A randomised double blind placebo controlled trial to determine the effect of soluble dietary fibre (inulin-type fructans) on disease progression and body composition of HIV positive ARV naive adults attending a wellness clinic in KZN South Africa

BY

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PREFACE

The research contained in this thesis was completed by the candidate while based in the Discipline of Dietetics and Human Nutrition, School of Agriculture, Earth and Environmental Sciences, College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg, South Africa from March 2013 to December 2013 under the supervision of Professor Anna Coutsoudis.

The research was financially supported by the International Atomic Energy Agency and the National Research Foundation.

The contents of this work have not been submitted in any other form to another university, and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

I, Professor Anna Coutsoudis, agree to the release of this thesis for examination.

Signed: Acoutsoudis Date: 16/11/2015

Professor Anna Coutsoudis (supervisor)

DECLARATION

I, Chara Biggs, declare that:

(i) the research reported in this dissertation, except where otherwise indicated or acknowledged, is my original work;

(ii) this dissertation has not been submitted in full or in part for any degree or examination to any other university;

(iii) this dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons;

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(v) where I have used material for which publications followed, I have indicated in detail my role in the work;

(vi) this dissertation is primarily a collection of material, prepared by myself, published as journal articles or presented as a poster and oral presentations at conferences. In some cases, additional material has been included;

(vii) this dissertation does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the dissertation and in the References sections.

Signed: CBiggs Date: 16/02/2016

Chara Biggs (candidate)

ABSTRACT

The prevalence of HIV in South Africa is one of the highest in the world. Although those with CD4 counts of ≤350 cells/mm^3 were eligible for the sophisticated antiretroviral program offered by the country, the execution has been poor with many of those entitled to antiretroviral therapy (ART) being unable to access it. Strategies therefore which prolong wellness and delay the need for the initiation of ART would reduce the burden on the public health care system. As ART does not fully suppress the state of chronic immune activation characteristic of HIV, which has been identified as the predominant driving force behind the progression to the acquired immunodeficiency syndrome (AIDS), strategies to attenuate this activation are critical. As effective viral load suppression does not prevent the chronic activation of the immune system, factors other than the HIV virus contribute significantly with the focus having recently shifted to the potential role of the gastrointestinal tract (GIT). The most extensive CD4 T cell depletion occurs in the GIT at all stages of HIV infection as the majority of these cells express the primary HIV co-receptor chemokine receptor 5 (CCR5) which permits HIV entry and viral replication in the cell. The current hypothesis suggests that this disruption in the permeability of the GIT results in increased microbial translocation in HIV which in turn drives the chronic immune system activation and subsequent progression to AIDS. Strategies which target the integrity of the GIT could reduce microbial translocation and the associated chronic immune activation thereby impeding the progression to AIDS. Inulin-type fructans, which are well researched reputable prebiotics, exert an established protective effect on the GIT as they prevent pathogenic adhesion, function in an antioxidant capacity and are both bifidogenic and butyrogenic. Although inulin-type fructans therefore may prevent microbial translocation by defending the integrity of the GIT, their effectiveness in HIV has not been investigated. Promising preliminary evidence in HIV has demonstrated that prebiotics other than inulin-type fructans manipulate the microbiome to a less pathogenic profile; reduce microbial translocation; and improve immunity. Research investigating the potential benefit of supplementation with inulin-type fructans is critical as they may offer a strategy to promote wellness delaying the initiation of ART as well as serving as a valuable adjunct to ART. The purpose of this research was to determine the impact of inulin-type fructans on nutritional status, immunity, morbidity, microbial translocation and inflammation both systemic and of the GIT. Permission to conduct the randomized double blind placebo controlled study was

granted by the eThekwini Municipality Health Unit and ethics approval was granted by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BFC 145/010). Asymptomatic HIV-infected ART naive black African males and females 18 years or older (CD4 count >350 cells/mm^3) were recruited from Lancers Road Clinic, Durban during March to December 2013. The participants were randomised to receive either 15 g of inulin-type fructans daily or placebo for a period of 3 months. Their nutritional status, as represented by changes in body composition and biochemical markers (haemoglobin, albumin) was monitored pre and post study. The advanced body composition method of deuterium dilution as determined by the International Atomic Energy Agency (IAEA) was used to determine changes in fat- and fat free-mass. Immunity was measured by changes in CD4 count; morbidity by changes in the incidence of infections and episodes of diarrhoea; microbial translocation by changes in sCD14; systemic inflammation by changes in C reactive protein (CRP); and GIT inflammation by changes in the novel analysis of genetic faecal messenger ribonucleic acid (mRNA) markers of inflammation. These markers included polymeric immunoglobulin receptor (PIGR), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), superoxide dismutase 2 (SOD2), interleukin-8 (IL8), interleukin 1 beta (IL1B) and faecal calprotectin (S100A8). Monthly changes in gastrointestinal symptoms were measured using the gastrointestinal symptom rating scale (GSRS) in combination with the Bristol Stool Chart. Forty one (48.8%) of the 84 participants received inulin-type fructans. The dietary mean intake of inulin-type fructans was 4.14 g/day (±1.51). The majority were female (77, 91.7%), mean age 35.2 years old (±9.3) with a mean CD4 count of 542.5 (±144.8) cells/mm^3. Study participants were overweight (21/84, 25.0%) or obese (39/84, 46.4%) and body composition comprised of 44.0% (±18.7) fat and 56.0 % (±20.8) fat-free mass. Most were constipated (33, 39.3%), only 9 (10.7%) reported diarrhoea. No study participants complained of chronic diarrhoea. There were no significant changes in the means ($p > 0.05$) pre and post study in the inulin-type fructans versus control group for albumin [35.8 (±3.0) to 36.3 (±4.1) g/l versus 36.6 (±4.4) to 36.5 (±3.7) g/l]; Hb [11.7 (±1.7) to 12.2 (±2.6) g/dl versus 12.3 (±1.5) to 12.7 (±1.3) g/dl]; CD4 [519.9 (±142.6) to 503.2 (±169.5) cells/mm^3 versus 564.2 (±145.2) to 529.9 (±170.3) cells/mm^3]; CRP [5.3 (±7.3) to 6.8 (±12.7) mg/dl versus 5.6 (±7.3) to 5.0 (±6.1) mg/dl]; and sCD14 [1.6104 (±0.5842) to 1.6175 (±0.6231) µg/ml versus 1.5665 (± 0.5897) to 1.6194 (±.5897) µg/ml]. There was no significant correlation between levels of CRP, albumin, Hb and CD4 count. Hypoalbuminemia was present in 27 (32.1%) and mild iron deficiency anaemia in 36 (42.8%).

Only 4 (4/41, 10%) faecal samples contained significant levels of GAPDH (≥25 copies) and 21 (51.3%) contained levels of ≥1.5 copies, which is the lowest level of detection. In this group of 21 stool samples, more in depth analysis of genetic faecal mRNA markers was undertaken. There were no significant changes ($p > 0.05$) in the mean values pre and post study for inulin type fructans versus controls in all markers studied: PIGR [4.5446 (±3.5382) to 3.0665 (±2.2868) versus 3.9296 (±3.2363) to 2.5989 (±2.9172)]; IL8 [0.8032 (±1.1261) to 0.5002 (±0.5760) versus 0.6665 (±0.9395) to 0.7452 (±1.1363)]; IL1β [0.7272 (±1.3149) to 0.1795 (±0.1410) versus 0.4288 (±0.5136) to 1.1589 (±1.8913)]; S100A8 [0.5369 (±0.9134) to 0.2111 (±0.1512) versus 0.3939 (±0.5213) to 0.5898 (±0.6870)]; and SOD2 [0.6463 (±1.5062) to 0.2308 (±0.2052) versus 0.5023 (±0.5631) to 1.3848 (±2.2656)]. Although there was no statistical difference those on inulin-type fructans showed a discernible trend to a reduction in inflammation while those on control mostly showed an increase. Nutritional status, as reflected by body composition, haemoglobin and serum albumin, was not altered by supplementation with inulin-type fructans. The high levels of both overweight and obesity reflected the trends of the general population in KZN questioning the assumption that involuntary weight loss is inevitable prior to ART. This asymptomatic group was at a deceptively high risk of progression to AIDS, morbidity and mortality as a consequence of the high prevalence of hypoalbuminemia, mild iron deficiency anaemia and raised levels of inflammation despite a mean CD4 count of above 500 cells/mm^3. Supplementation did not reduce systemic inflammation and microbial translocation nor improve immunity. Increased GIT permeability/inflammation was not demonstrated as the genetic inflammatory markers were not detected in significant quantities, there was no evidence of increased cell shedding and constipation, *not diarrhoea*, was the most common gastrointestinal complaint. These results do not support the current belief that increased GIT permeability/inflammation in asymptomatic HIV is an important contributing factor in AIDS progression. As the rationale was that supplementation with inulin-type fructans would offer a local protective effect on the GIT thereby reducing microbial translocation and the chronic immune stimulation, the absence of GIT permeability/inflammation would explain the lack of effect of inulin-type fructans. As a slight but discernible trend was seen in the reduction of mild inflammation despite the very small sample size, the focus of the research should probably shift to the role of inulin-type fructans in symptomatic HIV.

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DEDICATION

"Two people are better off than one, for they can help each other succeed.

If one person falls, the other can reach out and help.

But someone who falls alone is in real trouble.

A person standing alone can be attacked and defeated,

but two can stand back-to-back and conquer.

Three are even better,

for a triple-braided cord is not easily broken"

(Ecclesiastes 4:9-12).

This thesis is dedicated to Mandy Read, a true friend
who has stood the test of time and
a sister in Jesus, the third cord who surpasses.

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CHAPTER 1: INTRODUCTION, THE PROBLEM AND ITS SETTING

1.1 IMPORTANCE OF THE STUDY

South Africa has both the highest incidence of Human Immunodeficiency Virus/Acquired Immune Deficiency Syndrome (HIV/AIDS) and the largest antiretroviral therapy (ART) program globally (Johnson 2012) (Evans 2013) (UNAIDS 2013). Although theoretically access to ART has improved significantly, practically coverage is poor. Approximately one out of two of those eligible actually receive ART, due to significant obstacles to access including the increased burden on the public healthcare system (Herbst *et al* 2009) (Johnson 2012) (Evans 2013) (Nyasulu *et al* 2013). This delay in the introduction of ART, the well-documented side effects of ART as well as the potential for the development of resistant HIV strains makes it essential that safe, therapeutic options for prolonging wellness are investigated (González-Herrera *et al* 2015).

The chronic immune activation, characteristic of HIV (Sandler and Douek 2012), is suggested to be the prime factor that depletes CD4 levels, which results in the malfunctioning of the immune system, in turn driving the progression to AIDS (Brenchley *et al* 2006b). The translocation of microbial products across the HIV damaged gastrointestinal barrier (Haruta *et al* 2007) (Ziegler *et al* 2008) (Jiang *et al* 2009) (Clarke *et al* 2010) (Sandler and Douek 2012) binds to the innate immune cells activating a cascade which ultimately results in the production of the pro-inflammatory mediators which drive the chronic immune activation (Sandler and Douek 2012). Even in the presence of effective ART and suppressed viral load, chronic immune activation still persists and therefore adjunct therapy to address this is essential.

The gastrointestinal barrier is thought to be damaged by the HIV virus itself, by chronic gastrointestinal inflammation, recurrent episodes of diarrhoea, repeated use of antibiotics, decreased immunoglobulin A (IgA) concentrations in the intestinal lumen as well as an abnormal composition of the gastrointestinal microflora (Kotler *et al* 1984) (Budhraja *et al* 1987) (Brenchley *et al* 2006a) (Lauritano *et al* 2010) (Sandler and Douek 2012) (Mutlu *et al* 2014). Prebiotics such as inulin-type fructans have been shown to reduce gastrointestinal inflammation; reduce episodes of diarrhoea; increase faecal IgA levels; and improve the composition of the gastrointestinal

microbiome and therefore may have the potential to moderate the chronic systemic immune activation (Schaafsma and Slavin 2015). Inulin-type fructans have a well-established bifidogenic effect (González-Herrera *et al* 2015) (Schaafsma and Slavin 2015). *Bifidobacteria* in turn exert an antimicrobial effect by producing increased concentrations of short chain fatty acids (SCFA) which reduce colonic pH. Other effects include; the release of antimicrobial factors; immune modulation increasing the host resistance to pathogens; and restoration of the disturbed microbiome (Sekine *et al* 1985) (Gibson and Roberfroid 1995). Prebiotics therefore may prolong wellness by a localized protective effect on the gastrointestinal tract (GIT) thereby reducing microbial translocation and systemically stimulating the immune system.

The only two studies that investigate the role of prebiotics in the treatment of HIV demonstrate promising preliminary evidence that they may be a therapeutic option in antiretroviral naive (ARV naive) HIV-infected adults. The Italian Clinical Trial with Oligosaccharides Powder for Application in HIV-1 infection (COPA) showed a disturbed intestinal microbiome with an increased pathogenic profile early on in HIV infection (Gori *et al* 2008a). Supplementation with a synthetic prebiotic mixture fueled *Bifidobacteria* growth and decreased faecal pathogenic load in conjunction with an upregulation of the immune system and a reduction in the markers of microbial translocation. The more recent multicenter Blinded Nutritional Study for Immunity and Tolerance Evaluation (BITE) trial found that prebiotic supplementation significantly reduced the decline in CD4 cell count and lowered the levels of CD4 T cell activation (Cahn *et al* 2013). Since prebiotics may offer a safe, well tolerated intervention (González-Herrera *et al* 2015) that potentially protects and restores the HIV damaged gastrointestinal tract, it is essential that further research is conducted to determine the possible benefits of supplementation particularly as to date there have been very limited investigations.

Inulin-type fructans are the most well established prebiotics and modulate the immune system both locally and systemically (Roberfroid 2001) (Rossi *et al* 2005). Although research has clearly demonstrated their beneficial properties, they have never been evaluated in the context of HIV. The goal of this study was to investigate the effect of supplementation with inulin-type fructans on nutritional indices, immunity, morbidity and inflammation both systemically and locally in GIT in asymptomatic HIV-infected adults.

1.2 PURPOSE OF THE STUDY

To determine the effect of a soluble dietary fibre (inulin-type fructans) on disease progression and body composition of HIV positive ARV naive adults attending a wellness clinic in KwaZulu-Natal (KZN), South Africa.

1.3 TYPE OF STUDY

A double blind randomized placebo controlled trial (RCT).

1.4 STUDY OBJECTIVES

Primary objective:

1.4.1 To compare changes in microbial translocation by detecting changes in the concentrations of both lipopolysaccharide (LPS) and soluble CD14 (sCD14) in participants in the inulin-type fructans versus placebo group.

Secondary objectives:

1.4.2 To compare changes in GIT inflammation by detecting changes in selected genetic faecal messenger ribonucleic acid (mRNA) markers in the inulin-type fructans versus placebo group. The mRNA markers included polymeric immunoglobulin receptor (PIGR), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), superoxide dismutase 2 (SOD2), interleukin-8 (IL8), interleukin 1 beta (IL1B) and faecal calprotectin (S100A8).

1.4.3 To determine changes in nutritional status by comparing changes in body composition using anthropometry [to determine body mass index (BMI), mid upper arm circumference (MUAC) and triceps skinfold thickness (TST)] and isotope dilution methodology [to determine fat and fat free mass] and changes in levels of serum albumin and haemoglobin (Hb) in the inulin-type fructans versus placebo group.

1.4.4 To compare the change in the levels of CD4 count (immune marker) and C-reactive protein (CRP) (inflammatory marker) in the inulin-type fructans versus placebo group.

1.4.5 To compare morbidity (incidence of infections and diarrhoea) in the inulin-type fructans versus placebo group.

1.5 HYPOTHESES

1.5.1 Decreased GIT inflammation/permeability would result in decreased microbial translocation in those taking inulin-type fructans which would reduce the levels of LPS and sCD14 suppressing the chronic state of inflammation resulting in an increase in CD4 count and a decrease in serum CRP.

1.5.2 The mRNA markers of GIT inflammation would decrease due to the local protective effect on the GIT of the inulin-type fructans.

1.5.3 Fat free mass and serum albumin levels of those on inulin-type fructans would improve due to a decrease in the incidence of infections and diarrhoea because of the localized immune effect of inulin-type fructans on the GIT and the displacement of pathogens.

1.5.4 Serum Hb levels would increase due to an improvement in the absorption of iron from the modulation of GIT inflammation by the inulin-type fructans and because of a reduction in the incidence of infections.

1.6 STUDY PARAMETERS

All asymptomatic HIV-infected ARV naive adults who attended the Lancers Road Clinic, Durban, South Africa from March 2013 to December 2013 whose CD4 count was >350 cells/mm^3 and who complied with the inclusion and exclusion criteria were included.

1.7 STUDY LIMITATIONS

Financial constraints prevented a more inclusive analysis of markers of inflammation and GIT permeability as many of these were not routinely tested in South Africa and the cost of analysis overseas was prohibitive.

A further limitation was being unable to determine whether the participants did indeed routinely consume the inulin-type fructans on a daily basis during the course of the study since we had no objective measure and relied on participants report on adherence to the study product.

1.8 STUDY ASSUMPTIONS

1.8.1 That the GIT of the participants would be inflamed

1.8.2 That they would present with diarrhoea

1.8.3 That inulin would have a bifidogenic effect

1.8.4 That the procedures used to detect changes in inflammation accurately measured this

1.8.5 That the South African National Accreditation Service of South Africa (SANAS) credited laboratories who performed the CD4 count, albumin and CRP analysis followed standard operating procedures (SOP)

1.8.6 That the participant routinely consumed the inulin-type fructans/placebo for the duration of the study

1.8.7 That the stool samples received from the participant belonged to the participant as the actual defecation process was not overseen

1.9 DEFINITION OF TERMS

Adaptive immune system	Also known as the acquired immune system - creates a specific response to a specific pathogen which provides the host with a highly specific response to subsequent exposures to the pathogen via the lymphocytes (B cells and T cells) – the system includes both humoral and cell mediated immunity
Bifidobacteria	Gram positive, anaerobic bacteria from the phylum *Actinobacteria* which are capable of fermenting inulin-type fructans by breaking the glycosidic bonds (Ventura *et al* 2010)
Bifidogenic	A substrate that specifically promotes the growth of *Bifidobacteria* in the GIT tract of mammals
Commensal microbiota	Are the normal indigenous microbiota found on the epithelium exposed to the external environment (Tlaskalová-Hogenová *et al* 2004)
Degree of polymerization	Is usually defined as the average number of base units per molecule if the molecules are composed of regularly repeating units, or as the average number of monomeric units per molecule (Technology 2002)
Dysbiosis	Is where potentially pathogenic microbes are dominant predisposing to disease (Roberfroid *et al* 2010)
Endotoxin unit	Is a measure of the activity of the endotoxin as the activity of different endotoxin preparations may not be similar to another of the same weight. The use of an endotoxin unit therefore allows comparison between different endotoxin tests assayed at individual laboratories as the use of the unit deals with the challenge of varying potencies of different endotoxins. Results of tests in EU/ml is not converted to units of weight of endotoxin per ml.

