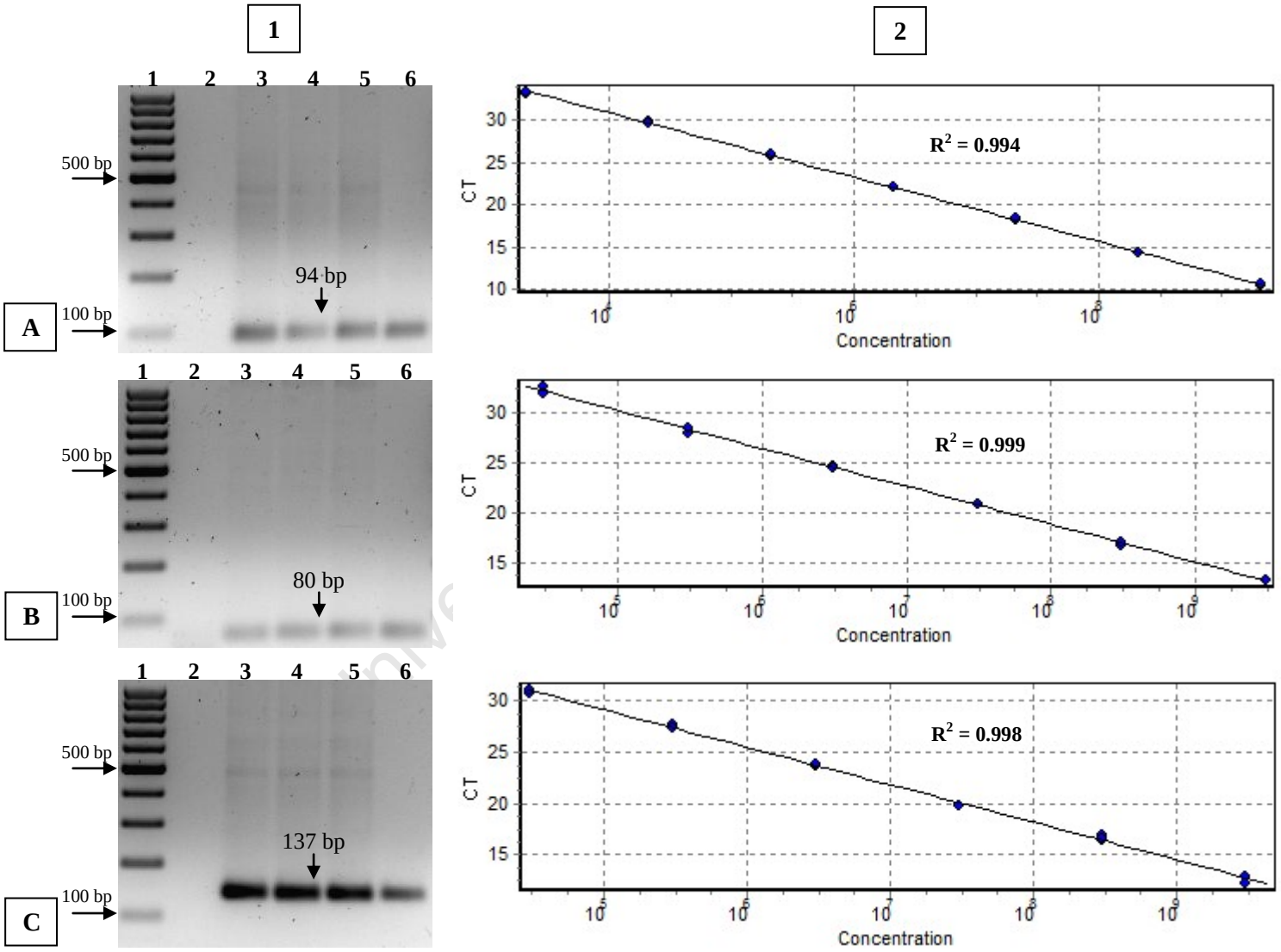



**Figure 5.4: Specificity (1) and detection range (2) of the qPCR primers employed for the *Taq*Man Method.** Lane 1: 100 bp molecular weight marker (Fermentas); Lane 2: Negative control (no DNA); Lanes 3-5: Clones 1, 2, 3 and Lane 6: Positive control (gDNA). (A): *S. enterica*; (B): *C. jejuni* and (C): *C. difficile.* [All standard curve dilutions were assayed in triplicate].

### 5.4.3 Quantitative analysis of gastrointestinal bacterial groups in faecal samples

SYBR Green qPCR was performed, to quantify and determine the abundance of eight specific dominant and sub-dominant gastrointestinal bacterial groups in the faecal samples of HIV-positive donors (prior to HAART) and HIV-negative donors. The results obtained for each bacterial group are presented in Tables 5.6 and 5.7, and are also graphically represented as box-and-whisker plots (Figure 5.5).

*5.4.3.1 Occurrence of major and minor groups in the HIV-negative donors*

The numbers of bacteria detected by qPCR in the healthy control group in this study are shown in Table 5.6, and the values were compared to other similar qPCR studies on healthy individuals reported in the literature. Published, peer-reviewed literature reflects a wide range of variation in the levels of bacterial numbers in healthy individuals detected using qPCR methods. A comparison of our data with four such articles (Table 5.6) showed that the values obtained for the *C. leptum, C. coccoides, Bacteroides*/*Prevotella, and Lactobacillus* groups were all within the range previously reported by other researchers using similar methods and primers (Larsen *et al*., 2010; Furet *et al*., 2009; Zhang *et al*., 2009; Bartosch *et al*., 2004). However, the estimation of the total bacterial numbers in this study appears to be towards the maximum levels reported in these published studies. This may be linked to the qPCR efficiency of 107% detected in the construction of the standard curve although this was still within the published recommended efficiency range (Stapleton *et al*., 2009). This higher estimation could result, perhaps, in a slight overestimation of the total numbers of bacteria (Figure 5.3A). Since the median values for all the other groups tested were very close to the published values, a calculation of the percentage representation of these groups, relative to the higher total bacterial value, would lead to the impression that the groups were under-represented. All attempts to improve the optimisation of the qPCR conditions as described earlier, did not change these values.

However, for the purposes of this study, the aim was to draw a direct comparison between the healthy and HIV-positive cohorts. The qPCR experiments for both groups were done within the study under identical conditions, and with the appropriate number of biological and technical replicates. The data is, therefore, sufficiently reliable to be used for a direct comparison between the bacterial numbers in the two donor groups.

**Table 5.6** Comparison between the qPCR data from this study (healthy volunteers) with four published studies

| Target Group | This Study | | Larsen *et al.*, (2010) | | Zhang *et al.*, (2009) | Furet *et al.*, (2009) | Bartosch *et al.*, (2004) |
|---|---|---|---|---|---|---|---|
| | Median† | Range† | Median† | Range† | Range† | Median† | Median† |
| Total bacteria | 11.86 | 10.3 – 13.1 | 10.5 | 10.0 – 11.3 | 10.2 - 11 | 11.5 | 12.0 |
| *C. leptum* | 9.73 | 8.4 – 10.8 | 9 | 7.5 – 10.5 | | 10.8 | 10.3 |
| *C. coccoides* | 8.52 | 7.7 – 10.4 | 9.9 | 6.5 – 10.5 | | 10.3 | |
| *Bacteroides*/*Prevotella* | 9.34 | 8.0 – 10.5 | 9.6 | 6.5 - 11 | | 10 | 10.5 |
| *Lactobacillus* group | 7.01 | 5.5 – 8.8 | 6.4 | 3.5 – 8.5 | | 7.6 | |

† Values expressed as $Log_{10}$ bacteria per g stool

*5.4.3.2 Comparison of HIV-positive (T0) with HIV-negative donors*

The numbers of bacteria detected by qPCR in the healthy control group and the HIV-positive cohort are shown in Table 5.7. The abundance of the total bacteria was slightly lower in the HIV-positive donors, before the commencement of HAART, than in the control HIV-negative donors, but this was not statistically significant ($p$ = 0.15). The most statistically significant difference between the HIV-positive and HIV-negative donors, at this time point, was seen for the *C. leptum* group ($p$ = 0.02), in that the number of 16S rRNA gene copies detected for this group was greatly reduced within the HIV-positive donors. There was a relatively lower abundance of the *Bacteroides* group within the HIV-positive donors, but this was not statistically significant ($p$ = 0.08). Species belonging to the *Enterococcus* group were not detected in either of the donor groups as, in most instances; the results that were recorded were below the minimum detection limit of the qPCR reaction.

The box-and-whisker plots shown in Figure 5.5 were generated for all seven bacterial groups comparing them in terms of HIV status. The quartile ranges (QR) of each box extend from the lower 25$^{th}$ percentile to the higher 75$^{th}$ percentile along with the median (50$^{th}$ percentile). The highest and lowest values recorded for each group are shown by the whiskers extending above and below each box. Statistical comparison between the numbers of the groups using this analysis confirmed the significant reduction of the *C. leptum* group in the HIV-positive cohort, as well as the trend towards a reduction in *Bacteroides* (although this was not significant) (Figure 5.5A [a]).

It is of interest that Larsen *et al.*, (2010) and Manichanh *et al.*, (2006) both found significant reductions to the *C. leptum* and *C. coccoides* populations using qPCR, in donors suffering from diabetes or Crohn's disease respectively. The *Bacteroides*, *C. leptum* and *C. coccoides*

**Table 5.7** Real-time PCR quantification of eight dominant, sub-dominant and three pathogenic gastrointestinal bacterial groups in the faecal samples of HIV-negative donors and HIV-positive donors at (T0) and (T4)

| Bacterial Group | HIV-Negative (n=12) | | | HIV-Positive T0 (n=12) | | | HIV-Positive T4 (n=8) | | | Significance | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | Min | Max | Mean | Min | Max | Mean | Min | Max | HIV-Negative vs. HIV-positive (T0) | HIV-positive (T0) vs. HIV-positive (T4)[a] | HIV-Negative vs. HIV-positive (T4) |
| Total Bacteria | 11.82 | 10.31 | 13.05 | 11.21 | 9.57 | 12.54 | 10.70 | 9.41 | 12.68 | $p = 0.15$ | $p = 0.60$ | $p = 0.03^*$ |
| *C. leptum* | 9.73 | 8.44 | 10.79 | 8.97 | 7.63 | 10.26 | 8.77 | 7.46 | 10.65 | $p = 0.02^*$ | $p = 0.83$ | $p = 0.05^*$ |
| *Bifidobacterium* | 7.88 | 5.92 | 10.86 | 8.35 | 6.97 | 10.19 | 8.10 | 6.42 | 10.04 | $p = 0.23$ | $p = 0.75$ | $p = 0.49$ |
| *C. coccoides* | 8.52 | 7.67 | 10.43 | 7.87 | 5.64 | 9.38 | 8.24 | 6.94 | 9.44 | $p = 0.42$ | $p = 0.14$ | $p = 0.82$ |
| *Bacteroides* | 9.34 | 8.03 | 10.50 | 8.52 | 6.65 | 10.57 | 7.94 | 6.87 | 9.77 | $p = 0.08$ | $p = 0.29$ | $p = 0.004^*$ |
| *E. coli* | 6.80 | 4.79 | 8.63 | 6.98 | 5.28 | 8.48 | 6.98 | 5.61 | 8.32 | $p = 0.67$ | $p = 0.83$ | $p = 0.76$ |
| *Lactobacillus* | 7.01 | 5.52 | 8.81 | 7.00 | 4.83 | 9.42 | 7.16 | 5.03 | 9.72 | $p = 0.82$ | $p = 0.67$ | $p = 0.88$ |
| *Enterococcus* | > LOD | | | > LOD | | | > LOD | | | | | |
| *S. enterica* | 5.45 | 4.13 | 6.14 | 5.04 | 4.52 | 5.57 | | | | $p = 0.02^*$ | | |
| *Clostridum* cluster XI | 5.92 | 4.11 | 7.51 | 6.19 | 4.70 | 7.75 | | | | $p = 0.43$ | | |
| *C. jejuni* | NDL | | | NDL | | | | | | | | |