Fructooligosaccharides	Are synthetic mixtures of short chain inulin-type fructans synthesized from sucrose. Each chain has a glucose molecule and between one to three fructose molecules with a maximum degree of polymerization (DP) of 4 and an average DP of 3.6. They consist therefore of very short chain lengths with a higher amount of glucose and free sugar content (Kelly 2008)
Frutafit® HD	Is a native inulin consisting of mainly linear fructose polymers mostly with a terminal glucose unit with an average DP of 8 to 13. It contains greater than 90% inulin-type fructans and less than 10% free sugars
Glyceraldehyde-3-phosphate dehydrogenase	A housekeeping gene/transcript that is present in every cell in reasonably consistent amounts
Immunosenescence	Natural gradual deterioration of the immune system
Innate immune system	Otherwise known as the nonspecific immune system as the cells respond to pathogens in a generic fashion without offering long-lasting or protective immunity which enables the provision of an immediate defense. The innate immune system consists of both humoral and cell mediated components
Interleukin	Any class of glycoproteins produced by the leucocytes for regulating the immune responses
Inulin	The native extract consists of 92% inulin-type fructans with chains of between 2 to 60 molecules in length (average DP 10 to 12). Approximately 10% has a DP less than 4, about 20% has a DP of 4 to 9 and the rest is above 9 (Kelly 2008). Six to ten percent consists of free sugars (fructose, glucose, sucrose) (Kelly 2008)
Inulin HP	High molecular weight inulin which has been further purified to contain only long chains of between 10 to 60 molecules with an average DP of 20 to 25. Sometimes referred to as long chain fructooligosaccharides (lcFOS) rather than inulin (Kelly 2008)

Inulin-type fructans	Inulin-type fructans are a mixture of oligosaccharides and polysaccharides containing two or more fructose units joined by a β(1→2) fructosyl-fructose glycosidic bond (Kelly 2008). There may be a terminal glucose molecule and the fructose polymers may be linear or branched (Kelly 2008)
IL1β	A cytokine which is an important mediator of the inflammatory response and the increased production of which leads to a variety of auto inflammatory syndromes
Interleukin 8	An important mediator of the innate immune response, which induces both chemotaxis and phagocytosis
Karnofsky performance scale/score	Is a tool used to assess the person's ability to function and perform daily living activities
Metagenomics	Study of genetic material recovered directly from environmental samples as opposed to studying cultivated clonal cultures as in traditional microbiology and microbial genome sequencing and genomics
Microbial translocation	Movement of microbial products (bacteria/peptidoglycan/ lipoteichoicacid/flagellin/rDNA/lipopolysaccharide/fungi) from the intestinal lumen across the GIT into the systemic circulation in the absence of overt bacteremia (Sandler and Douek 2012)
Microbiome	Refers to the microbiota, their genes and surrounding environmental conditions although the term is often interchanged with microbiota
Microbiota	Includes all forms of microbial life in the GIT
Native inulin	Minimally processed inulin extracted from the chicory root as opposed to other forms of inulin which have been processed to remove varying chain lengths or else purified to remove the free sugars
Oligofructose	Refers to the product obtained from the partial hydrolysis of inulin. Chain lengths are below 10 molecules and may or may not contain glucose (Kelly 2008). Despite clear distinctions

	between FOS and oligofructose the terms are often loosely interchanged in research leading to confusion when comparing outcomes (Kelly 2008)
Polymeric immunoglobulin receptor	An Fc receptor which is a protein found on the cell surface of certain immune system cells such as NK killer cells, macrophages and neutrophils. The Fc receptor binds to antibodies (immunoglobulin) attached to infected cells/invading pathogens in turn stimulating antibody-mediated phagocytosis/cell-mediated cytotoxicity
Prebiotic	An unabsorbed substrate which is selectively fermented in the colon resulting in beneficial health promoting changes in both the composition and activity of the hosts gastrointestinal microbiota by promoting the growth and activity of probiotic bacteria (Gibson and Roberfroid 1995)
Probiotic	Live microorganisms that offer health benefits to the host when consumed in adequate amounts (Sanders 2008). In essence the Greek work bios means life therefore the pre bios (prebiotic) is before life and the pro bios (probiotic) means supporting life. The prebiotic precedes the probiotic
Raftiline	Chicory derived inulin which has had the 8% sugars (fructose, glucose and sucrose) which are normally found in inulin removed – the product contains a longer chain 100% inulin with a DP of 22–25
Raftilose	Chicory derived oligofructose powder which is inulin based with a DP of 3–7
S100A8	Gene coding for faecal calprotectin which is made in large quantities by neutrophils and released into the intestines in excess when there is any inflammation present and is detectable in the stools as a marker of inflammation
Superoxide dismutase 2	Is an enzyme which catalyses the partitioning of the superoxide radical into either oxygen or hydrogen peroxide

1.10 ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
ART	Antiretroviral therapy
ARV	Antiretrovirals
BHT	Butylated hydroxytoluene
BITE	Blinded Nutritional Study for Immunity and Tolerance Evaluation
BMI	Body mass index
CCR5	Chemokine receptor 5
CDC	Centre for Disease Control
Cfu	Colony forming units
COPA	Clinical trial with Oligosaccharides Powder for Application in HIV-1 infection
CRP	C-reactive protein
CTCAE	Common terminology clinical adverse events
D_2O	Deuterium oxide
DDMRI	Doris Duke Medical Research Institute
DdPCR	Digital droplet polymerase chain reaction
Dl	Deciliter
DNA	Deoxyribonucleic acid
DP	Degree of polymerization
DRI	Dietary reference intake
DXA	Dual-Energy X-ray absorptiometry
EDTA	Ethylenediaminetetraacetic
ELISA	Enzyme-linked immunosorbent assay
EndoCab	Endotoxin-core antibody
EU	Endotoxin unit
FAM	6-carboxyflourescein
FAO	Food and Agriculture Organization of the United Nations
FIRST	Flexible Initial Retrovirus Suppression Therapies Trial
FISH	Fluorescence in situ hybridization

FOS	Fructooligosaccharides
FRAM	Fat Redistribution and Metabolic Change
FTIR	Fourier transform infrared spectroscopy
G	Grams
GALT	Gut associated lymphoid tissue
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GIT	Gastrointestinal tract
GOS	Galactooligosaccharides
GPR	G-protein-coupled receptors
G-protein	Guanine nucleotide-binding proteins
GRAS	Generally recognised as safe
GSRS	Gastrointestinal Symptom Rating Scale
H	Hydrogen
^{2}H	Deuterium
Hb	Haemoglobin
HIV	Human immune-deficiency virus
HLA	Human leukocyte antigen
IAEA	International Atomic Energy Agency
ICONA	Italian Cohort Naive Antiretroviral
I-FABP	Intestinal fatty acid binding protein
IFN	Interferon
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IgA	Immunoglobulin A
IL	Interleukin
IL1B	Interleukin 1 beta
INH	Isoniazid
IQR	Interquartile range
IRIS	Immune reconstitution inflammatory syndrome
Kg	Kilograms
KZN	KwaZulu-Natal

LAL	Limulus amebocyte lysate
lcFOS	Long chain fructooligosaccharides
LPS	Lipopolysaccharides
M	Meter
Mg	Milligrams
MIRA	Methods for Improving Reproductive Health in Africa
Ml	Microliters
Mm	Millimeters
Mmu	Milli mass unit
mRNA	Messenger ribonucleic acid
MUAC	Mid upper arm circumference
Ng	Nanogram
NHLS	National Health Laboratory Services
NI	Not included
NIDS	National Income Dynamics Study
NIH DAIDS	National Institute of Health, Division of AIDS
NK cell	Natural killer cell
Nm	Nanometer
NO	Nitrous oxide
NOD	Nucleotide-binding oligomerization domain
pAOS	Pectin hydrolysate
PCR-DGGE	Polymerase chain reaction denaturing gradient gel electrophoresis
Pg	Picogram
PI	Principal investigator
PIGR	Polymeric immunoglobulin receptor
PP	Peyer's patches
PTFE	Polytetrafluoroethylene
qPCR	Quantitative real-time polymerase chain reaction
RCT	Randomised controlled trial
rDNA	Ribosomal deoxyribonucleic acid
RIF	Rifampicin

RNA	Ribonucleic acid
Rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
RWISA	Rwandan Women's Interassociation Study and Assessment
S100A8	Faecal calprotectin
SALDRU	Southern Africa Labour and Development Research Unit
SANAS	South African National Accreditation System
SANHANES	South African National Health and Nutrition Examination Survey
SAS	Statistical Analysis Software
sCD14	Soluble CD14
SCFA	Short chain fatty acids
scFOS	Short chain fructooligosaccharides
scGOS	Short chain galactooligosaccharides
SCOPE	Study of the Consequences of the Protease Inhibitor Era Trial
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SHIME	Simulator of the human intestinal microbial ecosystem
SMART	Strategies for the Management of Anti-Retroviral Therapy
SOD2	Superoxide dismutase 2
SOP	Standard operating procedures
TB	Tuberculosis
TBARS	Thiobarbituric acid reactive substances
TBW	Total body water
TEER	Trans-epithelial electrical resistance
TGF	Transforming growth factor
THUSA	Transition and Health During Urbanisation of South Africans
TLR	Toll like receptor
TNF-α	Tumour necrosis factor-alpha
TST	Triceps skinfold thickness
UNAIDS	Joint United Nations Programme on HIV/AIDS
VCT	Voluntary counseling and testing

V_D	Volume of distribution
WHO	World Health Organization
WIHS	Women's Interagency HIV Study

CHAPTER 2: LITERATURE REVIEW

2.1 INTRODUCTION

South Africa has both the highest incidence of HIV/AIDS as well as the largest ART program globally (Johnson 2012) (Evans 2013) (UNAIDS 2013). Although theoretically access to ART has improved significantly, practically coverage is poor. Approximately one out of two of those eligible actually receive ART due to significant obstacles to access, including the increased burden on the public healthcare system (Herbst *et al* 2009) (Johnson 2012) (Nyasulu *et al* 2013) (Evans 2013). This delay in the introduction of ART, the well-documented side effects, as well as the potential for the development of resistant HIV strains makes it essential that safe therapeutic options (González-Herrera *et al* 2015) for prolonging wellness are investigated.

The chronic immune activation, which is characteristic of HIV infection (Sandler and Douek 2012), is proposed to be the prime factor that depletes the CD4 T cell levels, causing the malfunctioning of the immune system resulting in the progression to AIDS (Brenchley *et al* 2006b). One of the potential factors driving the chronic immune activation is thought to be the translocation of microbial products across the damaged gastrointestinal barrier (Jiang *et al* 2009) (Sandler and Douek 2012) which then bind to the innate immune cells activating a cascade which ultimately results in the production of the pro-inflammatory mediators (Sandler and Douek 2012). It has been recently proposed that preventing the microbial translocation across the damaged gastrointestinal barrier may be an effective strategy in treatment (Sandler and Douek 2012).

The gastrointestinal barrier is thought to be damaged by the HIV virus itself, the frequent state of chronic gastrointestinal inflammation, the recurrent episodes of diarrhoea, the repeated use of antibiotics, the decreased concentrations of faecal IgA, as well as an abnormal composition of the gastrointestinal micro flora (Kotler *et al* 1984) (Budhraja *et al* 1987) (Brenchley *et al* 2006a) (Lauritano *et al* 2010) (Sandler and Douek 2012) (Mutlu *et al* 2014). Prebiotics, such as inulin-type fructans, have the potential to moderate the chronic systemic immune activation by modifying the composition of the gastrointestinal flora as their bifidogenic and butyrogenic effect is well documented (Gori *et al* 2011) (González-Herrera *et al* 2015) (Schaafsma and Slavin 2015). Inulin-

type fructans modulate the local and systemic immune system both directly and indirectly to enhance immune protection (Roberfroid *et al* 2010) (Schaafsma and Slavin 2015). Emerging research of the therapeutic potential of inulin-type fructans is promising and the preliminary evidence indicates the potential to offer an affordable, safe and well tolerated adjunct therapy in HIV infection.

The scope of this literature review initially focusses on the human microbiome and the changes experienced in HIV infection. As prebiotics positively modulate the microbiome and could therefore be of benefit in the treatment of HIV infection, both the general and immune specific benefits of inulin-type fructans are reviewed. As inulin type fructans protect and promote intestinal health possibly preventing bacterial translocation, the evidence that bacterial translocation is a driving factor in the progression to AIDS is considered in HIV infection. The detrimental impact of HIV infection on the gut associated lymphoid tissue (GALT), GIT permeability and structural abnormalities is discussed in conjunction with the evidence from research studies that supplementation with pre and probiotics may have a role in modulating this damage.

2.2 GASTROINTESTINAL TRACT MICROBIOME

As HIV targets the GIT from early on in the disease (Brenchley and Douek 2012) and the complex interactions of the microbiome with its host promotes both the health of the GIT and general well-being (Servin 2004) (Apanavicius *et al* 2007), it is feasible that the colonic microbiome could play an important role in the course of HIV infection.

2.2.1 Colonic microbiome composition of a healthy adult

The colonic bacteria outnumber the cells in the human body by up to two fold and the concentration of bacterial genes is at least a hundred times greater than that of the human genome (Brenchley and Douek 2012). The microbes in the human GIT are comprised of over 400 species, most of which are situated in the colon (Servin 2004) (Brenchley and Douek 2012). The colon hosts approximately 10^{14} microorganisms with 10^{12} microbes per gram of colonic content (Brenchley and Douek 2012). At least 60% of the faecal weight consists of bacteria (Stephen and Cummings 1980). The microbiota composition of the faeces and the microbiota adherent to the mucosa are dissimilar (Zoetendal *et al* 2002) (Eckburg *et al* 2005) (McKenna *et al* 2008).

Most of the bacteria are strict anaerobes with up to 90% composed of the mostly gram positive phylum *Firmicutes*[1] and the gram negative genus *Bacteroides* (Gibson and Roberfroid 1995). Other common gram positive bacteria include *Lactobacilli, Eubacteria, Clostridia* and *Cocci* (Gibson and Roberfroid 1995) (Salminen *et al* 1998). *Bifidobacteria*[2] usually contribute up to 10% of the colon microbiota (Duncan *et al* 2003) with *Bifidobacterium adolescentis*, *B. longum* and *B. catenulatum* being the predominant species in the adult (Matsuki *et al* 1999).

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1 All bacteria belong to the kingdom Prokaryotae which is further divided into phyla which are subdivided into class, order, family, genus (eg *Bifidobacteria*) and finally species (eg *adolescentis*). The divisions are based on similarities in genetics, structure, chemistry, physiology and ecology.

2 *Bifidobacteria,* from the phylum *Actinobacteria* (a dominant sub group), are gram positive, non motile usually branched anaerobes some strains of which have been identified as important probiotics. The prebiotic action of inulin-type fructans is thought to partly exert its effect via promoting the growth of these bacteria therefore *Bifidobacteria* are an important part of this review.

Broadly the colonic bacteria are divided into those that are pathogenic and those that are beneficial. The pathogenic bacteria, which cause diarrhoea, infections, liver damage, carcinogenesis and intestinal putrefaction, usually belong to the genus *Clostridia* and *Bacteroides* (Roberfroid *et al* 2010). Those that are beneficial usually belong to the genus *Lactobacilli* and *Bifidobacteria* (Gibson and Roberfroid 1995). *Bifidobacteria* are considered to play an important role in the maintenance of a healthy and stable microbial ecosystem (Schaafsma and Slavin 2015) and concentrations of *Bifidobacteria* raised above the norm could even be regarded as a marker of intestinal health (Roberfroid *et al* 2010). The composition of the GIT microbiome should comprise of benign and potentially health promoting microbes rather than those pathogenically inclined (Roberfroid *et al* 2010). Each individual has their own widely varied unique composition of sub species although the core composition is normally stable (Simon and Gorbach 1984).

The microbiome is influenced by many known factors including the genetic makeup of the host, geography, diet, sex, age, pH, transit time, peristalsis, mucin secretions containing Ig, bacterial antagonism, medication and disease (Kerckhoffs *et al* 2006) (McKenna *et al* 2008) (Spor *et al* 2011) (Sharifuzzaman 2014). Suffering from a disease such as HIV, with the associated medications including ART and antibiotics, could detrimentally disrupt the microbiome.

2.2.2 Colonic microbiome composition in HIV

Little is known regarding the composition of the microbiome in HIV infection (Mutlu *et al* 2014). Comparison between studies is complex as some studies have analyzed faecal and others mucosal samples. The mucosal microbiome is varied at different sites on the colon so the area of biopsy may be a confounding factor (McKenna *et al* 2008). Some studies have focused on specific bacteria rather than the microbiome as a whole. More sophisticated sequencing techniques have recently become available which confuses the comparison with the earlier studies. The study populations were usually very small and included both ARV naive and those on ART.

2.2.2.1 Composition of the faecal microbiome in HIV

The COPA trial was one of the first to study the HIV microbiome in some detail using both fluorescence in situ hybridization (FISH) and quantitative real-time polymerase chain reaction (qPCR) (Gori *et al* 2008a). In a study population similar to that in our study, they found that the baseline faecal composition of 57 asymptomatic HIV-infected ARV naive adults (mean CD4 520 cells/mm^3) was disturbed from early on in HIV infection with an increased pathogenic profile when compared to other studies of healthy adults (Gori *et al* 2008a) (Table 1).[3] Of the faecal samples from those infected with HIV, 92% contained *Pseudomonas aeruginosa* (versus 20% in the general population) and all contained *Candida albicans* (Gori *et al* 2008a). Ten-fold higher concentrations of *P. aeruginosa*, 10 000-fold greater concentrations of *C. albicans*, half the expected levels of *Bifidobacteria* (2.5%) and almost undetectable levels of *Lactobacilli* (0.02%) were found in those infected with HIV (Gori *et al* 2008a). They concluded that the composition of the microbiome was both qualitatively and quantitatively altered in HIV with increased concentrations of opportunistic pathogens and decreased concentrations of both *Bifidobacteria* and *Lactobacilli,* both of which have been shown to benefit the mucosal immune function and GIT health (Gori *et al* 2008a). Strategies to increase the population of *Bifidobacteria* with supplementation of a known bifidogenic prebiotic such as inulin-type fructans may therefore promote wellness in those infected with HIV.