Results are expressed as log gene copies/g of stool. Min: minimum; Max: maximum
> LOD: lower than minimum level of detection
NDL: No detectable levels
* Indicates a significant difference between HIV-Positive and HIV-Negative samples ($p \leq 0.05$)
[a] Only the 8 longitudinal HIV-positive donors were compared at T0 and T4

groups are the most predominant populations found within the human intestinal tract (Eckburg *et al*., 2005; Sghir *et al*., 2000), and are considered to be important components of the intestinal microbiota (Lay *et al*., 2005; Duncan *et al*., 2002). Some species belonging to the *C. leptum* and *C. coccoides* groups are capable of producing butyrate, which is the preferred energy source within the colonic mucosa (Roediger, 1980) and has been shown to play a key role in reinforcing the colonic defence barrier, by increasing the production of mucins and antimicrobial peptides and reducing intestinal epithelial permeability (Van Immerseel *et al*., 2010). Members of the *Bacteroides* group also play an important role within the gastrointestinal tract, such as being able to utilise a wide variety of carbon sources (Macfarlane and Gibson, 1991). Any form of disruption or reduction in the number of butyrate producing and *Bacteroides* species could negatively affect these essential functions and networks established between the host's epithelial cells and the endogenous intestinal microbiota (Manichanh *et al*., 2006).

With regard to *Enterococcus*, Bartosch *et al*., (2004); Vanhoutte *et al*., (2004) and Marteau *et al*., (2001) also reported extremely low detection levels and abundance of the *Enterococcus* community in human faeces. The administration of Bactrim as a prophylactic treatment to HIV-positive patients in this study (discussed in Chapter 2), is associated with a reduction in both *Enterobacteriaceae* and *Enterococcus* species (Knothe, 1979). An important point to bear in mind is, however, that at T0, the recruited HIV-positive donors had only just started treatment with Bactrim and any major changes to the abundance of both *E. coli* and *Enterococcus* species was therefore not expected at this stage.

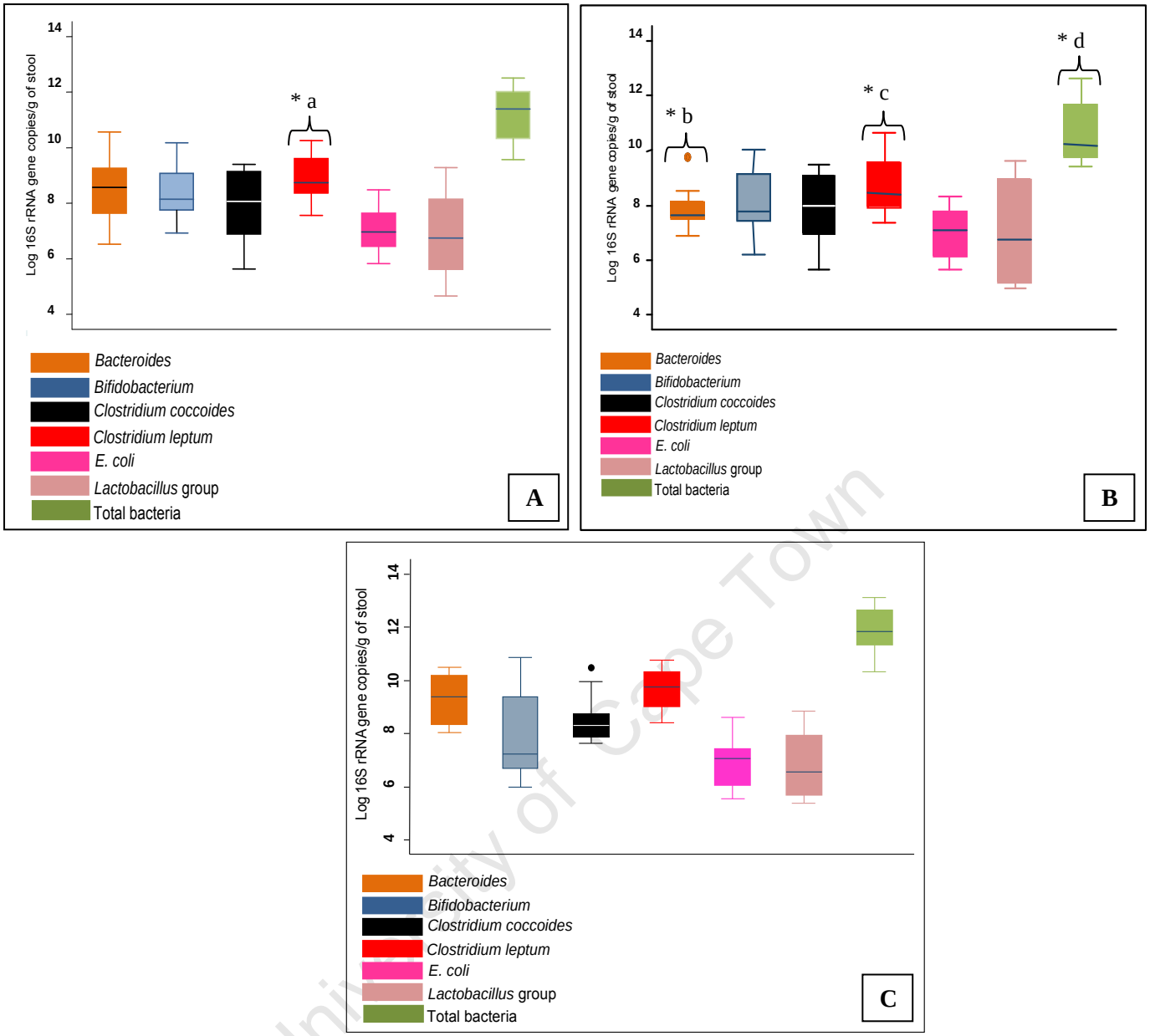





**Figure 5.5:** **Box-and-whisker plots of bacterial groups quantified using qPCR.** Bacterial groups were quantified using SYBR Green in (A) HIV-positive donors (HAART-naïve); (B) HIV-positive donors 6 months after initiating HAART and in (C) HIV-negative donors. Results are expressed as log 16S rRNA gene copies/g of stool while the black and orange dots indicate observations considered to be outliers.