A pilot study using 16S ribosomal deoxyribonucleic acid (rDNA) amplification on the faecal samples of 16 HIV-infected adults (ARV naive 10/16, CD4 418 cell/mm^3) reported a very weak trend to an increased population of the pro-inflammatory *Enterobacteriales* (includes *P. aeruginosa*) when compared to healthy controls (Ellis *et al* 2011). In the ARV naive only, the frequency of *Enterobacteriales* was approximately 10-fold higher when compared to controls which confirms the results of the COPA trial.

A South African study (Cape Town) analysed total bacterial diversity (RNA polymerase chain reaction denaturing gradient gel electrophoresis PCR-DGGE) then focused on selected bacterial species (qPCR) from the faecal samples of 12 HIV-infected adults (CD4 <200 cells/mm^3) prior to

[3] This table has been included as a summary at the end of this section.

initiating ART and 12 HIV-uninfected controls (Du Plessis 2012). A significant reduction in the total bacterial population diversity was found in the HIV-infected. In contrast to the COPA trial however, no significant difference was found in either the abundance or diversity of *Bifidobacterium* or *Lactobacillus* between those infected and the controls. They concluded that supplementation with *Bifidobacteria* therefore would not be beneficial. This observation however was based on the assumption that these concentrations are optimal for those suffering from HIV and that increasing them above the norm would offer no benefit.

The concentrations of the beneficial butyrate producing *Clostridium leptum* was significantly reduced in those infected with HIV. The faecal samples of 8 of these HIV-infected adults were reassessed after 6 months of successful ART and compared to 8 HIV-uninfected controls. No significant changes were detected in the populations of either *Bifidobacterium* or *Lactobacillus* although concentrations of *C. leptum* and *Bacteriodes*[4] was significantly reduced compared to controls. Supplementation with a butyrogenic prebiotic, such as the inulin-type fructans, may counteract the reduction in *C. leptum* concentrations as inulin-type fructans have been shown to increase *C. leptum* concentrations in rats (Parnell and Reimer 2012) and piglets (Paßlack *et al* 2015).

The faecal bacterial population of 13 recently HIV-infected men before the initiation of ART (CD4 559 cells/mm^3) and after 48 weeks on ART was pyrosequenced using bacterial 16S rDNA (Pérez-Santiago *et al* 2013). Before ART initiation, greater concentrations of *Lactobacillales*[5] in the distal gut was significantly associated with lower levels of sCD14, a marker of microbial translocation, lower viral loads, higher CD4/CD8 T-cell ratio, CD4% and CD4 count implying a protective effect of the bacteria. No association was found with LPS, an additional marker of microbial translocation. There was no apparent relationship between *Lactobacillales* and either human leukocyte antigen-DR+ (HLA-DR+) or CD38+ T cells (markers of lymphocyte activation) or percentage of Ki67+ of CD4 T cells (marker of CD4 lymphocytes). There were no significant changes during ART. The higher proportions of gut *Lactobacillales* therefore was associated with

4 Genus of gram-negative, obligate anaerobic bacteria which normally have a beneficial relationship with the host if they are contained in the GIT but cause significant pathology on translocation. They produce beneficial substances such as succinic acid.

5 Lactic acid bacteria (including *Lactobacillus)* which are generally regarded as safe for addition to food as probiotics.

less microbial translocation, less systemic immune activation, less GIT T lymphocyte proliferation, and a higher CD4% in the GIT. They concluded that manipulating the composition of the colonic microbiota could contribute to improved immune function in HIV (Pérez-Santiago *et al* 2013).

Faecal bacterial 16S rRNA sequencing in 25 HIV-infected adults (ARV naive 14/25 CD4 551 cell/mm^3) showed significant colonic bacterial changes (Lozupone *et al* 2013) the ARV naive, there was an increased diversity of bacterial composition with a significant decrease in *Bacteroides* and an increase in *Prevotella*[6] when compared to thirteen healthy controls. There was no consistent impact of ART on bacterial composition, as the microbiota of those on long term ART resembled either that of the ARV naive or the healthy controls (Lozupone *et al* 2013).

The preceding studies are summarized in Table 1 and although results are conflicting, in general there appeared to be a disturbed faecal microbiome with an increase in potential pathogens (*P. aeruginosa, C. albicans, Prevotella*) and a decrease in beneficial bacteria (*Bifidobacteria, Lactobacilli, C. leptum).* The genus *Lactobacillales* appeared to protect against microbial translocation and systemic immune activation. The impact of successful ART on the faecal microbiome varied. Supplementation with inulin-type fructans may improve the faecal microbiome in the ART naive, as well as those on ART, and therefore protect against microbial translocation.

[6] Gram negative obligate anaerobic who can be an opportunistic pathogen in humans.

Table 1: Comparison of the studies investigating the faecal microbiome in HIV-infected adults

Authors	Sample	CD4 (cells/mm^3)	*Pseudomonas aeruginosa*	*Candida albicans*	*Bifidobacteria*	*Lactobacilli/ Lactobacillales*	*Clostridium leptum*	*Bacteriodes*	*Prevotella*
(Gori *et al* 2008a)	ARV naive 57/57	520	Ten-fold higher	10 000-fold higher	Half that expected	Almost undetectable	NI*	NI	NI
(Ellis *et al* 2011)	ARV naive 10/16	418	Ten-fold higher in ART naive	NI	NI	NI	NI	NI	NI
(Du Plessis 2012)	ARV naive 12/12	<200	NI	NI	No difference	No difference	Reduced	NI	NI
	After 6 months ART		NI	NI	No difference	No difference	Reduced	Reduced	NI
(Pérez-Santiago *et al* 2013)	ARV naive 13/13	559	NI	NI	NI	Higher concentrations associated with less microbial translocation, systemic immune activation, GIT T lymphocyte proliferation, and a higher CD4% in GIT	NI	NI	NI
	48 weeks post ART		NI	NI	NI	No significant changes	NI	NI	NI
(Lozupone *et al* 2013)	ARV naive 14/25	551	NI	NI	NI	NI	NI	NI	Increased in ARV naive On ART resembled either ARV naive or healthy controls

*NI stands for not investigated

2.2.2.2 Composition of the mucosal microbiome in HIV

The composition of the rectal mucosal microbiota was analysed using barcoded 16S Illumina deep sequencing in 20 ARV naive adults (mean CD4 439 cells/mm^3), twenty on ART (mean CD4 534 cells/mm^3) and 20 healthy controls (McHardy *et al* 2013). The ARV naive had significantly increased concentrations of the opportunistic pathogens *Fusobacteria, Anaerococcus, Peptostreptococcus* and *Porphyromonas.*

There were significantly reduced concentrations of the commensal bacteria *Roseburia*[7], *Coprococcus, Ruminococcus, Eubacterium, Alistipes* and *Lachnospira.* Inulin-type fructan supplementation stimulates the growth of *Roseburia,* which are a genus of beneficial butyrate producing bacteria (De Vuyst *et al* 2014). A similar mucosal profile to the ARV naive was found in those on ART but this did not reach significance suggesting that ART impacted, but did not completely restore, the composition of the mucosal microbiota (McHardy *et al* 2013) (Table 2).

Vujkovic-Cvijin *et al* (2013) used high-resolution bacterial community profiling to identify bacteria adherent to the rectosigmoid mucosa of 25 HIV-infected adults (ARV naive 6/25, mean CD4 356.3 cell/mm^3, ART 18/25 mean CD4 374.5 cell/mm^3, non-progressor 1/25 mean CD4 505 cell/mm^3) and 9 healthy controls (Vujkovic-Cvijin *et al* 2013). Similarly to other studies, the total bacterial load did not differ significantly between the study groups but the microbiota composition of the ARV naive significantly differed to the controls. The ARV naive has increased numbers of the potential pathogens *Proteobacteria* (includes *Salmonella, Escherichia, Serratia, Shigella, Klebsiella, Vibrio, Helicobacter, Yersinia*) as well as *Staphylococcus, Pseudomonas* and *Campylobacter* at the expense of the *Bacteroidaceae.* Although not significantly associated with CD4 counts, GIT HIV RNA or DNA levels, the enriched taxa correlated with increased levels of T cell activation (particularly the *Enterobacteriaceae* family), soluble plasma markers of inflammation including interferon (IFN)-inducible protein-10, soluble tumour necrosis factor (TNF) receptor II and interleukin (IL)-6), diminished levels of mucosal T cells secreting IL-17 and IL-22 and increased tryptophan catabolism through the kynurenine pathway (particularly the *Staphylococcaceae*). Tryptophan catabolites via the kynurenine pathway of tryptophan

[7] *Roseburia* is a anaerobic gram positive butyrate releasing bacteria.

metabolism inhibits the differentiation of IL-17 secreting CD4+ T cells. Interleukin-17 helps promote gut integrity possible reducing microbial translocation. Raised kynurenine/tryptophan ratios found at baseline in 40 HIV-infected patients was positively associated with CD8+ T-cell activation and increased levels of inflammatory cytokines (IL-6, INF-γ–inducible protein 10, IL-18, TNF-α) and negatively associated with dendritic cell frequencies at baseline (Jenabian *et al* 2015). The key cytokines TNF-α, and IL-10 are crucial to the control of the magnitude of both the immune and inflammatory response to infections (Xiong *et al* 2004). In contrast the microbiota of those on ART in the study by Vujkovic-Cvijin et al (2013) was widely varied with some being similar to the controls and others to the ARV naive. Antiretroviral therapy therefore did not restore the colonic microbiome. The only one HIV-infected long-term non-progressor (stable peripheral blood CD4, 21 years infected) had a mucosal colonic microbiota comparable to the controls suggesting that non-progression may be linked to the composition of the GIT microbiome (Vujkovic-Cvijin *et al* 2013).

Mutlu *et al* (2014) used sequencing technologies to document the microbial composition of 21 HIV-infected adults on ART (mean CD4 425 cells/mm^3) versus HIV-uninfected controls (Mutlu *et al* 2014). In addition to faecal samples, the terminal ileum, right colon and left colon were sampled during a colonoscopy. The microbiome of those HIV-infected was found to be less diverse particularly on the mucosal surfaces. There was a significant increase in potentially pathogenic bacteria (*Brachyspira*, *Campylobacter*, *Catenibacterium*, *Escherichia, Enterobacteriaceae*, *Fusobacteriaceae*, *Mogibacterium*, *Prevotella* and *Ralstonia*) and a loss of a variety of the commensal bacterial genera.

Dillon *et al* (2015) found a significant decrease in concentrations of *Bacteroides* and increased concentrations of *Prevotella* at the genus level and increased amounts of *Proteobacteria* and reduced concentrations of *Firmicutes* at the phylum level in colon biopsies and faecal samples using bacterial 16S rDNA sequencing in 18 HIV-infected ARV naive adults (mean CD4 425 cells/mm^3) versus 14 healthy controls. The increase in *Prevotella* was significantly related to increased concentrations of activated colonic T cells and myeloid dendritic cells indicating increased mucosal cellular activation and blood T cell activation (Dillon *et al* 2015).

The preceding studies are summarized in Table 2 and generally show an increase in pathogenic bacteria and a decrease in beneficial and commensal bacteria on the mucosa of the HIV-infected adult which is not restored by ART. The increased concentrations of these pathogenic bacteria have been significantly associated with increased T cell activation and inflammation. Manipulation of the microbiome to a less pathogenic profile by supplementation with inulin-type fructans may therefore reduce T cell activation and inflammation and may offer an adjunct therapy to those on ART and the ARV naive (Vesterbacka *et al* 2013; Gori *et al* 2011).

Table 2: Comparison of the studies investigating the mucosal microbiome of HIV-infected adults

Authors	Sample	CD4 (cells/ mm³)	*Fusobacteria*	*Anaerococcus*	*Peptostreptococcus*	*Porphyromonas*	*Roseburia*	*Coprococcus*	*Ruminococcus*	*Eubacterium*	*Alistipes*	*Lachnospira*	*Proteobacteria*
McHardy *et al* 2013	ARV naive 20/40	439-534	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	NI
	ART 20/40		↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	NI
Vujkovic-Cvijin *et al* 2013	ARV naive 6/25	356	NI*	NI	NI	NI	NI	NI	NI	NI	NI	NI	↑
	ART 18/25	375	NI	NI	NI	NI	NI	NI	NI	NI	NI	Varied widely and either resembled the ART naive or the healthy controls	
	non-progressor 1/25	505	NI	NI	NI	NI	NI	NI	NI	NI	NI	Similar to the healthy controls	
Mutlu *et al* 2014	ART 21/21	425	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
Dillon *et al* 2014	ARV naive 18/18	425	↑	NI	NI	NI	NI	NI	NI	NI	NI	NI	↑

Authors	Sample	CD4 (cells/ mm³)	*Staphylococcus*	Pseudomonas	Campylobacter	*Bacteroidaceae*	*Brachyspira*	*Catenibacterium*	*Escherichia*	*Entero bacteriaceae,*	*Prevotella*	*Ralstonia*	*Firmicutes*
McHardy *et al* 2013	ARV naive 20/40	439-534	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
Vujkovic-Cvijin *et al* 2013	ARV naive 6/25	356	↑	↑	↑	↓	NI	NI	NI	NI	NI	NI	NI
	ART 18/25	375	Varied widely and either resembled the ART naive or the healthy controls				NI	NI	NI	NI	NI	NI	NI
	non-progressor 1/25	505	Similar to the healthy controls				NI	NI	NI	NI	NI	NI	NI
Mutlu *et al* 2014	ART 21/21	425	NI	NI	↑	NI	↑	↑	↑	↑	↑	↑	
Dillon *et al* 2014	ARV naive 18/18	425	NI	NI	NI	↓	NI	NI	NI	NI	↑		↓

* NI = not investigated. Direction of arrow indicates an increase ↑ or decrease ↓

2.2.2.3 Effect of antibiotics on the HIV microbiome

Antibiotics affect the colonic microbiome of HIV-uninfected adults (Jakobsson *et al* 2010). Due to opportunistic pathogens taking advantage of the immune suppression, antibiotics are commonly prescribed for those with HIV. The WHO and the Joint United Nations Programme on HIV/AIDS (WHO/UNAIDS) has recommended the use of the antibiotic co-trimoxazole in Africa for those who are classified as WHO clinical stages 2 to 4 or who have a CD4 count <500 cells/mm^3 or a total lymphocyte count equivalent, as a prophylactic strategy to reduce morbidity and mortality (World Health Organization 2000). The effect on the microbiome has not been well researched.

Overall, despite the difficulties in comparison between studies, the profile of the faecal and mucosal colonic microbiome of HIV-infected adults appears to have shifted towards a more pathogenic proinflammatory composition (Lozupone *et al* 2014) which has been associated with increased T cell activation and inappropriate tryptophan catabolism. Reduced concentrations of the commensals included those with established health benefits such as *Bifidobacteria, Lactobacillales* and the butyrate producers *Roseburia and C. Leptum.* These shifts in the microbiome were not reversed by successful ART. Restoring the profile of the microbiome could promote wellness in the HIV-infected by strengthening the GIT defenses (Wilson *et al* 2013).

Modulators of the microbiome, such as prebiotics and probiotics, may therefore offer a promising adjunct therapy to those on ART and the ARV naive (Vesterbacka *et al* 2013) (Gori *et al* 2011).

2.3. DEFINITION OF PREBIOTICS AND PROBIOTICS

A prebiotic is a unabsorbed substrate which is selectively fermented in the colon resulting in beneficial changes in both the composition and activity of the host's gastrointestinal microbiota by promoting the growth and activity of probiotic bacteria (Gibson and Roberfroid 1995). Probiotics are live microorganisms that offer health benefits to the host when consumed in adequate amounts (Sanders 2008). In essence the Greek work bios means life therefore the pre bios (prebiotic) is before life and the pro bios (probiotic) means supporting life. The prebiotic precedes the probiotic.

Prebiotic supplementation may offer an advantage over probiotic supplementation as the targeted bacteria should already be established in the colon. The bacteria in the probiotic however still have to survive the precarious passage down the GIT before reaching the colon (Hopkins *et al* 1998). When research involves an economically challenged community such as in this study population, the use of prebiotics is more practical as they do not require refrigeration or other specific storage needs. Inulin-type fructans are the most extensively researched prebiotic (Kolida and Gibson 2007).

2.4 INULIN-TYPE FRUCTANS

The inulin-type fructans meet all the criteria to permit classification as a prebiotic (Gibson and Roberfroid 1995), have scientifically well documented prebiotic properties (Rossi *et al* 2005) (Roberfroid *et al* 2010) (Franco-Robles and López 2015) and seem to produce the best prebiotic effects (Roberfroid 2001).

2.4.1 Definition, sources and metabolism

Understanding the structure of the various inulin-type fructans is essential as this determines their beneficial impact in the colon. Inulin-type fructans are a mixture of oligosaccharides and polysaccharides containing two or more fructose units joined by a β(2→1) fructosyl-fructose glycosidic bond (Kelly 2008) (Figure 1). There may be a terminal glucose molecule and the fructose polymers may be linear or branched (Kelly 2008).

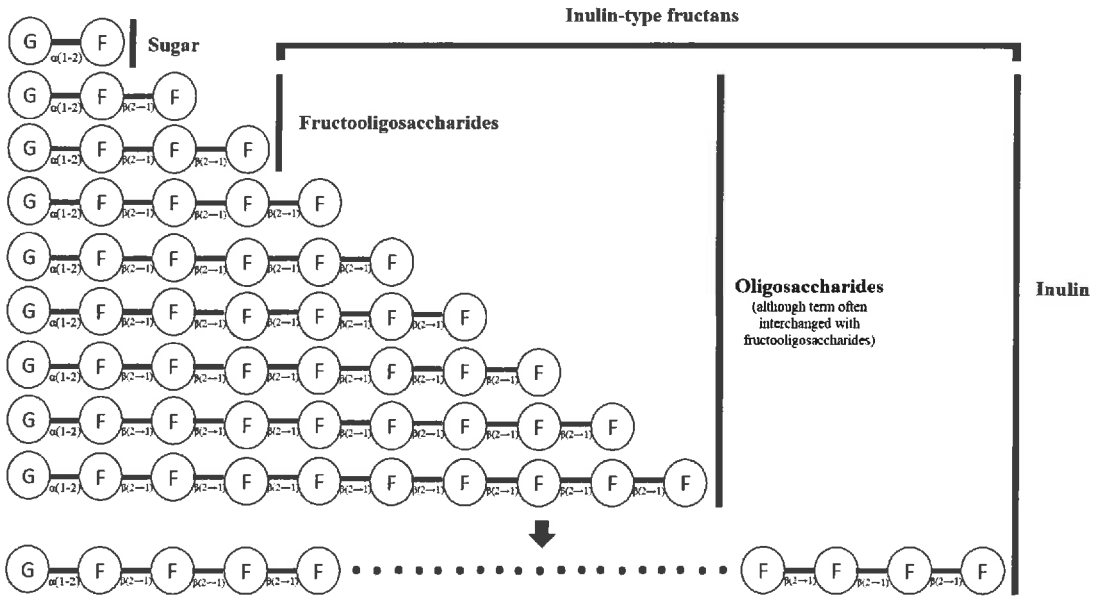

Figure 1: Schematic representation of inulin-type fructans

A variety of nomenclature exists for inulin-type fructans and no consensus has been reached regarding standard terminology (Kelly 2008). Generally the term inulin-type fructans refers to fructooligosaccharides (FOS), oligofructose and inulin. The classification is primarily based on chain length which is referred to as the DP.