* Significant differences between:

[a]: HIV-positive (T0) vs. HIV-negative (*C. leptum*, $p = 0.02$);

[b]: HIV-positive (T4) vs. HIV-negative (*Bacteroides*, $p = 0.004$);

[c]: HIV-positive (T4) vs. HIV-negative (*C. leptum*, $p = 0.05$) and

[d]: HIV-positive (T4) vs. HIV-negative (Total bacteria, $p = 0.03$)

*5.4.3.3 Longitudinal evaluation of bacterial numbers in HIV-positive donors, 6 months after HAART initiation*

The influence of HAART administration on the abundance of the seven selected major or minor bacterial groups over a 6 month period was measured using qPCR. The numbers of the bacteria in HIV-positive donors prior to HAART (T0) were compared to those present in the same donors at 6 months after HAART initiation (T4), and the results of this comparison are summarised in Table 5.7 and Figure 5.5A and B. After 6 months of HAART no significant changes in abundance of any of the seven investigated bacterial groups were seen.

The difference in the abundance of the *E. coli* group over 6 months was not significant (Table 5.7). Due to the fact that the HIV-positive donors had been continuously administered Bactrim over the 6 month period, a marked difference between the T0 and T4 time intervals was expected. However, several investigations have shown that the resistance of *E. coli* species, in HIV-positive patients, towards Bactrim has increased significantly, with reports of 24% (Murray *et al*., 1982), 76% (Martin *et al*., 1999) and 85% (Hamel *et al*., 2008) resistance. As a consequence, the persistence of this bacterium may be attributable to an increase in the resistance of *E. coli* to Bactrim, following 6 months of continuous administration. Manichanh *et al*., (2006) found that, in donor's with Crohn's disease, an increase in the number of Gram-negative bacteria colonising the intestinal tract occurred, which they attributed to a reduction in the Firmicutes within the gastrointestinal tract.

There were, however, significant changes seen in the abundance of each of the investigated bacterial groups when the HIV-positive donors at T4 (6 months of HAART) were compared to the control HIV-negative donors (Table 5.7 and Figure 5.5B and C). This comparison showed significant reductions in the total bacteria ($p$ = 0.03), as well as the dominant *C. leptum* ($p$ = 0.05) and *Bacteroides* ($p$ = 0.004) groups. This result suggests that alterations in certain important members of the HIV-positive microbiome exist, relative to the control group, even when patients have received HAART and show signs of clinical improvement. There was, however, no significant change in the abundance of the *Bifidobacterium* and *Lactobacillus* populations, once again suggesting that these probiotic strains are not affected by the disease or the administration of HAART. This is in contrast to the results reported by Gori *et al*., (2008), who found that the relative proportions of both the *Bifidobacterium* and *Lactobacillus* populations in Italian, HIV-positive, HAART-naïve patients with average $CD_4^+$

T cell counts of 520 cells/mm$^3$ and plasma HIV RNA levels of 28 393 copies/ml were reduced to approximately 2.5% and 0.02% of the total bacterial population respectively. This study, however, used FISH analysis, and so cannot be directly compared to the data reported here.

### 5.4.4 Quantitative analysis of potential intestinal pathogens in faecal samples

The abundance of three intestinal pathogens within the faecal samples of the recruited HIV negative and HIV-positive donors was also determined. These particular pathogens were selected since they had been previously detected in the faeces of HIV positive patients with diarrhoea (Obi et al., 2007; Sanchez *et al*., 2005). Previously designed oligonucleotide primer pairs and hydrolysis probes specific for *S. enterica* (Malorny *et al*., 2004), *C. jejuni* (Iijima *et al*., 2004) and *Clostridium* cluster XI (*C. difficile*) (Song *et al*., 2004) were used. The detection levels for each of the three pathogenic bacteria are shown in Table 5.7.

*S. enterica* was detected significantly more frequently within the HIV-negative donor group ($p$ = 0.02) (Table 5.7), while no differences between the two donor groups were seen for the *Clostridium* cluster XI. Furthermore, no detectable levels of *C. jejuni* were found in either the HIV-positive or HIV-negative donors. The prevalence of enteric bacterial pathogens in the stool samples of South African HIV-positive and HIV-negative donors was determined, using standard culture techniques, in a study conducted by Obi *et al*., (2007). The findings of that particular investigation showed that the most frequently isolated pathogens amongst HIV-positive donors experiencing diarrhoea were *E. coli* (21.5%) and *Campylobacter* (20.5%) species, while *E. coli* was also significantly more common amongst HIV-positive donors without diarrhoea. In the light of these findings, the absence of *C. jejuni* in all HIV-positive donors recruited for the current study was not surprising given that all of these donors had no clinical signs or symptoms of diarrhoea. HIV-positive donors presenting with diarrhoea, were excluded from participating in this study (discussed in Chapter 2), thereby ensuring that the baseline comparisons obtained were not affected by secondary factors such as antibiotic treatment for diarrhoea.

Even though the detection of *S. enterica* and species belonging to the *Clostridium* cluster XI was successful, several limitations encountered during this study need to addressed in future work. The first limitation of this study was the poor primer specificity of CXI-F1 and CXI-R2

used for the detection of *C. difficile*. Although the primers used were originally reported to detect *C. difficile* (Song *et al*., 2004), they are also capable of detecting other *Clostridium* species belonging to the *Clostridium* cluster XI. *Clostridium* cluster XI is a taxonomically heterogeneous group including species such as *C. difficile*, *Clostridium lituseburense* and *Clostridium bifermentans,* which are all endogenous to the intestinal tract (Collins *et al*., 1994). The low primer specificity shown by this primer set, taken together with the fact that *C. difficile* has approximately 10 to 12 *rrn* copies, suggests possibly that the result reflected in Table 5.7 may not be an exact reflection of the abundance of *C. difficile*. Thus, to achieve a more accurate reflection, the above limitation needs to be addressed by selecting a more suitable primer and probe set that is specific for *C. difficile* and does not target the 16S rRNA gene. Recently, Luna *et al*., (2011) successfully detected toxigenic *C. difficile* strains, in human faecal samples, using *Taq*Man qPCR that targeted the toxin genes *tcd*A and *tcd*B.

The second limitation of this study was attempting to determine the number of detectable pathogens present in the stool sample by means of copy number. A problem associated with the use of copy number in the detection of pathogens is that it does not give an indication of an infective dose or what the minimum level of detection is for that specific pathogen. In most instances, studies utilising *Taq*Man qPCR for the detection of pathogens determine the probability of detecting the pathogen in the presence of background microbiota by generating a standard curve based on CFU/ml. To establish this, a stool sample is spiked with a known concentration of the specific pathogen, following which, DNA is then extracted from the spiked sample and used for qPCR. The detection probability of the specific pathogen at a certain concentration can, therefore, be determined. An attempt was made in this study to determine the minimum level of detection for all three of the investigated pathogens. However, due to several culture and growth technicalities encountered, the minimum detectable level of *C. jejuni* could not be determined. Nevertheless, the minimum detectable levels of *S. enterica* and *C. difficile* were concluded to be around $10^3$ CFU/ml and $10^4$ CFU/ml respectively (data not shown). These detection limits were in accordance with two previously published studies by Malorny *et* al., (2004) and Rinttilä *et al*., (2004).

The selected target region used for the detection of *Salmonella* species was the *ttrRSBCA* locus, located on the *Salmonella* Pathogenicity Island 2, which is critical for the proliferation of *S. enterica* (Hensel, 2000) while also playing an important role in tetrathionate respiration and intestinal colonisation (Rohmer *et al*., 2011). The composition of the endogenous

gastrointestinal microbiota has been shown to influence enteric pathogen colonisation. Sekirov *et al*., (2008) and Lupp *et al*., (2007) both showed that colonisation by *S. enterica serovar typhimurium* increased when the overall Firmicutes to *Bacteroides* ratio had been significantly altered, as a result of antibiotic treatment. Although the results obtained for this study are preliminary, (due to the limitations identified above), the detection of significantly less *S. enterica* in the HIV-positive donors could be due to Bactrim administration. Hamel *et al*., (2008) found that monitoring the number of *E. coli* species resistant to Bactrim was necessary, as it served as a marker for possible resistance amongst other Enterobacteria species such as *Salmonella* and *Shigella*. An increase in the number of resistant *E. coli* species, together with efficient horizontal gene transfer between *E. coli* and Enterobacteria species such as *Salmonella*, could result in an increase in the resistance of Enterobacteria, not only to Bactrim, but to other antibiotics such as ampicillin, cefazolin and gentamicin, which has been shown by Sibanda *et al*., (2011) and Martin *et al*., (1999). The possible development of multiple drug resistance, especially within the context of this study, is of concern, in that one of the recruited donors was receiving a multiple antibiotic treatment plan, in addition to Bactrim and HAART (Chapter 2).

Real-time PCR employing *Taq*Man probes, is a useful method in determining the presence of pathogens present in very low numbers. In order to achieve an accurate result, appropriate gene targets and standard curves are important. Future work should, therefore, involve accurately determining the number of enteric bacterial pathogens present within HIV-positive donors, using more specific primers for *C. difficile* together with a more robust standard curve. Furthermore, in light of the results recorded by Obi *et al*., (2007), future qPCR investigations should consider determining the presence of additional pathogens such as *E. coli*, *Shigella* and *Aeromonas hydrophila* within South African, HIV-positive donors who have not been excluded due to diarrhoea.

## 5.5 CONCLUSION

The results generated in this chapter clearly show that both the SYBR Green and *Taq*Man assays could be successfully used in the quantification of bacterial groups, as well as intestinal pathogens, and provide a representation of the differences in abundance of specific intestinal groups. The results reported in this chapter indicate that prior to the initiation of HAART, the abundance of the *C. leptum* group was significantly reduced in the HIV-positive donors, when compared with the HIV-negative control group ($p = 0.02$). There were, however, no significant differences in the abundance of the *C. coccoides* ($p = 0.42$), *Lactobacillus* ($p = 0.91$), *E. coli* ($p = 0.49$) and *Bifidobacterium* ($p = 0.27$) groups in either donor group.

After 6 months of HAART, a statistically significant reduction in the abundance of certain bacterial groups, relative to the control HIV-negative group, was observed. This may possibly be an indicator of poor viral suppression and inefficient $CD_4^+$ T cell replenishment within the gastrointestinal tract. Future investigations, in particular, for the longitudinal study, should attempt to recruit a larger donor group in conjunction with extending the sampling period to 24 months after HAART initiation. A smaller sample size is affected by host specificity, while a larger donor group would ensure that any individual host variations affecting the outcome of the results generated and conclusions drawn in this section would be eliminated. The study published by Guadalupe *et al*., (2006) showed that, even after 24 months of HAART, HIV-positive patients experienced a minimal increase in $CD_4^+$ T cell levels and viral suppression within the gastrointestinal tract. Therefore, as an extension of this current study, future studies could determine whether the abundance of the dominant groups, which were further reduced after 6 months HAART in this study, is affected further after 24 months of HAART. Furthermore, establishing closer sampling time intervals may assist in identifying significant differences as well as drawing specific conclusions.

As discussed in Chapter 4, Guadalupe *et al*., (2006) showed that inefficient viral suppression and delayed restoration of $CD_4^+$ T cells within the GALT occurred, despite a subsequent decrease in the viral load and increase of $CD_4^+$ T cells in the blood of HIV-positive donors, one year after initiating HAART. In addition, genes associated with inflammation, apoptosis and immune response were significantly upregulated, while a decrease in the expression of genes involved in regulating the epithelial barrier, mucosal repair and regeneration as well as lipid metabolism and nutrient absorption, was reported (Sankaran *et al*., 2005; Guadalupe *et al*., 2003). Continued intestinal inflammation and disruption to the mucosal lining could

continue to affect the dominant bacterial groups within the gastrointestinal tract, despite the fact that patients were being administered HAART and showed indications of improved blood $CD_4^+$ T cell levels and reduced viral load. In light of the above, it may be a worthwhile option to investigate specific immunological markers such as mucosal T lymphocytes, cytokine levels, and peripheral blood mononuclear cells (PBMC) in both faecal and mucosal biopsy samples, while, at the same time, sampling the faecal material.