Fructooligosaccharides are synthetic mixtures of short chain inulin-type fructans synthesized from sucrose. Each chain has a glucose molecule and between one to three fructose molecules with a maximum DP of 4 and an average DP of 3.6. They consist therefore of very short chain lengths with a higher amount of glucose and free sugar content (Kelly 2008). Oligofructose refers to the product obtained from the partial hydrolysis of native inulin. Chain lengths are below 10 molecules and may or may not contain glucose (Kelly 2008). Despite clear distinctions between FOS and oligofructose the terms are often loosely interchanged in research leading to confusion when comparing outcomes (Kelly 2008).

Native inulin is hot water extracted from chicory roots (Gibson and Roberfroid 1995) although onion, banana, Jerusalem artichoke, cereal plants and some grasses such as wheat also contain

inulin in reasonable quantities (Van Loo *et al* 1995) (Moshfegh *et al* 1999). This minimally processed inulin contains chains with and without glucose. The extract consists of 92% inulin-type fructans with chains of between 2 to 60 molecules in length (average DP 10 to 12). Approximately 10% has a DP less than 4, about 20% has a DP of 4 to 9 and the rest is above 9 (Kelly 2008). Six to ten percent consists of free sugars (fructose, glucose, sucrose) (Kelly 2008).

The study product, Frutafit® HD, is a native inulin consisting of mainly linear fructose polymers mostly with a terminal glucose unit with an average DP of 8 to 13 (Appendix A). It contains greater than 90% inulin-type fructans and less than 10% free sugars. Frutafit has been classified as Generally Recognised As Safe (GRAS) by the Food and Drug Administration (Food and Drug Administration 2002).

Highly processed purified inulin contains 99% as opposed to 92% inulin-type fructans, and although different, both are termed inulin. High molecular weight inulin (inulin HP) has been further purified to contain only long chains of between 10 to 60 molecules with an average DP of 20 to 25. Confusingly this is sometimes referred to as lcFOS rather than inulin (Kelly 2008). Other commercial mixtures are available where inulin or inulin HP has been enriched with FOS and there is no consensus regarding the nomenclature.

An understanding of the physiological impact of inulin-type fructans is complex, as their effect depends on chain length which determines their site of fermentation and therefore where they exert their effect both locally on the mucosa and on the microbiome (Rossi *et al* 2005) (Franco-Robles and López 2015). Although structurally similar, the chain length of FOS (short chain), oligofructose (short to medium chain) and inulin (mainly long chain) determines the rate of the saccharolytic fermentation (Franco-Robles and López 2015). When using in vitro-cultured colon microbiota from the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) reactor, FOS was more rapidly metabolized in the proximal colon while inulin was metabolized in both the proximal and distal colon (Van de Wiele *et al* 2007). Inulin (particularly high molecular weight) is fermented more slowly. The slow release is thought to encourage a prolonged beneficial effect in the distal colon (Den Hond *et al* 2000) (Schaafsma and Slavin 2015) potentially discouraging harmful proteolytic fermentation (Schaafsma and Slavin 2015). Inulin from different

manufacturers have been shown to exert different effects as some have higher molecular weights and differing degrees of purification.

The inconsistent use of terms, as well as the use of a wide variety of inulin-type fructans at different doses for different lengths of time and the wide range of parameters measured compounds the difficulties in interpreting the scientific literature (Rycroft *et al* 2001) (Kelly 2008). Despite this both the bifidogenic and butyrogenic effects (Guigoz *et al* 2002) (Gibson *et al* 2004) (Macfarlane *et al* 2006) (Schaafsma and Slavin 2015) (De Vuyst *et al* 2014) and the resulting positive impact on human health is backed by robust evidence (De Vuyst *et al* 2014).

2.4.2 Bifidogenic and butyrogenic effect

Inulin-type fructans are unique in that on entering the colon intact, they are fermented by selective probiotic bacteria capable of breaking the glycosidic linkage (Roberfroid 1993) (Delzenne and Roberfroid 1994) (Roberfroid *et al* 1998) (Roberfroid 1999). The glycosidic linkage therefore enables the growth of specific microbes to be targeted.

All inulin-type fructans promote the growth of most strains of *Bifidobacteria* (Wang and Gibson 1993) hence the term "bifidogenic effect" although an individual's response to the identical dose of the same inulin-type fructan differs with respect to the increase in number and strains of *Bifidobacteria* (Kelly 2008). The varying chain lengths however influence the ability of different strains of *Bifidobacteria* to utilize the inulin-type fructans which is probably a mechanism to avoid competition between each other and other species (McKellar and Modler 1989) (Rossi *et al* 2005). Inulin (Raftiline) for example stimulated the growth of *B. longum* to a greater degree than *B. pseudolongum* and *B. catenulatum* (Hopkins *et al* 1998). Most *Bifidobacteria* appear to prefer FOS while only some utilize inulin (McKellar and Modler 1989) (Rossi *et al* 2005). The inulin degraders release FOS and fructose which cross feeds the *Bifidobacteria* of other strains (Rossi *et al* 2005) (Huebner *et al* 2007). It has become apparent that both inulin and FOS selectively stimulate the growth of other intestinal species including *Lactobacilli*, *Bacteroides*

Faecalibacterium prausnitzii[8] and *Roseburia* (Rossi *et al* 2005) (Van der Meulen *et al* 2006) (Huebner *et al* 2007) (Ramirez-Farias *et al* 2009). As *Bifidobacteria* themselves do not produce butyrate, it is assumed that the butyrogenic effect of the inulin-type fructans comes from enhancing the growth of the butyrate releasing bacteria such as *Roseburia* (De Vuyst *et al* 2014). Bacteria utilizing FOS tend to release large amounts of acetate which also cross feeds the butyrate producing bacteria (De Vuyst *et al* 2014).

The bifidogenic and butyrogenic effects offers both general health benefits and those specific to immune defense, many of which are pertinent to those suffering from HIV.

2.4.3 Health benefits

The effects need to be considered as a whole, as it is very difficult to separate the contribution of the inulin-type fructans themselves or the microbes they stimulate or the butyrate released. Often studies investigating the benefits of the inulin-type fructans did not concurrently investigate the impact on the microbiome to determine which was exerting the effect.

2.4.3.1 General health benefits

There are many general health benefits which include:

- Restoring the microbiome to a more health promoting profile (Roberfroid *et al* 2010)
- Stimulating salt and water absorption in the colon (Schaafsma and Slavin 2015)
- Suppressing proteolytic fermentation therefore decreasing the formation of toxic endpoints such as ammonia and p-cresol (De Preter *et al* 2008)
- Decreasing the concentrations of secondary bile acids (Welters *et al* 2002)
- Enhancing vitamin synthesis and dietary mineral absorption (calcium, magnesium, iron) which impacts bone health (Ohta *et al* 1993) (Miyazawa *et al* 1996) (Younes *et al* 2001) and which may impact iron deficiency anaemia (O'Brien *et al* 2005). This may be of particular importance in countries like South Africa where many cannot afford to regularly

[8] One of the most highly metabolic and abundant bacteria in the intestinal microbiota of healthy adults and is considered to be an important component of a healthy microbiota.

consume calcium and iron rich foods in sufficient amounts (Charlton *et al* 2005) (Napier and Oldewage-Theron 2015)

- Lowering of plasma triglycerides and low density lipoprotein cholesterol and enhancing secretions of both the satiety hormones and GIT peptides, potentially lowering the risk of atherosclerotic disease and obesity. As ART is known to predispose to cardiac disease and a large percentage of the South African population is obese, this may be particularly beneficial (Sartorius *et al* 2015)
- Improving bowel function and modulating intestinal motility (Dass *et al* 2007). Many with HIV experience GIT related disturbances such as diarrhoea, bloating and flatulence (Wilson *et al* 2013) which inulin-type fructans may resolve

2.4.3.2 Immune specific health benefits

Inulin-type fructans appear to enhance immune protection by modulating both the local and systemic immune system (Roberfroid *et al* 2010) (Schaafsma and Slavin 2015). They seem able to exert a direct effect on the immune system in their own capacity, in addition to their bifidogenic and butyrogenic effects (Roberfroid *et al* 2010) (Nauta and Garssen 2013). The immune specific benefits include their capacity to function as an antioxidant, increased pathogen resistance and the production of SCFA. What follows is a brief discussion of the immune specific benefits which is elaborated further in the text specifically in relation to HIV.

2.4.3.2.1 Antioxidant activity

Inulin-type fructans scavenge reactive oxygen species[9] and so may protect the colonic mucosa from oxidative attack (Franco-Robles and López 2015). A 30% oligosaccharide mixture demonstrated dose dependent anti-oxidant capacity in vitro when compared to the well-established action of the antioxidants butylated hydroxytoluene (BHT) and α-tocopherol (Li *et al* 2007). The percent inhibition of the superoxide radical generation was as follows: BHT (85%)> α-tocopherol

[9] Reactive oxygen species include superoxide anion, hydroxyl radical and non-radical molecules such as hydrogen peroxide and singlet oxygen.

(67%) > oligosaccharides (47%). The scavenging activity on 2,2-diphenyl-1-picrylhydrazyl[10] was oligosaccharides (87%) > BHT (71%) > α-tocopherol (36%) and on hydrogen peroxide was BHT (83%) > oligosaccharides (69%) > α-tocopherol (56%) (Li *et al* 2007). In aged Kunming mice oligosaccharide supplementation demonstrated a strong free radical scavenging capacity by significantly decreasing lipid peroxidation, increasing the total antioxidant capacity as well as increasing the activity of superoxide dismutase and glutathione peroxidase in all organs (Li *et al* 2007). Rats supplemented over a 4 week period with a massive amount[11] of FOS (Raftilose P95 DP 4.8) showed significantly lower indirect measures of oxidative stress as the Thiobarbituric Acid Reactive Substances (TBARS) values were reduced in both plasma and urine (Busserolles *et al* 2003).

Increased production of nitrous oxide (NO) facilitates the antimicrobial activity of activated macrophages. Murine macrophage-like RAW 264.7 cells pretreated with interferon-γ (IFN-γ) showed an increased production of NO, tumour necrosis factor-alpha (TNF-α) and inducible NO synthase expression when incubated with inulin (Koo *et al* 2003).

2.4.3.2.2 Increased resistance to pathogens

The increased resistance to pathogens is a result of the release of antimicrobial factors such as SCFA, lactic acid and bacteriocins[12] (Brenchley and Douek 2012); reduced luminal pH as a consequence of SCFA generation and competition for both growth substrates and for adherence sites to the epithelial barrier (Roberfroid 1993) (Delzenne and Roberfroid 1994) (Roberfroid *et al* 1998) (Roberfroid 1999) (Servin 2004) (Brenchley and Douek 2012). In vitro evidence using caco-2 cells and mucus secreted by the HT29-MTX cells has demonstrated that the adhesiveness properties of some strains of *Bifidobacteria* prevents the adhesion of bacterial pathogens onto

[10] A stable lipophilic free radical.

[11] The rats were given 10 g per 100 g body weight per day. The inulin-type fructan supplementation in this current study was 15 g per adult per day.

[12] Bacteriocins are similar to narrow spectrum antibiotics and are toxins released by bacteria to kill other species of bacteria in the nearby environment.

cultured human intestinal cells possibly by competition for the same carbohydrate receptors (Bibiloni *et al* 2001) (Servin 2004).

2.4.3.2.3 Effect of short chain fatty acids

The inulin-type fructans causes marked fluctuations of SCFA (particularly butyrate) in the intestinal lumen (Sanderson 2007). The microbes that prefer FOS tend to release acetate and lactate and those which prefer inulin tend to release butyrate (Rossi *et al* 2005) (De Vuyst *et al* 2014) demonstrating that these structurally related carbohydrates of different lengths produce different physiological responses (Rossi *et al* 2005). The roles of SCFA are considerable and vary according to the type. Acetate is largely utilized in the muscle, propionate is metabolized by the liver and adipose tissue while butyrate is largely metabolized by the colonocytes (Mortensen and Clausen 1996) (Reshef *et al* 1967) (Wong *et al* 2006) (Schaafsma and Slavin 2015).

Short chain fatty acids appear to directly interact with the immune cells (Karaki *et al* 2008) (Tazoe *et al* 2009). They are involved in metabolic regulation by signaling through guanine nucleotide-binding proteins (G-proteins)[13] which are activated by G protein-coupled receptors (GPR) (Vinolo *et al* 2009). G-protein-coupled receptors (GPR41, GPR3) receptive to SCFA have been identified on leukocytes, especially polymorphonuclear cells (Le Poul *et al* 2003) (Nilsson *et al* 2003), enterocytes and enteroendocrine cells in the human colon (Brown *et al* 2003) (Covington *et al* 2006) (Karaki *et al* 2008) (Tazoe *et al* 2009) (Maslowski *et al* 2009). Growing evidence suggests that SCFA, particularly butyrate, impacts the immune system beneficially by acting on leucocytes, intestinal cells as well as endothelial cells via the inhibition of the histone deacetylases (Canani *et al* 2011) (Canani *et al* 2012) which acts on G proteins. Histone deacetylases removes acetyl groups. Deoxyribonucleic acid expression is regulated by acetylation and de-acetylation. The inhibition of histone deacetylase activity in the enterocyte increases the production of IL-8 (Sanderson 2007) which can result in the migration of the leukocytes to the area of inflammation (Le Poul *et al* 2003) as well as suppress the release of proinflammatory cytokines and chemokines (Maslowski *et al* 2009) (Vinolo *et al* 2009).

[13] The role of G-proteins is to modulate cell functions including transcription, motility, contractility, and secretion as well as to control metabolic enzymes and ion channels.

The butyrogenic effect of inulin-type fructans is particularly important for intestinal health and therefore may protect against the intestinal onslaught of HIV.

Butyrate:

- decreases the pH of the colon which inhibits the growth of both putrefactive and pathogenic bacteria such as *Escherichia coli* and *Salmonella* (Cherrington *et al* 1991) (Blaut 2002) (Rossi *et al* 2005) increased concentrations of which have been found in those infected with HIV (Gori *et al* 2008b)
- is an energy source for epithelial cells (Cummings *et al* 1987) (Engelhardt *et al* 1991) (Vogt and Wolever 2003) and stimulates epithelial cell proliferation and differentiation in the large and small bowel and appears to be able to both up and down regulate gene expression in the epithelial cells (Sanderson 2007)
- appears to control the expression of the protein resulting in the myofibroblast modulation which heightens the epithelial chemotactic signals (Frankel *et al* 1994) (Sanderson 2007)
- reinforces the intestinal barrier functions (Canani *et al* 2011) (Canani *et al* 2012) (Tremaroli and Bäckhed 2012) against the translocation of LPS and peptidoglycans possibly explaining its anti-inflammatory effect (Canani *et al* 2012)

Inulin-type fructans are well established prebiotics, supplementation of which may improve the disturbed microbiome found in HIV-infected adults. The benefits and area of impact in the GIT is determined largely by chain length. The protective effect exerted by the inulin-type fructans on the intestine as a result of their ability to function as GIT antioxidants, to prevent pathogenic adhesion and to produce butyrate may prevent or reduce bacterial translocation. The translocation of microbes and their products has been implicated as an important factor driving the progression to AIDS. Supplementation with inulin-type fructans therefore may play an important role in reducing the progression to AIDS.

2.5 FACTORS DRIVING THE PROGRESSION TO AIDS

After the discovery of the HIV virus and its deleterious impact on CD4 cells, it was initially assumed that uncontrolled viral replication drove the progression to AIDS and that the disease was quiescent after the successful suppression of the viral load following ART (Bharaj and Chahar 2015). If the only driving factor was the HIV virus, intervention with ART would suppress the activated immune response (Stephen and Cummings 1980). Effective ART however does not eliminate the immune system activation (Chege *et al* 2011) (Sandler and Douek 2012) as demonstrated by B and T cell activation (Hazenberg *et al* 2000), high T cell turnovers (Hellerstein *et al* 1999) as well as increased levels of pro-inflammatory and pro-fibrotic mediators which are still present after viral suppression (Kuller *et al* 2008) (Lederman *et al* 2011b) (Sandler *et al* 2011).

Although the HIV virus is the causative factor of the HIV infection, it is currently thought that the progression to AIDS is multifactorial and not primarily dependent on the virus itself (Bharaj and Chahar 2015). In fact, the chronic systemic immune activation characteristic of HIV (Brenchley *et al* 2006b) (Sandler and Douek 2012) appears to be a more accurate reflection of the progression to AIDS irrespective of plasma viral load. The forces driving the chronic immune activation are not fully understood (Brenchley *et al* 2006b) (Bharaj and Chahar 2015) but probably include the influence of the HIV virus itself which, via a variety of toll like receptors (TLR), stimulates both the innate and adaptive immune response (Tremaroli and Bäckhed 2012). Additional contributing factors potentially include pathogens and opportunistic infections (Tremaroli and Bäckhed 2012), co-morbidities, premature or abnormal immunosenescence and microbial translocation from the GIT (Stein *et al* 1997) (Salminen *et al* 1998) (Brenchley *et al* 2006a).

The concept of microbial translocation across the GIT mucosal wall as a driving factor in the progression to AIDS has attracted significant research investigating the impact of HIV on the GIT.