A drawback of using quantitative real-time PCR is selecting an appropriate gene target and, despite the obvious limitations associated with using the 16S rRNA gene, numerous qPCR based studies have conducted successful investigations with primers designed to target the 16S rRNA gene (Gueimonde *et al*., 2010; Furet *et al*., 2009; Rinttilä *et al*., 2004; Huijsdens *et al*., 2002). Nevertheless, certain studies have opted to focus rather on a single bacterial species from a particular group, thereby eliminating any under or overestimation that may be caused as a result of the number of *rrn* copies. However, since the genome size and copy number of *rrn* operons is limited for the vast majority of species, it can therefore be concluded that in most instances, when using qPCR targeting the 16S rRNA gene, only general estimations and conclusions regarding the population can be made (Vaughan *et al*., 2000; Farrelly *et al*., 1995). The detection of absolute numbers of total bacteria might be improved by employing other previously published primers (Larsen *et al*., 2010).

*Taq*Man qPCR showed that the inclusion of hydrolysis probes increases the overall specificity and sensitivity of the reaction, which is necessary when detecting specific species such as enteric pathogens, present in relatively low numbers. This study also showed that previously published *Taq*Man probes successfully detected two intestinal pathogens present in subclinical levels within HIV-positive donors. Unfortunately, specific conclusions regarding the abundance of *C. difficile* within the donor groups could not be made, due to the fact that the primer set used for the detection of *C. difficile* was not specific. More specific primers are now available and should be used in future studies (Matsuda *et al*., 2012; Luna *et al*., 2011).

The noticeable reduction of *Salmonell*a within the HIV-positive donors, together with the relatively stable abundance of *E. coli* species, 6 months after HAART, suggests a possible increase in the resistance of *E. coli* to Bactrim. While outside the scope of this study, it would be interesting to determine and monitor whether *E. coli*, in addition to *Salmonella*, found within the recruited HIV-positive donors, showed increased resistance to Bactrim and antibiotics such as

ampicillin and gentamicin, which could possibly indicate the transferral of resistance from *E. coli*.

Finally, the significant reduction in the abundance of total bacteria, *C. leptum* and *Bacteroides* groups in the HIV-positive cohort after 6 months of HAART, suggests that probiotic supplementation with specific bacteria from within these groups may be a therapeutic direction to be explored in assisting in normalising the gut microbiota. Species belonging to these dominant bacterial groups could be considered as possible probiotic candidates, as increasing the abundance of these groups within the intestinal tract, may counteract and reduce the disruption and inflammation caused by the replication of HIV. For example, *Faecalibacterium prausnitzii*, a member of the *C. leptum* group, is estimated to make up approximately 10% of the human faecal bacteria (Ramirez-Farias *et al*., 2009; Eckburg *et al*., 2005) and has been shown to be significantly reduced in the gastrointestinal tracts of IBD patients (Van Immerseel *et al*., 2010). The probiotic potential of *F. prausnitzii* has been previously suggested and investigated, as a result of its known anti-inflammatory properties for use in patients suffering from Crohn's disease or IBD (Sokol *et al*., 2008). However, species belonging to the *C. leptum* group within the gastrointestinal tract are phylogenetically diverse and it has not yet been determined which of these species are reduced in the HIV-positive patients evaluated in this study. Therefore, changes to the diversity and abundance of *F. prausnitzii* should be monitored, in the HIV-positive donors, using both PCR-DGGE and qPCR, prior to HAART and 24 months after HAART initiation, in order to determine whether the administration of *F. prausnitzii* might be beneficial in patients if they showed significantly reduced levels.

# CHAPTER SIX

## General Conclusions

The endogenous microbiota of the gastrointestinal tract is a large and complex community that plays an essential role in the maintenance and regular function of the intestinal tract (Ponnusamy *et al*., 2011), development of the host immune system (Kassinen *et al*., 2007) and various other functions involved in human health, such as complex food digestion and the production of essential vitamins (Ponnusamy *et al*., 2011). The structure of the gastrointestinal microbiota of humans has been extensively studied and characterised, using both culture-dependent and culture-independent methods, including PCR-DGGE (Muyzer *et al*., 1993), TGGE (Zoetendal *et al*., 1998), FISH (Franks *et al*., 1998) and more recently, the HITChip (Rajilić-Stojanović *et al*., 2010) and metagenomics (Qin *et al*., 2010). Characterising the endogenous microbial diversity allows the identification of species that can be classified as belonging to the normal microbiota and, in turn, provides a better understanding of changes and imbalances in either the diversity or composition brought about by various diseases. At the start of this project, very little scientific data and information was available regarding the effect of HIV on the intestinal tract and the associated endogenous microbiota. This study utilised some of the abovementioned molecular techniques to analyse aspects of the microbiota found within HIV-positive and HIV-negative donors. This research project is the first investigation to determine the effect of HIV and HAART on selected bacterial species in the gastrointestinal tracts of South African, HIV-positive donors. The results generated have increased the understanding of how HIV may affect specific groups within the endogenous microbiota.

The first objective of this project was to establish if the microbial community of HIV-positive donors differed from HIV-negative donors in terms of diversity and composition. Donor recruitment and sample collection were successfully conducted for both donor groups, providing an accurate representation of the socio-demographic profile of the South African population, as well as the HIV-positive population. Establishing a baseline comparison between the 12 HIV-positive and 12 HIV-negative donors revealed a significant reduction in the diversity of the total bacterial population present in HIV-positive donors. PCR-DGGE analysis of the *Bifidobacterium* and *Lactobacillus* groups showed that the diversity of the *Bifidobacterium* population was higher in the HIV-positive donors than the control group, but

that this difference was not statistically significant. However, a study of a larger cohort would be needed to confirm the validity of this observation. The detection of bacterial species normally closely associated with the intestinal epithelial lining (such as *L. mucosae*), might point towards a disrupted mucosal lining caused by the replication of HIV. To verify whether the mucosal lining of HIV-positive donors is compromised, future investigations should attempt to compare the diversity and abundance of selected bacterial groups in both faecal and biopsy samples. Unfortunately, this particular aspect of work will only be able to be conducted following ethical approval and the implementation of specific safety criteria, both within the laboratory and in the clinic setting.

The second objective of this project was to determine the effect of HIV and HAART on *Bifidobacterium* and *Lactobacillus* species in HIV-positive donors during a 6 month longitudinal analysis. Data generated in this study, using PCR-DGGE, showed that the *Lactobacillus* population was temporally more unstable than the *Bifidobacterium* population in both donor groups. After 6 months of HAART, no significant changes in either the *Bifidobacterium* or *Lactobacillus* populations were seen. However, due to the continuous, disrupted state of the gastrointestinal tract during HAART, as reported by Guadalupe *et al*., (2003), the results generated also emphasised the importance of investigating the composition of and changes in other bacterial groups of the endogenous microbiota. The comparative DGGE study should, therefore, be expanded to include a longitudinal diversity analysis of the total bacterial population as well as other specific bacterial groups of interest.

The third objective of the study was to establish if HIV replication, within the gastrointestinal tract, and HAART have an effect on the abundance of the endogenous microbiota in HIV-positive patients. A quantitative analysis using qPCR was undertaken in order to obtain an overview of the abundance of selected bacterial groups. The key observation made, following analysis of the qPCR data, was that the abundance of the total bacteria as well as two dominant bacterial groups, *C. leptum* and *Bacteroides* was significantly reduced in the HIV-positive donors after 6 months of HAART relative to the HIV-negative donor group. Bacterial species belonging to the *C. leptum* group are actively involved in the production of butyric acid, which serves as an essential energy source for the colonic mucosa (Lara-Villoslada *et al*., 2006; Roediger, 1980), as well as enhancing the integrity of the epithelial barrier (Van Immerseel *et al*., 2010) and modulating the intestinal immune system (Lara-Villoslada *et al*., 2006). A major disruption to this group, as seen in the HIV-positive donors, could potentially

result in the establishment of an imbalanced microbiota composition. Consequently, all beneficial and vital functions performed by this group may be reduced or no longer provided. It was interesting to note that there were no significant changes in the abundance of the *Bifidobacterium* and *Lactobacillus* species throughout the study period. This indicates that these commonly used probiotic candidates would not necessarily improve the microbial composition within the gastrointestinal tract of HIV-positive patients, and that the lack of other key bacteria might point towards identifying more appropriate probiotics. Targeted probiotic supplementation with such bacteria might assist in restoring the beneficial functions and integrity of the gastrointestinal tract.

A review of the relevant literature reveals that several research studies have been conducted, employing similar qualitative and quantitative molecular techniques, to provide further insights into the structure and exact role of the endogenous microbiota in human health and diseases, such as Crohn's disease, IBS and diabetes. A summary, representing a few of these studies, together with the results generated, is briefly discussed below.

Taken together, it is clear that the results obtained in these studies depend to a large degree on the method used. An example of this was clearly seen in the studies of Sghir *et al*., (2000) and Franks *et al*., (1998) who both characterised similar bacterial groups within healthy adult donors, but utilized dot blot hybridization and FISH respectively. The use of two different methods produced varying results when comparing the groups with each other, with results ranging from 0.7 to 3% having been recorded for the *Bifidobacterium* population. The studies conducted by Kerckhoffs *et al*., (2009) and Manichanh *et al*., (2006), both showed a reduction in the abundance of *C. leptum*, *C. coccoides* and *Bifidobacterium*, when analysing the microbial conditions present during IBS and Crohn's disease respectively. A similar trend was shown by Bartosch *et al*., (2004), who also reported significant reductions in the total bacteria, as well as the *Bacteroides* and *C. leptum* groups, when using qPCR to investigate the effect that aging and a weakened immune system have on the endogenous microbiota. Although not technically a "disease condition", the process of aging has been shown to seriously affect the composition of the endogenous microbiota (Biagi *et al*., 2010). Type 2 diabetes was also associated with considerable changes in the composition of the intestinal microbiota (Larsen *et al*., 2010). The abundance of the Firmicutes was significantly lower in the diabetic patients relative to the non-diabetic group. Conversely, the Bacteroidetes and Proteobacteria were present in a much higher abundance. The abundance of *F. prausnitzii*, a

member of the *C. leptum* group, was also found to be significantly reduced in the gastrointestinal tracts of IBD and Crohn's patients as well as the elderly (Van Immerseel *et al*., 2010). The probiotic potential of *F. prausnitzii* has been previously suggested and widely investigated, for use in patients suffering from Crohn's disease or IBD, due to its known anti-inflammatory properties (Sokol *et al*., 2008). In light of this, future studies focusing on the microbiota of HIV-positive patients should evaluate the levels of *F. prausnitzii* with a view to determining whether increasing the abundance of this species within the intestinal tract of HIV-positive patients, may counteract and reduce the disruption and inflammation caused by the replication of HIV.

The fourth objective of this project was to detect any pathogen activity within the HIV-positive donors, due to the fact that the microbial composition has been altered and may no longer be functioning as a protective barrier. Preliminary analysis indicated that the *Taq*Man probes successfully detected the presence of *Salmonella* species in both groups of donors, but at a higher level in the HIV-negative cohort. It would appear, however, that the levels were not high enough to cause diarrhoea in either group. A future aspect of this work would be to investigate the presence and activity of other bacterial pathogens in HIV-positive patients with diarrhoea symptoms and in those without diarrhoea. Secondly, it is imperative that the increasing resistance of the intestinal microbiota, as well as intestinal pathogens, towards antibiotics such as Bactrim, doxycycline, ampicillin and gentamicin, within HIV-positive donors, be carefully monitored, especially in view of the fact that doctors working at local South African provincial clinics will continue to administer these antibiotics for treatment purposes.