2.5.1 Impact of human immunodeficiency virus on gut associated lymphoid tissue

A massive, rapid depletion and infection of CD4 T cells in the GALT has been shown to occur in HIV. Brenchley *et al* (2004) studied 14 HIV-infected ARV naive and 7 healthy adults to determine the effect of the HIV virus on specific subsets of CD4 T and CD8 T cells in the blood, GIT and lymph nodes. In comparison to the lymph nodes and blood, the most extensive CD4 T cell depletion occurred in the GIT at all stages of HIV disease (Brenchley *et al* 2004). There were higher concentrations of activated CD4 T cells in the GIT (Brenchley *et al* 2004) which are the most susceptible to HIV infection as the activation increases co-receptor expression (Cullen and Greene 1989). The majority of the GIT mucosal CD4 T cells are chemokine receptor 5 (CCR5) activated memory CD4 T cells (Mehandru *et al* 2004) (Brenchley *et al* 2004). As CCR5 is the primary HIV co-receptor which allows HIV entry and viral replication in the cell (Douek 2007) (Novati *et al* 2015), the CCR5 activated memory CD4 T cells were the most substantially depleted. A sub population of CD4 T cells, the Th17 cells were also substantially decreased (Kanwar *et al* 2010). As Th17 cells secrete IL-17 which promotes the proliferation of epithelial cells, recruits neutrophils to the GALT to clear pathogens and related byproducts and promotes the expression of antibacterial defensin (Chege *et al* 2011) (Bixler and Mattapallil 2013) (Kim *et al* 2013), Th17 cells probably play an important role in preventing microbial translocation (Kanwar *et al* 2010). Chege *et al* (2011) found that although Th17 dysregulation was evident in the blood only at the later stages of HIV infection, the sigmoid gastrointestinal Th17 was preferentially depleted much earlier on (Chege *et al* 2011) potentially increasing the risk of microbial translocation.

A study in California involving 15 HIV-infected ARV naive adults, 8 healthy adults and 4 long term non-progressors concluded, that the long term non-progressors did not experience CD4 T cell depletion in the mucosal components (Sankaran *et al* 2005). Those that were infected however, showed severe CD4 T cell depletion and low HIV specific mucosal CD8 T cell responses (Sankaran *et al* 2005). In the intestinal mucosa of those HIV-infected, but not in the non-progressors, there was a significant increase in the gene expression regulating immune activation, cell trafficking and the inflammatory response including the genes associated with the maintenance of epithelial cell barriers (Sankaran *et al* 2005). This suggested that the GALT was experiencing ongoing immune activation which in turn could result in epithelial barrier injury (Sankaran *et al*

2005). As inulin-type fructans protects the epithelial barrier and has a bifidogenic effect, supplementation may improve CD4 T cell concentrations in the GALT.

2.5.2 Impact of *Bifidobacteria* supplementation on gut associated lymphoid tissue

Neonatal rats fed with 1×10^{10} colony forming units (cfu) of *B. longum* for 6 weeks demonstrated improved systemic and intestinal maturity early in life via the promotion of dendritic cell maturation in PP, up-regulation of IL-10, IL-12, IFN-γ mRNA and the IFN-γ/IL-4 ratio in the intestinal mucosa and both increased IFN-γ gene expression and raised IgM secretion in cultured peripheral blood mononuclear cells (Dong *et al* 2010). A trial on gnotobiotic germ free mice showed a strain specific effect of *Bifidobacterium* (*B.bifidum*, *B. dentium, B. longum*) on the immune system via activation of the Th1 and Th2 cytokines raising levels of IL-4, IL-10, IFN-γ and TNF-α at both the intestinal and systemic levels (Ménard *et al* 2008).

Supplementation with inulin-type fructans with the resultant increase in *Bifidobacteria* may counteract the impact of HIV on the GALT.

In general in HIV the net result is a massive depletion of CD4 T cells in the GIT (Dandekar 2007) (Novati *et al* 2015). The GIT mucosa therefore is a major site of viral replication and CD4 cell depletion and, as such, is a large reservoir of both target and infected cells (Douek 2007). It is thought that this massive depletion and possible immune activation results in microbial translocation where microbes originating in the GIT translocate to the systemic circulation without the presence of overt bacteremia (Brenchley *et al* 2006b) (Douek 2007) (Sandler *et al* 2011) (Sandler and Douek 2012) (Novati *et al* 2015).

2.5.3 Microbial translocation

The microbiota inhabiting the human GIT forms a complex, heterogeneous ecosystem which is essential for healthy gut function and general well-being provided that the microbes remain resident in the GIT (Servin 2004) (Brenchley and Douek 2012) (Kelly *et al* 2015). Disruption of this relationship could encourage microbes and/or their byproducts to relocate into the systemic

system with potentially disastrous consequences (Brenchley and Douek 2012). Microbial byproducts include LPS (Ziegler *et al* 2008), peptidoglycans (Clarke *et al* 2010), lipoteichoic acid (Haruta *et al* 2007), flagellin (Ziegler *et al* 2008), ribosomal deoxyribonucleic acid (rDNA) (Jiang *et al* 2009) and unmethylated CpG-containing DNA (Francés *et al* 2008). On translocation they act via a number of receptors including nucleotide-binding oligomerization domain 1 (NOD1) and NOD2, as well as TLR2, TLR4, TLR5, TLR6 and TLR9 (Sandler and Douek 2012). Stimulation of these receptors found in innate immune cells such as monocytes, macrophages and dendritic cells activates a cascade of signals ultimately resulting in the release of the pro-inflammatory cytokines IL-1, IL-6, TNF and type I IFN (Sandler and Douek 2012). These, although beneficial in acute infection, can be detrimental in chronic infections such as HIV (Sandler and Douek 2012). Although microbial translocation naturally occurs in healthy individuals, damage to the intestinal barrier results in increased translocation (Kelly *et al* 2012). As the intestine is one of the first sites that the HIV virus attacks, the intestinal barrier may be disturbed from early on in the infection (Gori *et al* 2011).

The human GIT is very difficult to access therefore ex vivo systemic immune markers are used and their interpretation is limited (Roberfroid *et al* 2010).[14] Commonly used markers of microbial translocation in HIV includes LPS, sCD14 and endotoxin-core antibodies (EndoCab) (Brenchley *et al* 2006b). Lipopolysaccharide is a major component of both commensal and pathogenic gram negative bacterial cell walls but is not found in gram positive bacteria such as *Bifidobacteria* (Novati *et al* 2015). Lipopolysaccharide binds to CD14 which exists as both membrane bound and in a soluble form – CD14 then transfers the LPS to TLR4/MD-2 complex in the plasma membrane which in turn triggers the release of pro-inflammatory cytokines and type I IFN (Płóciennikowska *et al* 2015). Soluble CD14 therefore is used as a marker of LPS stimulated monocyte or macrophage activation (Landmann *et al* 1996) (Hiki *et al* 1998). Endotoxin-core antibodies clear LPS from the circulation. As LPS levels rise, there should be a concurrent increase in sCD14 and a subsequent decrease in the levels of EndoCab although this had not always been supported by research. In the early stages of HIV the host may initially compensate as CD14 and

[14] The common markers of microbial translocation include LPS, sCD14 and EndoCab.

EndoCab should control the LPS levels so there could be normal levels of LPS but raised levels of CD14 and decreased levels of EndoCab indicating that microbial translocation is occurring.

Plasma LPS, as well as sCD14 levels, have been shown to be increased in nearly all studies on HIV-infected individuals living in diverse environments including Africa (Redd *et al* 2009a) (Lester *et al* 2009) (Nowroozalizadeh *et al* 2010) (Cassol *et al* 2010), the United States (Brenchley *et al* 2006b) (Ancuta *et al* 2008) (Hunt *et al* 2008) (Papasavvas *et al* 2009) (Sun *et al* 2010), Australia (Rajasuriar *et al* 2010) and Europe (d'Ettorre *et al* 2011) (Vesterbacka *et al* 2013) (Novati *et al* 2015) providing evidence that bacterial translocation is a common denominator in HIV.

2.5.3.1 African studies

A Ugandan cohort study of a heterosexual population of 107 HIV-infected ARV naive adults (non progressors 27/107, standard progressors 41/107, rapid progressors 39/107) explored the longitudinal relationship between microbial translocation (LPS, sCD14, EndoCab) and circulating inflammatory cytokine responses (Redd *et al* 2009a). The markers were similar between groups and did not significantly change during the progression to AIDS. They concluded that although microbial translocation was present, it did not play a role in HIV disease progression in Africa. They suggested that this could be a consequence of the different study populations as those in Europe and the United States consisted mainly of males who were either homosexuals or who had contracted HIV though intravenous drug use. When compared to samples from the United States, the Ugandan individuals had significantly greater levels of EndoCab as well as other differences in various cytokine levels prior to being infected. It was thought that this was probably a consequence of environmental exposure to intestinal infections which may have altered their response to the HIV virus. In contrast African studies published later found increased LPS levels to be significant markers of disease progression.

A cohort of 57 HIV-infected female sex workers (ARV naive 38/57; ART 19/57) showed significantly higher mean plasma LPS levels (ART 0.0249 EU/ml[15] > ARV naive 0.0188 EU/m > uninfected 0.0119 EU/ml) when compared to 31 uninfected female sex-workers in Nairobi, Kenya indicative of microbial translocation which was not impacted by ART (Lester *et al* 2009). No correlation was found between LPS levels and plasma viral load or peripheral CD4 count.

A study in Guinea-Bissau involving 166 HIV-infected ARV nreaive and 69 healthy adults found significantly raised median LPS levels (>140 pg/ml) compared to healthy controls (>110 pg/ml) (Nowroozalizadeh *et al* 2010). A CD4 count <500 cells/mm^3 was significantly inversely related to LPS as was viral load.

A South African study (Pretoria) included 80 HIV-infected adults (ART 20/80) and 10 healthy African controls (Cassol *et al* 2010). Levels of LPS were significantly higher in those infected versus controls (mean 2.14 vs 1.10 EU/ml). Levels of sCD14 (mean 2.47 vs 1.61 μg/ml) and TNF (90.14 vs 11.40 pg/ml) were significantly raised in the ART-naive adults compared to controls (Cassol *et al* 2010). Those on ART at baseline had lower mean LPS levels (1.66 EU/ml) but despite undetectable viremia, these levels were not normalised after a further year on ART (1.10 EU/ml) nor were the mean levels of sCD14 or TNF. This is indicative of ongoing microbial translocation and immune activation despite viral suppression. Levels of LPS and sCD14 were positively correlated in those with no opportunistic infections suggesting that the sCD14 levels were mainly a consequence of microbial translocation and that the LPS was bioactive.

2.5.3.2 International studies

A nested case-control study (ARV naive 117/275) within the international Strategies for the Management of Anti-Retroviral Therapy (SMART) study (North America, United States, Europe, Africa, Asia, South America) showed that higher median levels were found in the HIV-infected

[15] EU is an endotoxin unit. The use of an endotoxin unit therefore allows comparison between different endotoxin tests assayed at individual laboratories as the use of the unit deals with the challenge of varying potencies of different endotoxins. Results of tests in EU/ml is not converted to units of weight of endotoxin per ml.

versus healthy controls respectively for LPS (32.5 pg/ml vs 25.0 pg/ml) and sCD14 (2.34 x 10^6 pg/ml vs 1.66 x 10^6 pg/ml) and lower median levels for EndoCab (121 mmu/ml vs 197.3 mmu/ml) (Sandler *et al* 2011). Although not normalised, the levels of LPS and sCD14 tended to be lower and EndoCab levels higher in those on ART. There was a lack of association between LPS and sCD14 which could be explained by the difficulties of measuring LPS.

Brenchley *et al* (2006) found significantly raised LPS and sCD14 levels in 205 HIV-infected adults compared to 47 healthy controls (United States and France) (Brenchley *et al* 2006b). There was a significant correlation between the raised LPS and sCD14 levels. The significant inverse correlation between the low EndoCab levels and LPS implied that the EndoCab concentration was insufficient to appropriately neutralize the LPS and thereby prevent immune system activation. As they found no increase in plasma LPS in the first month after seroconversion they suggested that damage to the mucosa appears later or that the individual compensates and is able to initially control the LPS levels. They claimed that the high levels of LPS and sCD14 in combination with low levels of EndoCab supplied evidence of increased microbial translocation which contributed to the chronic immune activation in HIV.

A study in the United States on 94 HIV-infected adults (ART failures 61/94, ART 11/94, ARV naive 14/94) demonstrated that both LPS and sCD14 median levels were significantly higher than healthy controls (Ancuta *et al* 2008). The positive correlation between the median LPS and sCD14 levels implied that the LPS was bioactive (Ancuta *et al* 2008). The Study of the Consequences of the Protease Inhibitor Era (SCOPE) trial (San Francisco) found that the raised median LPS levels in a group of 30 controllers[16] (61 pg/ml) and 66 HIV-infected ARV naive adults (71 pg/ml) were not significantly different but were significantly higher than the 47 healthy controls (28 pg/ml) (Hunt *et al* 2008). In San Francisco 44 HIV-infected adults had significantly higher median plasma LPS (3.63 EU/ml vs 1.85 EU/ml) and sCD14 levels (2.47 μg/ml vs 1.66 μg/ml) than 11 healthy controls (Sun *et al* 2010). A Philadelphian study reported that the median LPS levels of 10 HIV-infected ARV naive participants was 20 EU/ml versus 10 EU/ml in 26 healthy controls (Papasavvas *et al* 2009).

[16] Controllers referred to a HIV-infected adults who maintained undetectable plasma HIV RNA levels without treatment as opposed to the ART-naive who had detectable plasma HIV RNA levels.

Significantly higher LPS and sCD14 levels were found in 96 HIV-infected participants prior to ART living in Melbourne Australia. These levels were significantly reduced on ART but remained higher than the controls (Rajasuriar *et al* 2010).

A study in Europe (Rome) including 22 HIV-infected adults (ART 14/22) and 10 healthy controls found that the median plasma LPS of the ARV naive (8.8 EU/ml) and those on ART (8.7 EU/ml) were significantly higher than the HIV-uninfected (6.4 EU/ml) (d'Ettorre *et al* 2011).

The impact of microbial translocation (LPS, sCD14, EndoCab) hastening disease progression (AIDS, death, CD4 <200 cells/mm^3, ART initiation) in 379 HIV-infected adults participating in the Italian Cohort Naive Antiretroviral (ICONA) study was assessed over an average of 3 years post HIV seroconversion (Marchetti *et al* 2011). They concluded that the raised median LPS levels (110 pg/ml) but not the raised median levels of sCD14 (3.3 μg/ml) was the only marker associated with outcome independent of age, HIV-RNA and CD4 (Marchetti *et al* 2011). This is in direct contradiction to the SMART study who found that sCD14 was the only marker associated with outcome (Sandler *et al* 2011) and the longitudinal study in Uganda who found that raised LPS was not associated with outcome (Redd *et al* 2009a).

A longitudinal study in Italy investigated 44 HIV-infected ARV naive adults starting ART and 13 healthy controls (Merlini *et al* 2011). Those infected had higher median levels of sCD14 before ART (3.07 μg/ml) and after ART (4.26 μg/ml) versus the controls (1.96 μg/ml). After 12 months of stable ART, it was found that the partial immunological responders (baseline 75 pg/ml, end 75 pg/ml) and immunological non responders (baseline 75 pg/ml, end 75 pg/ml) had similar raised median levels of LPS. Antiretroviral therapy did not improve either sCD14 or LPS levels. Further evidence of microbial translocation was demonstrated by the presence of polymicrobic peripheral blood microbiota which included pathogenic and symbiotic bacteria both before and after 12 months of ART. There was a difference in the microbiota in that the immunological non-responders had no probiotic *Lactobacillaceae* at baseline and at the end (Merlini *et al* 2011) implying that bacteria may play a role in the response to ART.

A Scandinavian study by Vesterbacka *et al* (2013) randomised HIV-infected adults to either Efavirenz combined with two non-nucleoside reverse-transcriptase inhibitors once daily (37/71) or Ritonavir-boosted Lopinavir in combination with two non-nucleoside reverse-transcriptase inhibitors twice daily (34/71) to investigate the effects of different ART regimes on microbial translocation (LPS, sCD14, anti-flagellin antibodies) after 1.5 years on therapy (Vesterbacka *et al* 2013). In contrast to the study by Merlini *et al* (2011) both regimes significantly reduced the raised median LPS (157.5 pg/ml vs 140.0 pg/ml) and median sCD14 levels (3.13 μg/ml vs 2.85 μg/ml). Antiretroviral therapy therefore reduced the markers of microbial translocation and enterocyte damage although not to normal levels.

Although in these studies LPS has primarily been used as a marker of microbial translocation, it has been shown to be significantly related to immune activation (Novati *et al* 2015). The response to LPS is disturbed in HIV infection as there is an increased secretion of TNF-α, IL-6 and IL-8 (pro-inflammatory) and the reduced secretion of IL-10 and IL-1 receptor antagonist (less inflammatory) when compared to healthy controls (da Silva *et al* 1999). In addition the percentage of CCR5 activated memory cells is increased by LPS so raised LPS levels may increase the number of cells that the HIV virus replicates in (Juffermans *et al* 2000) (Ebert and McColl 2002) (Sandler and Douek 2012).

Raised markers of microbial translocation in HIV have been demonstrated in most studies across the world implying that microbial translocation is an integral part of the HIV disease process and disease progression. The permeability of the GIT is therefore disrupted to allow translocation.

2.5.4 Permeability of the gastrointestinal tract in human immunodeficiency virus

The GIT mucosa is a barrier formed by a continuous closed-lining monolayer of columnar epithelial cells (Epple *et al* 2009) (Novati *et al* 2015) which plays an important role in relaying information from the GIT to the mucosal immune system (Sanderson 2007). This mechanical barrier is responsible for the physical and functional integrity of the GIT. It passively prevents microbes and their byproducts from infiltrating the body (Vroling *et al* 2008) and actively plays a role in microbial defense and in controlling the local immune response (Vroling *et al* 2008).

Soon after the initial discovery of the HIV virus, a wide variety of techniques has been used to study the altered permeability of the GIT. One of the earliest case control studies by Kotler *et al* (1984) involving 25 HIV-infected malnourished men found that those with diarrhoea (7/12) had significantly lower serum d-xylose concentrations and urinary d-xylose excretion demonstrating increased intestinal permeability when compared to healthy controls (Kotler *et al* 1984). Similarly Gillin *et al* (1985) demonstrated that the absorption of C-glycerol tripalmitin[17] was decreased as a consequence of increased permeability in 20 HIV-infected adults with diarrhoea (Gillin *et al* 1985). Stockmann *et al* (1988) used lactulose mannitol flux to show that HIV-infected adults suffering from diarrhoea (8/21) had a significantly increased flux (Stockmann *et al* 1998). Kapembwa *et al* (1991) concluded that those with advanced HIV infection had an increased ratio of urinary lactulose to mannitol recovery (Kapembwa *et al* 1991). Lim *et al* (1993) using D-xylose, lactulose, L-rhamnose and 3-O-methyl-D-glucose in 51 HIV-infected adults concluded that abnormal permeability and reduced intestinal absorption capacity were common in HIV-infected adults at all stages of HIV disease, especially in the presence of diarrhoea (Lim *et al* 1993).

Keating *et al* (1995) demonstrated that malabsorption occurred in HIV-infected symptomatic males (63/88) using 3-O-methyl-D-glucose, D-xylose, L-rhamnose, and lactulose (Keating *et al* 1995). Bjarnason *et al* (1996) who used the gold standard (111-indium leukocytes) to measure intestinal permeability in 30 HIV-infected males found impaired intestinal permeability on progression to AIDS (Bjarnason *et al* 1996). In 1999, Sharpstone *et al*, who measured 3-O-methyl-D-glucose, D-xylose, L-rhamnose and lactulose absorption in 60 adults with AIDS, found that intestinal permeability was significantly increased in those who were symptomatic (Sharpstone *et al* 1999). Stein *et al* (1997) measured urinary butyrate to assess GIT permeability and concluded that the excretion was significantly higher in the AIDS group with weight loss (14/66) (Stein *et al* 1997). Pernet *et al* (1999) measured lactulose and mannitol and absorption of D-xylose in 96 HIV-infected adults and concluded that a reduction in intestinal functional absorptive surface occurs as the disease progresses possibly from the early stages (Pernet *et al* 1999).

[17] C-glycerol tripalmitin is a dietary neutral fat that is labeled with a non-radioactive isotope so after consumption its presence can be measured in stools as a marker of fat digestion and malabsorption.

More recent studies have used citrulline and faecal calprotectin as markers of intestinal damage and inflammation. As the small intestinal enterocytes produce the most citrulline, the measurement of citrulline concentrations is used as an indirect marker of epithelial damage. Crenn *et al* (2009) investigated citrulline concentrations in HIV-infected adults and found raised levels in those with diarrhoea (62/115) compared to healthy controls indicating increased intestinal permeability (Crenn *et al* 2009).

Increased levels of faecal calprotectin are a reflection of the migration of neutrophils to the intestinal mucosa which happens during intestinal inflammation. Faecal calprotectin as a marker of gut inflammation has been strongly correlated with the gold standard of 111-indium labelled leucocytes (Costa *et al* 2003). Raised faecal calprotectin levels were found in well asymptomatic HIV infected adults (Gori *et al* 2008a). Fifty percent (27/53) had raised faecal calprotectin, a third of which were very high levels leading investigators to conclude that significant chronic inflammation was present in the GIT early on in HIV disease and that this reduced intestinal barrier function allowing bacterial translocation which resulted in the chronic systemic immune response and the resultant progression to AIDS.

Enterocyte loss results in increased intestinal permeability. Enterocyte destruction may be a consequence of the actual virus (Heise *et al* 1991) (Asmuth *et al* 1994) (Maresca *et al* 2003) (Canani *et al* 2006) (Nazli *et al* 2010) possibly because of the reduced uptake of glucose by the enterocytes (Maresca *et al* 2003) or because of the release of pro-inflammatory cytokines such as TNF as increased local and systemic inflammation is associated with the loss of enterocytes in those infected with HIV (Sandler and Douek 2012). The SMART nested case-control study (ARV naive 117/275) found that raised intestinal fatty acid binding protein (I-FABP)[18] levels were more commonly found in those HIV infected versus controls (61.8% vs 26.8%) and that the median I-FABP levels were higher (139.8 pg/mL vs 20.0 pg/ml) (Sandler *et al* 2011). Lower baseline CD4 counts were associated with detectable I-FABP levels (579 vs 659 cells/mm^3) implying that enterocyte damage appears more likely with lower CD4 counts. Antiretroviral therapy did not impact the I-FABP levels (Sandler *et al* 2011). The Scandinavian study comparing the effects of

[18] Intestinal fatty acid binding protein is released from damaged enterocytes and is used as a measure of enterocyte loss.

different regimes of ART on intestinal permeability found that during the 1.5 years of treatment, the median I-FABP levels tended to increase (2.26 ng/ml vs 3.13 ng/ml) but this only reached significance in the Efavirenz group (Vesterbacka *et al* 2015). ART therefore did not improve enterocyte loss and restore the permeability of the intestine. Inulin-type fructan supplementation may reduce enterocyte loss (Apanavicius *et al* 2007).

Increased intestinal permeability, probably as a result of structural abnormalities (Kotler *et al* 1984) (Gillin *et al* 1985) (Batman *et al* 1989) (Greenson *et al* 1991), disrupted tight cell junctions (Epple *et al* 2009), enterocyte loss (Vesterbacka *et al* 2015), a disturbed mucus layer, decreased IgA secretions (Kotler *et al* 1984) and decreased numbers of dendritic cells (Serei *et al* 2015), has been well established in symptomatic HIV-infected individuals using a plethora of techniques (Epple *et al* 2010) (Nazli *et al* 2010) (Marchetti *et al* 2013).

Increased intestinal permeability permitting microbial translocation which may drive the progression to AIDS has been demonstrated in HIV. Inulin-type fructans and/or supplementation with *Bifidobacteria* in both animal and HIV-uninfected human studies, and in vitro, has been shown to tighten tight cell junctions, inhibit enterocyte loss, protect the mucus layer, increase IgA secretion and increase the population of dendritic cells potentially protecting the integrity of the intestine (Table 3) (Gaskins *et al* 1996) (Qiao *et al* 2002) (Hosono *et al* 2003) (Nakamura *et al* 2004) (Commane *et al* 2005) (Bakker-Zierikzee *et al* 2006) (Winkler *et al* 2007) (Apanavicius *et al* 2007) (Benyacoub *et al* 2008) (Scholtens *et al* 2008) (Ryz *et al* 2009) (Kabeerdoss *et al* 2011) (Delgado *et al* 2012) (Vogt *et al* 2014) (Vogt *et al* 2015). Very few studies investigating the impact of pre and probiotic supplementation has been conducted in HIV.

Table 3: Summary of studies demonstrating that prebiotics/probiotics potentially protect intestinal permeability

Authors	Population	Intervention	Outcome
Commane *et al* (2005)	Caco-2 human adenocarcinoma cell	Inulin-type fructans (Raftilose and Raftiline) *B. animalis ssp. lactis; Bifidobacteria species* 420; *L.* GG	Tightened cell junctions
Vogt *et al* (2014)	T84 human intestinal epithelial cell monolayers	Inulin type fructans[19]	Tightened cell junctions
Winkler *et al* (2007)	Mice with colitis	FOS (DP 8)	Increased mucin-rich crypts and longer crypt length
Apanavicius *et al* (2007)	Puppies	scFOS or inulin	Reduced enterocyte sloughing during *S. typhimurium* DT104 infection Greater concentration of faecal acetate and other SCFA Increase in *Lactobacillus*
Qiao *et al* (2002)	Healthy BALB/c mice pups	*B. bifidum* and *B. infantis*	Raised faecal IgA levels before rotavirus induced diarrhoea. Earlier resolution of diarrhoea.
Hosono *et al* (2003)	BALB/c mice	scFOS (Meioligo-P®)	Upregulated IgA secretion by PP Increased production of IFN-δ and IL-10 from PP
Nakamura *et al* (2004)	Newborn BALB/c mice	scFOS (Meioligo-P®)	Increased faecal IgA
Delgado *et al* (2012)	BALB/c mice	FOS	Increased faecal IgA Reduced proinflammatory cytokine IL-1β
Benyacoub *et al* (2008)	BALB/c mice	FOS:inulin mix	Increased peritoneal macrophage phagocytic activity Increased faecal IgA concentrations Increased survival from *S. typhimurium* infection
Bakker-Zierikzee *et al* (2006)	Newborn human infants	1 scGOS/lcFOS	Increased faecal IgA levels
Scholtens *et al* (2008)	Infants	scGOS/lcFOS	Increased faecal IgA and percent of faecal *Bifidobacteria* Reduced percent of the *Clostridium* species
Kabeerdoss *et al* (2011)	Adults	B. lactis Bb12®	Increased faecal IgA levels
Gaskins *et al* (1996)	C57BL/6NHsd mice	FOS	Increased numbers of dendritic cells in lamina propria of the caecum Decreased prostaglandin E2 in the small intestine, colon, and caecum
Ryz *et al* (2009)	Rats	Inulin (Orafti Beneo HP DP 23)	Increased dendritic cells in the PP and increased ex vivo secretion of IL-2, IL-10 and IFN-γ from both the spleen and MLN

[19] These formulations included FOS (DP $\leq$ 10) or inulin enriched with FOS (DP mostly $\leq$ 10 but up to 60) or inulin (DP mostly 10 to 60 but some under 10) and pure inulin (DP almost entirely 10 to 60).

2.6 PREBIOTICS IN HUMAN IMMUNODEFICIENCY VIRUS

Although there are no studies primarily investigating supplementation with inulin-type fructans, there is promising preliminary evidence that prebiotics may offer a therapeutic option in ARV naive HIV-infected adults. The RCT Italian COPA pilot trial supplemented 57 asymptomatic HIV-infected ARV naive individuals (mean CD4 520 cells/mm^3) with either 15 g or 30 g of a synthetic prebiotic mixture of scGOS/lcFOS/ pAOS for a period of 3 months (Gori *et al* 2011). The full dose however was only reached at the third week as the intake was gradually increased to avoid the potential side effect of increased flatulence. Supplementation significantly fueled *Bifidobacteria* growth (Gori *et al* 2011). For those on 15 g the median percent of *Bifidobacteria* increased from 2.8% to 15.7% and for those taking 30 g from 3.6 to 18.9%. For both supplemented groups there was a significant decrease in median concentrations of the *C. coccoides/Eubacterium rectale* group as well *as C. lituseburense* and *C. histolyticum* although there was no full reduction in the total pathogenic load. The populations of *P. aeruginosa*, *C. albicans*, *Bacteroides, Prevotella*, *Lactobacilli*, *E. coli* and *Atopobiu*[20] remained unchanged. Mean levels of sCD14 were significantly lowered by week 4 in the 15 g group compared to control (9,952 pg/ml vs 11,237 pg/ml) and at week twelve (9,720 pg/ml vs 11,302 pg/ml)[21] although not in the 30 g/day group (11 244 pg/ml). Only those on 15 g/day showed a slight but significant decrease in mean plasma LPS levels compared to baseline (519.5 pg/ml vs. 588.2 pg/ml). The CD4 T cell count did not vary significantly between the groups at baseline to the end of the study although the 30 g group showed a significantly lower CD4 T cell activation (CD25 expression) at week 12. Natural killer cell activity was significantly increased in the 15 g group. The 15 g dose appeared more beneficial than the 30 g dose. Supplementation with a synthetic prebiotic mixture which contained inulin HP (high molecular weight) improved the colonic microbiome in HIV, decreased microbial translocation (sCD14, LPS) and enhanced immunity (NK cell).

The nutritional product NR100157 contains the synthetic prebiotic mixture used in the COPA trial plus eicosapentaenoic acid, docosahexaenoic acid, gamma-linolenic acid, bovine colostrum

[20] *Atopobiu* is a gram positive facultative anaerobic bacteria which is associated with bacterial vaginosis.

[21] These are the values stated in the publication.

protein and cysteine. In a multicenter RCT trial, 340 HIV-infected ARV naive adults (CD4 <800/mm^3) consumed either NR100157 or an isocaloric isonitrogenous control for 52 weeks (Cahn *et al* 2013). The decline in mean peripheral CD4 count was significantly reduced in those on the nutritional product versus controls (−28 vs −68 cells/mm^3). There was no change in viral load and the mean percentage of CD8+CD38+ levels remained unchanged. Those on NR100157 had significantly lower percentages of CD4+CD25+ indicating lower CD4 T cell activation. NR100157 significantly slowed the decline in CD4 count and appeared to lower CD4 T cell activation.

Prebiotic mixes, including inulin-type fructans, may improve the colonic microbiome, decrease microbial translocation, decrease CD4 T cell activation, enhance NK cell activity and prevent a decline in peripheral CD4 count. As prebiotics stimulate the growth of probiotics, studies investigating probiotic supplementation in HIV are relevant.

2.7 PROBIOTICS IN HUMAN IMMUNODEFICIENCY VIRUS

There are very limited studies researching probiotics in HIV and even fewer investigating *Bifidobacteria*. Initially there was caution regarding their use as even nonpathogenic bacteria in the arena of immunosuppression could become potentially pathogenic. The initial studies therefore focused on safety. One of the first studies was that by Wolf *et al* (1998) who concluded that no clinically significant changes were seen in serum chemistry, haematology, immune profile, urinalysis, physical examination and gastrointestinal tolerance in 39 HIV-infected adults ingesting *L. reuteri* for a period of 3 weeks (Wolf *et al* 1998). The faecal levels of *L. reuteri* significantly increased demonstrating for the first time that manipulation of the microbiome by the intake of a probiotic was possible in HIV (Wolf *et al* 1998). Since this initial study, the safety of probiotics has been established in a number of other HIV studies (Salminen *et al* 2015). In a recent analysis of 57 clinical studies by Van den Nieuwboer (2015), the safety of both probiotics and symbiotics in immune compromised adults, including those with HIV, was evaluated using the Common Terminology Clinical Adverse Events (CTCAE version 4.0) classification (Van den Nieuwboer *et al* 2015). They concluded that the current dosages and duration of use with the existing probiotics and/or symbiotics was safe as there were no serious adverse events related to these

products (Anukam *et al* 2008) (Hummelen *et al* 2011) (Schunter *et al* 2012) (Hemsworth *et al* 2012) (Yang *et al* 2014) (Schaafsma and Slavin 2015) (Van den Nieuwboer *et al* 2015). In fact adverse events tended to occur more frequently in the control groups rather than in the probiotics/symbiotics groups. Overall the products were well tolerated with minimal side effects.

2.7.1 *Bifidobacteria*

Inulin-type fructans have a bifidogenic effect. In a randomised double blind uncontrolled study 20 asymptomatic ARV naive adults (CD4 >350 cells/mm^3) were randomized to either a probiotic (*L. rhamnosus* HN001 plus *B. lactis* Bi-07 at 10^9 cfu/ml) or a prebiotic (10 g of agavins[22]) or both for 16 weeks (González-Hernández *et al* 2012). Bacterial translocation was measured by 16S rRNA levels in plasma, and the total bacterial load was determined by 16S rRNA levels in the faeces. The probiotic and symbiotic group had a significantly decreased total bacterial load in the faeces demonstrating that the microbiome had been altered. There was no decrease in viral load in any of the groups. Only the symbiotic group had a statistically significant reduction in plasma bacterial DNA concentrations indicting reduced translocation and a significant increase in mean CD4 count (+102 cells/mm^3) and a significant decrease in proinflammatory IL-6. There was no difference between the groups at baseline and end study for TNF-α, IL-1β, and IL-10. The combination of pre and probiotics appeared to reduce microbial translocation and improve immunity and reduce inflammation.

A recent longitudinal study which was neither randomized or controlled (Probio-HIV) in Rome investigated the effect of probiotic supplementation for 48 weeks on inflammatory and immunological markers in 20 HIV-infected adults (mean CD4 542 cells/mm^3, HIV RNA <50 copies/ml) who had been stable on combination ART for at least 3 years (d'Ettorre *et al* 2015). The supplement contained a cocktail of probiotics[23] which included *S. salivarius* ssp. Termophilus, *B. breve, B. infantis, B. longum, L. acidophilus, L. plantarum, L. casei, L. delbrueckii ssp.*

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[22] Agavins are fructans from the Agave tequilana plant.

[23] Each packet contained *S. salivarius* ssp. *Termophilus* (>204 billion CFU), *Bifidobacteria* (> 93 billion CFU), *L. acidophilus* (>2 billion CFU), *L. plantarum* (>220 million CFU), *L. casei* (<220 million CFU), *L. delbrueckii* ssp. *Bulgaricus* (>300 million CFU) and *S. faecium* (>30 million CFU). Two packets were taken daily.

Bulgaricus and *S. faecium*. There was a non-significant increase in mean CD4 count (28 cells/mm^3) and mean CD4 percentages (26%). Markers of CD4 T-lymphocyte activation, CD38 and HLA-DR, showed a statistically significant reduction post probiotic supplementation as did mean high sensitivity CRP (baseline 3.36 mg/l, end 1.625 mg/l) indicating an improvement in GIT immunity.

The other trials have investigated a range of different probiotics mostly from the *Lactobacillus* genus.

2.7.2 *Lactobacillus*

In a randomised uncontrolled double blind parallel pilot study, 33 asymptomatic adult females (mean CD4 >200 cells/mm^3) who had been on ART for 7 to 9 years (some with detectible viral loads) were randomised to either a probiotic/prebiotic mixture (Synbiotic 2000W)[24] or the prebiotic (nondigestible fermentable dietary fibres) only for 4 weeks (Schunter *et al* 2012). Real-time qPCR was used to determine whether the concentrations of the supplemented bacteria increased. Of these only *L. plantarum* and *P. pentosaceus* showed significant increases in concentration. Although they were supplemented with a very modest dose of inulin, the *Bifidobacteria* concentrations were not monitored. There was a slight but significant increase in mean HLA-DR expression on a minor population of CD4 T-cells that were negative for CD38 and PD-1 but no other detectible differences were found. No significant changes were found for mean CRP, sCD14, TNF-α and IFN-γ. The study indicates that supplementation with symbiotics can modify the microbiome in those infected with HIV although there did not appear to be a change in the microbial translocation or inflammatory markers (Schunter *et al* 2012).

In a RCT study over 25 weeks, 55 ARV naive women (mean $\leq$ 350 cells/mm^3 33/55) were supplemented with *L. rhamnosus GR-1* and *L. reuteri RC*-14 at 2 x 10^9 twice daily or a placebo (Hummelen *et al* 2011). From baseline to end study there was a slight trend to a higher mean CD4 count in the group on probiotics (46 cells/mm^3) versus those on controls (19 cells/mm^3). No

[24] Synbiotic 2000W contained 4 strains of probiotic bacteria (10^{10} each) *Pediococcus pentosaceus* 5–33:3, *Leuconostoc mesenteroides* 32–77:1, *L. paracasei subsp paracasei* 19 and *L. plantarum* 2362 plus 2.5 g of each of the following four nondigestible, fermentable dietary fibers: betaglucan, inulin; pectin and resistant starch.

differences were found in other immune markers (IgG, IgE, IFN-γ and IL-10). There was no significant impact of the probiotic on immunity.