The findings reported in this thesis show that differences do indeed exist in the microbiota of HIV-positive and HIV-negative donors and, in most cases, the disrupted microbial conditions found within the gastrointestinal tract did not improve, even after 6 months of HAART. It is important to remember that this particular project was the starting point of a projected larger investigation. The results obtained thus far have identified the need for additional investigative studies into the intestinal microbiota of HIV-positive patients. Notwithstanding certain shortcomings identified in this project, the data generated can be used as a foundation upon which future characterisation studies can build. The combination of PCR-DGGE and qPCR produced reliable and accurate comparative results within the study regarding the relative composition of the microbiota of South African HIV-positive donors as compared to

HIV-negative individuals. However, in order to verify our findings and acquire a more robust and in depth analysis of the intestinal microbiota, it would be valuable to complement these two methods with other profiling techniques such as the HITChip and high throughput sequencing. This research project could also be further expanded to include samples collected from HIV-positive donors residing outside of the Western Cape, such as the remaining eight South African provincial provinces or internationally. The main motivation for this would be to determine if diet and lifestyle, which are both known to influence the structure and diversity of the gastrointestinal microbiota, will affect the differences identified in 12 HIV-positive donors from a single study site in the Western Cape. Additionally, it may also be worthwhile extending the longitudinal study to cover a longer time period, for example 2 to 3 years after HAART initiation, along with shorter, more consistent sampling points.

The final and most important aim of this project was to identify any significant changes in the intestinal microbiota profile that could necessitate the administration of probiotics. The results showing the lack of improvement in the abundance of specific dominant bacterial groups after 6 months of HAART, suggest that probiotic supplementation may well assist in normalising the intestinal microbiota and could possibly re-establish a healthy gastrointestinal balance. However, before proceeding with any form of probiotic supplementation, prior investigations into the diversity and abundance of several probiotic candidates, for example *F. prausnitzii*, need to be conducted prior to HAART and during HAART. Furthermore, a particular stage at which the probiotic can be safely administered, as well as the mode of delivery to be used, needs to be identified. Upon successful establishment of these criteria, controlled clinical trials involving HIV-positive volunteers could be initiated.

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# APPENDIX A

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# APPENDIX B

**Patient Information Sheet:**
Investigation of qualitative and quantitative changes in selected gut microbiota of HIV positive patients during HAART treatment.

**Investigators:** A. Prof VR Abratt [1], A. Prof SJ Reid[1], Dr M Mendelson[2], Dr M. Pandie[2], Dr M. Handschur[1]
[1] Department of Molecular and Cell Biology, University of Cape Town; [2] Department of Medicine, University of Cape Town

**What are we trying to do?**

- We are researchers from Groote Schuur Hospital and the University of Cape Town. We are interested in finding out ways of helping people suffering from HIV by protecting them from developing the unpleasant and painful symptoms of diarrhoea ("runny tummy").
- In order to do this, we need to understand what bacteria (germs) in the body cause these symptoms and what good bacteria might help to prevent the bad bacteria from making people sick.
- We know that these good bacteria are important for our health, and we think that people who are suffering from HIV and AIDS may have lost their good bacteria because of the HIV infection and treatment with antibiotics. We think that during antiretroviral treatment, the good bacteria may once again begin to grow as the virus is killed, and that these bacteria can help a person recover.
- We would, therefore, like to look more closely at the bacteria in your gut before and during your antiretroviral treatment to see if the good bacteria begin growing again.
- The only way we can do this is to find them in your stool sample. This may seem unpleasant, but it is a very helpful way to see what is going on in your gut.
- The results will help us find out which good bacteria are missing from people who have AIDS. In future, these good bacteria might be given in food supplements to people who have AIDS to help them recover faster during their antiretroviral treatment.

**What will you be asked to do?**

- Your doctor will tell you about the study and ask you if you would like to take part.
- If you agree to this, you will be asked some general questions about your age and medical history.
- You will be given a large, very clean tin container in which to collect your stool on the day of your clinic visit. This container is big enough to sit on and can be tightly sealed so there will be no mess or bad smell.
- You will also be given an attractive cooler bag in which to transport your tin so it is kept private.

**Are there any risks involved for you if you take part in the study?**

- You will not be asked to change your treatment in any way.
- There are no risks involved.
- There will be a minimum of discomfort by having to use the tin to collect your stool.
- All the stool samples will be destroyed after we have finished studying them.
- No-one will know your results or your personal information. Your name will not be given to anyone. The results of the study will be made available to the scientific community but no names will be linked to any of the results.
- You can change your mind about taking part in the study at any time without giving a reason and this will not affect the quality of your HAART treatment in any way. You are not obliged to take part in the study.

**What will you get if you take part?**

- There is no payment or reward for taking part in the study but you will be given R20 to help you with transport when you bring your sample to the clinic
- You will be helping us understand why AIDS sufferers get such severe "runny tummies". We hope that what we learn will help in the treatment of this condition.

**Patient Consent Sheet**

**Do you agree to participate in this trial?**

**Please ask any questions you have about the study.**

**Your contact person for this study is Dr Marc Mendelson: Tel 021 4066079.**

I _____(**Name in block capitals**) have carefully read and understood the Patient Information Sheet and Informed Consent or have had these read and explained to me. All my questions about the study have been answered to my complete satisfaction. I understand that I can keep copies of these documents. I was given enough time to decide about taking part in the study.

By signing the Informed Consent Sheet, I declare that I take part in this study of my own free will and without being persuaded or pressurised by another person. I understand that I can withdraw from the study at any time without giving a reason.

I document my informed consent by signing my name below:

_____ _____ _____

Participant Signature Date Place

I _____(**Name of researcher or his/her delegate**) have fully informed the above person of the procedures, aims and risks of the study and answered all questions truthfully.

_____ _____ _____

Researcher/delegate Signature Date Place