In an observational uncontrolled retrospective study, Irvine *et al* (2010) found that yogurt supplemented with *L. rhamnosus Fiti* in 68 HIV-infected adults (ART and ARV naive) resulted in a significant increase in mean CD4 count after controlling for the effect of ART (Irvine *et al* 2010). As the 82 healthy adults serving as the control group did not receive yoghurt, it is not possible to claim that the result was the consequence of the probiotic as it may have been the impact of the yoghurt alone or the combination.

Twenty four HIV-infected ARV naive adults (CD4 >200 cells/mm^3) with moderate diarrhoea taking part in a RCT trial were assigned to either a conventional yoghurt fermented with *L. delbruekii* var *bulgaricus* and *S. thermophiles* or to the same fermented yoghurt additionally supplemented with *L. rhamnosus* GR-1 and *L. reuteri* RC-14 for a 15 day period (Anukam *et al* 2008). There was no significant change in the haematological parameters[25] from baseline to end study. The mean CD4 count remained the same or increased in 11/12 on probiotics versus 3/12 in the control. At the end of the study 10/11 in the probiotic group had a significant increase in CD4 counts which was a 3 to 4 fold improvement when compared to controls. GI symptoms (diarrhoea, flatulence and nausea) resolved in all on the probiotic within a period of 2 days versus only 2 out of 12 in the control group (Anukam *et al* 2008). The supplementation improved peripheral CD4 count and resolved common gastrointestinal challenges in HIV.

In a RCT cross over trial 21 HIV-infected adults (CD4 579 cells/mm^3) receiving ART consumed yoghurt supplemented with micronutrients and *L. rhamnosus* CAN-1 at 10^9 cfu/ml for 30 days or yoghurt supplemented with micronutrients only for 30 days or yoghurt supplemented with probiotics. There was a 2 week washout period between each (Hemsworth *et al* 2012). The mean increase in CD4 was yoghurt plus micronutrient (+41 cells/mm^3) > yoghurt plus micronutrient and probiotic (+19 cells/mm^3) > yoghurt plus probiotic (-7 cells/mm^3) although this did not reach

[25] These included white blood cells, red blood cells, haemoglobin, haematocrit, pack cell volume, mean cell volume, mean cell hb, mean cell hb concentration, red blood cell distribution width and platelets.

significance. No significant change was found in inflammation using CRP as a marker (Hemsworth *et al* 2012).

2.7.3 *Bacillus*

Supplementation with *Bacillus coagulans* GBI-30, 6086 capsule over a 3 month period in a RCT study in ten HIV-infected adults on ART versus controls found that chronic GI symptoms as rated by the Gastrointestinal Symptom Rating Scale (GSRS) appeared to improve (Yang *et al* 2014). The percent of peripheral CD4 T cells significantly improved versus controls (+2.8% versus −1.8%). Soluble CD14, I-FABP, sCD163, D-dimer, CRP, IL-8 and TNF-α levels did not change from baseline in any group. Supplementation improved gastrointestinal symptoms and immunity (CD4 count) but not microbial translocation (sCD14, I-FABP, sCD163) or inflammation (D-dimer, CRP, IL-8, TNF-α).

2.7.4 *Saccharomyces boulardii*

A RCT trial supplementing with the yeast *Saccharomyces boulardii* for 12 weeks in 44 HIV-infected adults on ART (Villar-García *et al* 2015) reported that the median values of LPS-binding protein (−0.30 vs +0.70 pg/ml) and pro-inflammatory IL-6 (−0.60 vs +0.78 pg/ml) were significantly decreased in the probiotic versus the control group. There was no significant change in median sCD14, TNF-α, IFN-γ and CRP levels. They concluded that supplementation with *S. boulardii* decreased microbial translocation as indicated by LPS-binding protein and reduced the inflammatory markers (IL-6) in HIV-infected patients with long-term virologic suppression.

Interpretation is fraught with difficulty as diverse pre and probiotics have been investigated in HIV in vastly different combinations and doses measuring widely different parameters on very diverse study populations have been investigated. The physiological impact of the inulin-type fructans is dependent on chain length. The effect of a probiotic genera such as *Bifidobacteria* or *Lactobacillus* can be specific to an actual strain rather than even the species. The benefits of one strain may not be able to be extrapolated to another (Wilson *et al* 2013). Combining a variety of pre and probiotics limits the interpretation as to which, if any, was the effective agent in HIV and assumes

that the combination would be synergistic and not antagonistic. Overall the potential seems to exist for prebiotics and probiotics to modify the colonic microbiome in HIV to a less pathogenic more beneficial profile, decrease microbial translocation, enhance immunity and reduce inflammation.

2.8 CONCLUSION

It is proposed that immune and structural damage to the GIT epithelium in HIV, promotes microbial translocation which triggers the release of pro-inflammatory cytokines and type I IFN resulting in the characteristic chronic stimulation of the immune system driving the progression to AIDS.

The microbiome in HIV shifts towards a more pathogenic proinflammatory profile with a reduction in the probiotic species, including the butyrate producing bacteria, which is not restored on effective ART. These pathogenic shifts have been associated with increased levels of T cell activation and increased tryptophan catabolism through the detrimental kynurenine pathway. The bacteria found in diminished concentrations are those that are thought to reduce microbial translocation and systemic immune activation. Prebiotics and probiotics which modulate the microbiome could therefore offer a promising adjunct therapy in the treatment of HIV.

Indirect evidence of increased microbial translocation has been demonstrated by raised levels of LPS and/or sCD14 in most international studies. Increased intestinal permeability from structural abnormalities, loss of tight cell junctions, decreased numbers of dendritic cells, disruption of mucus and reduced faecal IgA secretion has been documented. Inulin-type fructans and/or supplementation with *Bifidobacteria* in vitro and in animal and human models has been shown to enhance epithelial health, tighten tight cell junctions, increase dendritic cell populations and stimulate both mucus and faecal IgA secretion.

Inulin-type fructans indirectly enhance immunity via bifidogenic and butyrogenic effects and directly enhance immunity by interacting with the immune system cells. Although they are the most extensively researched prebiotic there is no research conducted in HIV where they have been used as the sole intervention. Where combined with other prebiotics they have been shown to stimulate the growth of *Bifidobacteria* and improve the colonic microbiome, decrease the colonic pathogenic load, reduce markers of bacterial translocation, lower T cell activation, reduce the decline in peripheral CD4 count and increase natural killer cell activity.

As inulin-type fructans are a safe, tolerable and non-invasive intervention, have known immunomodulatory properties, promote a favourable microbiome and enhance epithelial health, it is urgent that research is conducted to determine whether they could indeed be a useful adjunct therapy as a sole intervention.

CHAPTER 3: METHODOLOGY

3.1 STUDY DESIGN

A double blind RCT conducted in HIV-infected ARV naive black African males and females 18 years or older recruited from Lancers Road Clinic, Durban, South Africa over a 3 month period.

3.2 SAMPLE SELECTION

All black African adults who were either attending the Lancers Road Wellness Clinic or who came for voluntary counseling and testing (VCT) in 2013, and who complied with the inclusion/exclusion criteria, were offered the opportunity to be included in the study.
Inclusion criteria:

- HIV-infected ARV naive adults 18 years or older
- CD4 count of >350 cells/mm^3 [26]

Exclusion criteria:

- CD4 count ≤350 cells/mm^3
- Taking ART
- Under the age of 18 years
- Pregnant/lactating women
- Tuberculosis or isoniazid (INH) prophylaxis
- Hepatic/renal insufficiency
- Chronic disease and WHO AIDS stage 2 – 4 (Novitsky 1994)
- Taking statins[27]

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[26] During the study period those with CD4 counts of ≤ 350 cells/mm^3 were eligible for ART in South Africa. Currently those with CD4 counts ≤ 500 cells/mm^3 are eligible.

[27] Statins lower CRP levels.

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- Taking heparin[28]
- Taking antibiotics for up to 1 month prior[29]
- Consuming dietary supplements with significant amounts of prebiotics including inulin-type fructans or probiotics or synbiotics for up to 1 month prior[30]
- Allergic to either maize or soya[31]

The sample size calculation was estimated on an expected greater reduction in LPS concentration in the intervention group as found in the results of the COPA study (Gori et al 2011). At the time of planning our study this was the only comparable study measuring the effect of a prebiotic mixture on intestinal permeability in HIV-infected, ARV naive subjects. On the assumption that the mean LPS values at 12 weeks in the intervention group were 519.5 ± 59.2 pg/ml compared to 588.2 ± 104.5 pg/ml in the control group the sample size was calculated at 40 per group. This sample size gave 80% power to detect a 2-sided difference with significance at the 95% level. The sample size was also sufficient to detect a difference in mean sCD14 values at 12 weeks between the 2 groups using the mean values reported in the COPA trial viz. 9.72 ± 1.44 μg/ml and 11.30 ± 1.72 μg/ml. To allow for loss to follow up the sample size was increased to 100. One hundred were recruited, eighty four completed the study.

Randomisation was executed by the statistician from the Biostatistics Unit of the Medical Research Council (Tarylee Reddy, Tarylee.Reddy@mrc.ac.za) using the program nQuery Advisor 7.0. Block randomization was used with random block sizes. The study subjects were divided into a large number of blocks of sizes 2, 4, 6 or 8 and simple randomization was performed within each block. The randomization code was kept by an independent researcher administrator (Charlene Baxendale, baxendale@ukzn.ac.za) and blinding was broken only after the completion of data analysis.

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[28] Heparin interferes with the analysis of LPS.

[29] Antibiotics prior to the trial would have disturbed the microbiome.

[30] The prior consumption of prebiotics, probiotics and synbiotics would have altered the microbiome.

[31] The ingredients of the nutritional supplement included corn, soya, sucrose, maltodextrin, soya milk powder, salt and flavouring.

The study site was not randomly selected. A clinic setting rather than a hospital setting was chosen as clinics are the primary entry point into the health system and where VCT occurs in South Africa. The eThekwini Lancers Road Clinic situated at Warwick Junction was selected because it is one of the largest clinic in KZN treating between 200 to 400 people per day, and therefore offered the best opportunity for adequate enrolment within a year. Warwick junction is the primary public transport interchange of Durban with an estimated 450 000 people passing through per day. Although the clinic is only intended to serve those living in the immediate vicinity, in reality many who travel on public transport use this clinic because of convenience. The clinic is open 5 days per week and staffed by nurses and assistants with a clinician attending one session per week. The study provided additional research staff (clinician, phlebotomist, research assistant) so as not to burden the clinic. The population attending the Lancers Road Clinic were mostly Zulu speaking black Africans with very few Indian/Caucasian participants.

3.3 OUTCOME VARIABLES

These included demographics (age, race, sex, pregnant/lactating), clinical history, medication, tuberculosis screening, clinical symptoms (diarrhoea, constipation, ability to control defecation, difficulty of defecation, nausea, vomiting, burping, flatulence, stomach pain, borborygmus, bloating, heartburn, acid reflux), use of soluble dietary fibre/prebiotics/probiotics/synbiotics, blood indicators (albumin, CRP, CD4 count, haemoglobin), body composition (weight, height, MUAC, TST, fat mass, fat free mass), microbial translocation (LPS, sCD14) and gastrointestinal inflammation (mRNA).

3.4 CONFOUNDING VARIABLES

These included the intake of soluble dietary fibre/prebiotics/probiotics as well as the use of antibiotics and other medications (including traditional medicines) that potentially affected the gastrointestinal microbiota.

3.5 DATA COLLECTION

Permission to conduct the study at Lancers Road Clinic was obtained from the eThekwini Municipality Health Unit in December 2012 (Appendix B). Ethics approval was granted by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BFC 145/010) in February 2013 (Appendix C).

Data was collected over a ten month period from March to December 2013.

Those who tested HIV positive when undergoing VCT were referred to the Zulu speaking research assistant (phlebotomist/HIV counselor) who drew blood (4 ml ethylenediaminetetraacetic acid K2EDTA) which was sent to the National Health Laboratory Services (NHLS) for a CD4 count. The NHLS laboratories have been accredited by SANAS which has reciprocity to international bodies (SANAS 2013). The participants returned to the research assistant the next week to collect their results. Those whose CD4 count was >350 cells/mm^3 and who met the inclusion and exclusion criteria were invited to participate in the study. Adults already attending the wellness clinic whose CD4 count was >350 cells/mm^3 were also invited to participate.

3.5.1 Visit one (enrolment)

If the person was interested, the research assistant explained to them verbally in their home language the purpose of the study and what their involvement would entail. They were then asked to read the information sheet (Appendix D). The research assistant then asked them to explain what they thought their involvement with the study required. If they showed good understanding of the study and the process, they were asked to read and sign the informed consent (Appendix E). They were then given a copy of both the information sheet and the informed consent to keep and the original informed consent was placed in their file. Once enrolled, the research assistant administered a closed ended questionnaire to confirm that the participant complied with both the inclusion and exclusion criteria (Appendix F). If the participant had recently completed an extended course of antibiotics or was just finishing INH prophylaxis they were asked to come back in 1 month to enroll or in 2 weeks if a short course of 5 to 7 days of antibiotics had just been

completed. The study clinician then conducted a clinical examination to assess health status and to confirm that the participant was in Stage 1 of the disease (Appendix G). In compliance with the Department of Health guidelines which require that all HIV-infected individuals be tested for tuberculosis (TB) every 6 months, sputum samples were sent to the NHLS for real time PCR for *Mycobacterium tuberculosis* (GeneXpert MTB/RIF) and for TB culture (auramine O stain). If TB positive they were informed of the result and excluded from the study. To establish baseline inulin-type fructans/other prebiotics/probiotic intake a food frequency questionnaire was administered by the principal investigator (PI) (Appendix H). The date for visit two was made within the week and the participant was instructed not to eat for one hour prior to the visit. Finally they were given an appointment card, the contact telephone number of the research assistant as well as a sample of the study supplement to ensure that they would be willing to consume the supplement (EXCEL Future Life©) for a period of 3 months. They were paid transport money of R100. The initial visit lasted approximately one hour.

3.5.2 Visit two (baseline)

This took approximately 5 hours. On arrival the participant was allocated the next consecutive study number. They were then asked to urinate and to collect a stool sample. If unable to produce a stool sample they were requested to produce one before the end of the fasting period. The faeces were packed by the PI into six 1.8 ml cryovials (NUNC™ CryoTube™ Vials) which were stored within 6 hours at -75°C (ultra low temperature New Brunswick Scientific Freezer) at the Doris Duke Medical Research Institute (DDMRI) for later analysis of mRNA. Following urination and defecation, the participant was weighed (Masskot scale 50 g to 150 kg) in minimal clothing (underwear) and the values recorded on the data collection sheet (Appendix I). After confirming that they had not eaten for one hour prior, a baseline saliva sample was obtained by asking the participant to suck on dental cotton wool swabs (Henry Schein Dental Cotton Rolls) until they were wet with saliva. The saliva filled swabs were transferred into a 20 ml syringe (Healthease Plus LUER SLIP) and the plunger depressed so that the saliva was syringed into a 4.5 ml cryovial (NUNC™ CryoTube™ Vials). Once 4.5 ml of saliva had been collected, the participant drank a solution containing an accurately measured, approximately 30 g of deuterium oxide (SERCON Ltd UK Lot EB2039) and the bottle containing the dose was rinsed twice with 50 ml of water to

ensure complete consumption of the dose. The time of dose administration was recorded on the data collection sheet and the participant was instructed not to eat or drink anything (including water and chewing gum) for the next 4 hours. Their height (height rod Seca 213, 0 to 198cm), MUAC (MABIS anthropometry tape) and TST (LANGE calipers) was measured by the PI. All measurements were repeated twice. Baseline bloods were drawn into one 5 ml serum separator tube (BD vacutainer SST II advance ref 367995 5 ml) for albumin and CRP analysis and one 4 ml K2EDTA tubes (BD vacutainer K2E 7.2 mg ref 368861) for LPS and sCD14 analysis. Within 6 hours the blood was centrifuged (ALC PK 121 R multispeed refrigerated centrifuge) and stored at -75°C (ultra-low temperature New Brunswick Scientific Freezer) in DDMRI for later analysis. A microcuvette (HemoCue© Hb201) was used at the puncture site to obtain venous blood to measure Hb. If the Hb level was below 10 g/dl the test was repeated on a separate machine. If the Hb was confirmed to be <10 g/dl, the clinician prescribed iron (Gulf ferrous sulphate FeSO4 170 mg, manganese sulphate 2.5 mg, copper sulphate 2.5 mg) three times per day and folic acid (5 mg) to be taken once per day at a separate time from the iron. The Hb was repeated the next month and further supplementation prescribed as required. Recommendations were made by the PI (dietitian) of food sources high in iron which were both affordable and culturally acceptable (Appendix J). The GSRS (Appendix K) was used in combination with the Bristol Stool Chart (Appendix L) to establish baseline gastrointestinal symptoms. After completion of the four hour fast the saliva was resampled. The participant was then given a meal and fluids. After eating, the research assistant issued them with a box containing a month's supply of supplement[32] containing inulin-type fructans (Frutafit®) or supplement without the active ingredient. The participant was given instructions on how to consume the supplement such as what it could be mixed with. To introduce the inulin-type fructans in a graded dose to avoid possible gastrointestinal side effects, all participants were instructed to eat only half the contents of a packet (7.5 g Frutafit®) per day for the first eight days and then to eat the entire contents of a packet (15 g Frutafit®) per day for the rest of the study. They were paid transport money of R100. The date of the next visit a month later was filled in on their appointment card. The participants were asked to bring all remaining sachets of supplement at each visit.

[32] The supplement in this study refers to an energy drink which is described in section 3.8. As both groups received the identical amounts of supplement, the effect of the additional nutritional content was controlled for.

3.5.3 Visit three and visit four

There was a month between each study visit. Each follow up visit lasted approximately 30 minutes. The PI counted and recorded the left over sachets of supplement, confirmed that the participant was willing to continue the study and that they still complied with both the inclusion and exclusion criteria (Appendix F). Participants that were sick were treated by the clinician. After administering the GSRS, the Bristol stool chart and the food frequency questionnaire, the PI issued the supplement for the next month and booked the next visit. They were paid transport money of R100. The same procedure was followed during the second follow up visit. Participants who failed to keep appointments were phoned by the research assistant. If the participant no longer wanted to continue in the study the reason was determined. If they had forgotten their appointment another appointment date was booked before the participant had finished the supplement.

3.5.4 Visit five (exit)

This lasted approximately 5 hours and was a combination of the enrolment and baseline visit. The participant arrived at the clinic after having fasted for at least one hour. They were asked to urinate and collect a stool sample. Their body weight was measured. Saliva samples were taken and the deuterium dose administered. Repeat bloods were drawn. The clinician repeated the clinical examination. Mid upper arm circumference and TST measurements were repeated. The food frequency questionnaire, the GSRS and Bristol stool chart were administered. After four hours the saliva was resampled. They were then given a meal and fluids. The participant was then thanked for being in the study and was given a small gift of appreciation such as body lotion. They were paid transport money of R150. An appointment was made for the following week with the research assistant (HIV counselor) so they could collect their repeat CD4 results. If the CD4 count was $\leq$350 cell/mm^3 the participant was counseled and then referred to the clinic for initiation of ART. Those whose CD4 count was >350 cell/mm^3 were booked a return visit at the wellness clinic for 6 months' time (Figure 2).

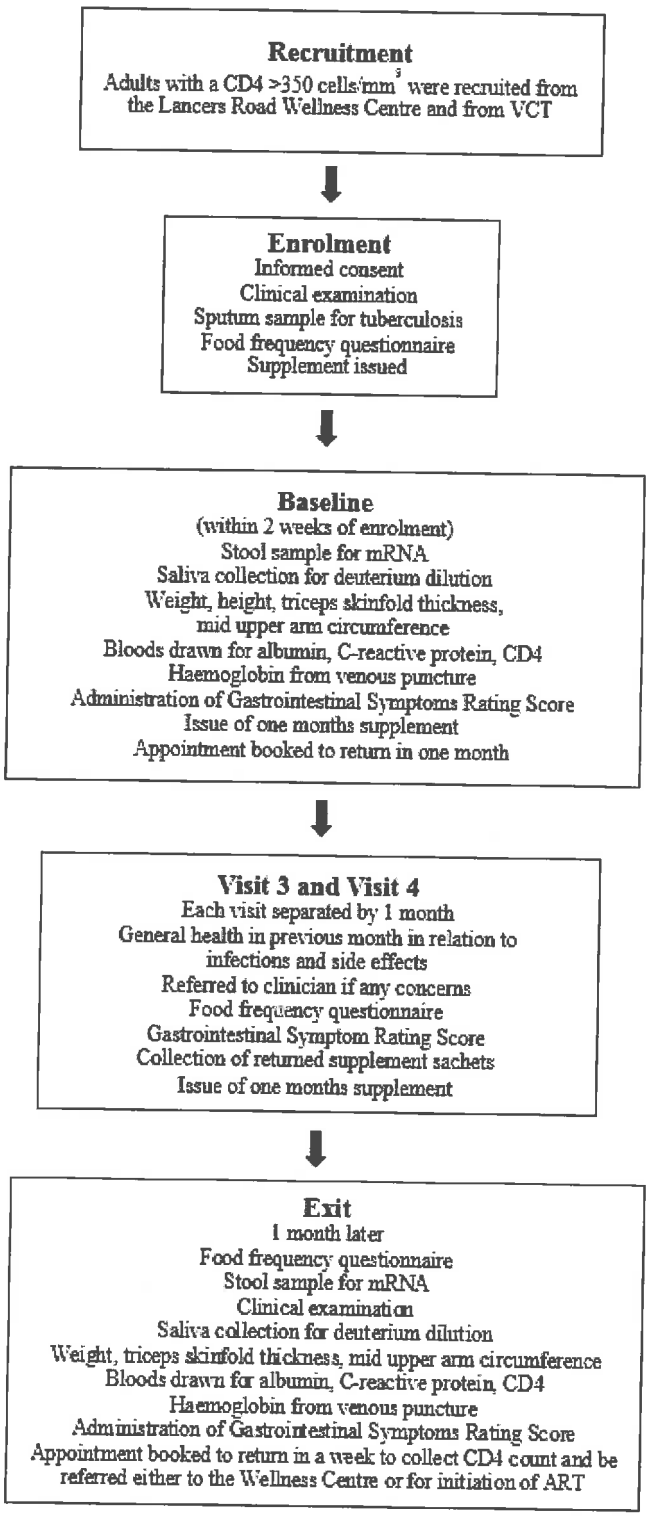

Figure 2: Flow diagram of study methodology

3.6 DATA COLLECTION INSTRUMENTS AND TECHNIQUES

A screening closed ended questionnaire was used to confirm that the participants complied with both the inclusion and exclusion criteria. The clinician conducted the medical examination and completed a standard form to determine whether the participant was in good health and to calculate the Karnofsky performance scale/score (Appendix G). TB status was determined from sputum samples. The prebiotic/probiotic intake was determined using a closed ended food frequency questionnaire. Weight and height was used to calculate BMI. Mid arm tapes and calipers were used to determine MUAC and TST. Fat and fat free mass was measured using the deuterium dilution method of the International Atomic Energy Agency (IAEA) (International Atomic Energy Agency 2010). The fat and fat mass index was calculated in relation to height. Messenger RNA was determined from the faeces. Blood was analysed for absolute CD4 count, albumin, CRP, haemoglobin, LPS and sCD14. The GSRS in combination with the Bristol Stool Chart was used to assess changes in gastrointestinal symptoms. Compliance was monitored by a monthly count of surplus sachets of supplement. An open ended questionnaire was asked monthly to determine the continued acceptability of the product, whether the participant still wanted to continue, whether they had been healthy or had been to a doctor/clinic or changed their medications and to determine whether the inclusion and exclusion criteria were still being met.

3.6.1 Questionnaires

During the detailed process of informed consent, the research assistant briefly assessed the participant for eligibility according to the inclusion and exclusion criteria. After signing informed consent, the research assistant used a screening form to confirm that the participant was suitable for inclusion and that no reason for exclusion existed (Appendix F). At each follow up visit an open ended questionnaire was asked to confirm continued eligibility and to determine whether the participant was willing to continue with the study (Appendix M).

A closed ended food frequency questionnaire was administered by the same dietitian (PI) at each visit to determine both the intake of inulin-type fructans (prebiotics) and probiotics found naturally in food as well as the intake from foods supplemented with these (Appendix H). The foods

included were those known to contain inulin-type fructans (Van Loo *et al* 1995) (Campbell *et al* 1997) (Moshfegh *et al* 1999) which were locally available and culturally acceptable. The questionnaire was repeated at each visit to monitor the change in the dietary intake of pre and probiotics. Each questionnaire was compared to the previous visit and any differences in intake were confirmed with the participant to ensure that the changes were genuine.

The GSRS was administered at each visit by the PI to monitor changes in gastrointestinal symptoms (Appendix K). The Bristol Stool Chart was used to identify stool consistency (Appendix L). To confirm that any changes in symptoms were genuine, the GSRS was compared to the previous visit and any discrepancies were confirmed with the participant.

3.6.2 Weight

A portable Masskot scale (50 g to 150 kg) was used to determine weight. The scales were calibrated prior to the study by SA Scales and were regularly checked during the study using a known 5 kg calibration weight (Avery). The participants were weighed in the morning in minimal clothing[33] after urination and defecation and prior to eating and drinking. They were instructed to step onto the center of the scale by the PI, with their weight evenly distributed on both feet, looking directly ahead (Norton and Olds 1996). The weight was recorded on the data collection sheet (Appendix I). The participant was asked to step off the scale. The scale was zeroed and the participant reweighed. If the readings differed by 100 g, the participant was weighed a third time and the average was taken of the two closest readings.

3.6.3 Height

The participants were asked to remove their shoes, socks and head gear and then to stand on the baseboard of the stadiometer with their back against the stadiometer (height rod Seca 213 0 to 198cm) so that they were facing forward (Norton and Olds 1996). They stood erect with their feet together and flat on the centre of the base plate with their arms hanging naturally by their side.

[33] They were weighed in a private room in their underwear.

Their heels, buttock, upper part of the back, as well as the back of the head were in contact with the stadiometer. The head was positioned in the vertical Frankfort plane by the PI. The participants were asked to deeply inhale and the headpiece was lowered onto their vertex. The reading was recorded in the data collection sheet (Appendix I). They were then asked to exhale and the measurement was repeated. If there was a difference of 0.2 cm, the reading was repeated a third time and the average of the two closest readings were used.

3.6.4 Body Mass Indices

The BMI was calculated as weight in kilograms divided by height in meters squared. The BMI was interpreted using the categories in the table which follows (Table 4) (WHO 1998).

Table 4: Body mass index classification of nutritional status

BMI (kg/m^2)	Interpretation
<16	Severe Malnutrition
16 – <17	Moderate malnutrition
17 – <18.5	Mild Malnutrition
18.5– <2524.9	Normal weight
25 – <30	Pre-Obese
30 – <35	Obese Class I
35 – 40	Obese Class II

Fat free mass and fat mass is related to height and should be interpreted as an index rather than the actual value (Kyle *et al* 2003). As there are no reference tables for HIV-infected individuals, the NHANES-1 tables for healthy adults was used (Kelly *et al* 2009). The body fat mass index was calculated as body fat mass (kg) divided by the height (m) squared (Table 5) and the body fat free mass index was calculated as fat free mass (kg) divided by height (m) squared (Table 6). The NHANES-1 references values for % body fat was used (Table 7). As many of the published studies had not reported the fat mass or fat free mass index this was calculated from the data in the publication to enable more accurate comparisons.

Table 5: NHANES-1 Body Fat Mass Index (kg/m^2) versus age in adult subjects

Males

	White			Black			Mexican American		
Age	M	Σ	L	M	Σ	L	M	σ	L
20	5.95	2.59	-0.144	4.82	2.49	-0.378	5.89	2.16	-0.238
25	6.37	2.69	-0.114	5.59	2.81	-0.299	6.80	2.44	-0.173
30	6.78	2.77	-0.084	6.17	3.02	-0.220	7.45	2.60	-0.108
35	7.19	2.84	-0.054	6.56	3.11	-0.141	7.84	2.67	-0.044
40	7.57	2.89	-0.024	6.81	3.14	-0.062	8.06	2.67	0.019
45	7.92	2.92	0.006	6.98	3.12	0.017	8.19	2.64	0.080
50	8.21	2.91	0.037	7.15	3.10	0.095	8.35	2.62	0.141
55	8.49	2.90	0.067	7.42	3.12	0.174	8.51	2.60	0.200
60	8.71	2.86	0.097	7.72	3.14	0.252	8.64	2.57	0.260
65	8.84	2.78	0.127	7.95	3.13	0.331	8.67	2.50	0.319
70	8.82	2.66	0.157	8.10	3.08	0.409	8.54	2.39	0.378
75	8.68	2.50	0.187	8.16	2.99	0.488	8.30	2.26	0.437
80	8.46	2.32	0.217	8.17	2.89	0.567	8.02	2.11	0.496
85	8.20	2.14	0.248	8.15	2.78	0.640	7.72	1.97	0.556

Females

	White			Black			Mexican American		
Age	M	Σ	L	M	Σ	L	M	σ	L
20	8.48	3.80	-0.310	10.02	4.56	-0.048	9.89	3.54	-0.073
25	8.90	3.89	-0.249	10.87	4.81	0.003	10.51	3.70	-0.047
30	9.35	3.98	-0.188	11.59	4.98	0.054	11.07	3.82	-0.021
35	9.82	4.07	-0.126	12.09	5.05	0.106	11.52	3.90	0.005
40	10.27	4.13	-0.064	12.59	5.10	0.157	11.98	3.98	0.031
45	10.72	4.19	-0.003	13.01	5.11	0.210	12.46	4.06	0.057
50	11.20	4.25	0.059	13.33	5.08	0.262	12.80	4.09	0.083
55	11.67	4.29	0.121	13.57	5.00	0.316	12.91	4.04	0.109
60	12.03	4.28	0.183	13.68	4.88	0.369	12.88	3.95	0.135
65	12.14	4.17	0.245	13.64	4.70	0.424	12.73	3.82	0.161
70	12.02	3.99	0.308	13.46	4.48	0.478	12.48	3.67	0.187
75	11.70	3.74	0.370	13.04	4.18	0.533	12.16	3.49	0.213
80	11.27	3.47	0.433	12.30	3.80	0.587	11.79	3.31	0.239
85	10.79	3.19	0.495	11.46	3.42	0.639	11.45	3.15	0.262

M = Median, σ = Standard Deviation, L = Skewness

Table 6: NHANES-1 Body Fat Free Index (kg/m^2) versus age in adult subjects

Males

	White			Black			Mexican American		
Ag	M	Σ	L	M	Σ	L	M	σ	L
20	18.98	2.50	-1.115	19.50	2.98	-1.103	18.78	2.22	-0.738
25	19.31	2.52	-1.022	19.97	3.01	-0.969	19.36	2.23	-0.703
30	19.60	2.54	-0.929	20.33	3.01	-0.835	19.83	2.24	-0.667
35	19.85	2.56	-0.835	20.54	2.98	-0.701	20.16	2.25	-0.633
40	20.04	2.56	-0.740	20.60	2.93	-0.567	20.34	2.26	-0.600
45	20.15	2.55	-0.643	20.56	2.88	-0.434	20.37	2.26	-0.571
50	20.15	2.53	-0.546	20.47	2.85	-0.301	20.31	2.25	-0.543
55	20.07	2.50	-0.447	20.35	2.83	-0.169	20.15	2.21	-0.517
60	19.91	2.44	-0.348	20.19	2.80	-0.037	19.93	2.16	-0.492
65	19.67	2.36	-0.248	19.95	2.77	0.096	19.63	2.10	-0.467
70	19.36	2.26	-0.148	19.65	2.71	0.228	19.26	2.02	-0.442
75	18.98	2.15	-0.047	19.30	2.65	0.360	18.84	1.94	-0.418
80	18.58	2.03	0.053	18.93	2.58	0.493	18.39	1.85	-0.393
85	18.16	1.92	0.154	18.59	2.51	0.616	17.93	1.76	-0.369

Females

	White			Black			Mexican American		
Ag	M	Σ	L	M	Σ	L	M	σ	L
20	15.60	2.01	-1.404	17.24	2.74	-0.924	15.82	2.08	-1.510
25	15.83	2.10	-1.378	17.60	2.80	-0.911	16.24	2.18	-1.411
30	16.03	2.18	-1.352	17.87	2.84	-0.898	16.58	2.28	-1.311
35	16.19	2.26	-1.326	18.03	2.86	-0.885	16.83	2.36	-1.212
40	16.30	2.32	-1.299	18.12	2.86	-0.872	17.02	2.42	-1.112
45	16.36	2.35	-1.272	18.13	2.84	-0.859	17.14	2.45	-1.013
50	16.35	2.36	-1.244	18.07	2.80	-0.844	17.13	2.46	-0.912
55	16.30	2.35	-1.216	17.97	2.73	-0.830	17.00	2.43	-0.811
60	16.21	2.30	-1.188	17.88	2.66	-0.816	16.81	2.39	-0.710
65	16.08	2.24	-1.160	17.77	2.56	-0.803	16.57	2.35	-0.607
70	15.92	2.16	-1.132	17.61	2.46	-0.789	16.28	2.30	-0.505
75	15.73	2.07	-1.104	17.39	2.35	-0.776	15.97	2.25	-0.402
80	15.53	1.98	-1.076	17.12	2.24	-0.763	15.63	2.19	-0.299
85	15.32	1.89	-1.049	16.85	2.13	-0.750	15.34	2.14	-0.210

M = Median, σ = Standard Deviation, L = Skewness

Table 7: NHANES-1 Fat (%) versus age in adult subjects

Males

Ag	White			Black			Mexican American		
	M	Σ	L	M	Σ	L	M	σ	L
20	23.4	6.68	0.221	19.8	6.60	-0.311	24.4	6.09	0.075
25	24.6	6.49	0.325	21.7	6.89	-0.123	26.0	5.68	0.238
30	25.7	6.25	0.428	23.1	6.92	0.064	27.2	5.26	0.400
35	26.6	5.97	0.530	24.2	6.75	0.250	27.9	5.00	0.561
40	27.5	5.68	0.631	25.0	6.52	0.434	28.4	4.93	0.720
45	28.2	5.44	0.732	25.6	6.33	0.617	28.8	4.96	0.877
50	29.0	5.30	0.831	26.1	6.23	0.799	29.2	5.02	1.033
55	29.8	5.23	0.930	26.9	6.22	0.980	29.8	5.03	1.188
60	30.5	5.19	1.028	27.7	6.22	1.160	30.2	4.99	1.343
65	31.1	5.16	1.126	28.6	6.14	1.339	30.6	4.88	1.497
70	31.4	5.11	1.223	29.3	5.97	1.519	30.7	4.73	1.652
75	31.6	5.02	1.320	30.0	5.68	1.698	30.7	4.55	1.806
80	31.6	4.88	1.418	30.5	5.29	1.877	30.7	4.36	1.960
85	31.6	4.73	1.515	31.0	4.89	2.045	30.7	4.18	2.114

Females

Ag	White			Black			Mexican American		
	M	Σ	L	M	Σ	L	M	σ	L
20	35.1	7.22	0.361	36.0	7.78	0.955	38.0	6.30	1.126
25	36.0	7.23	0.573	37.8	7.59	1.127	39.2	6.11	1.237
30	37.0	7.21	0.785	39.2	7.31	1.300	40.0	5.83	1.347
35	38.0	7.12	0.996	40.1	6.91	1.471	40.6	5.53	1.456
40	38.9	6.96	1.207	41.0	6.48	1.641	41.2	5.29	1.564
45	39.8	6.73	1.417	41.8	6.10	1.811	42.1	5.11	1.672
50	40.8	6.46	1.626	42.4	5.82	1.980	42.9	4.98	1.779
55	41.7	6.16	1.833	43.0	5.68	2.149	43.4	4.87	1.887
60	42.5	5.86	2.041	43.3	5.63	2.318	43.5	4.82	1.994
65	43.0	5.58	2.247	43.3	5.63	2.487	43.6	4.89	2.102
70	43.0	5.37	2.453	43.1	5.65	2.655	43.5	5.07	2.210
75	42.9	5.24	2.660	42.7	5.64	2.824	43.3	5.31	2.319
80	42.5	5.14	2.866	42.0	5.55	2.992	43.2	5.58	2.427
85	42.1	5.08	3.072	41.1	5.43	3.153	43.0	5.82	2.522

M = Median, σ = Standard Deviation, L = Skewness (see LMS description in Methods)

3.6.5 Mid Upper Arm Circumference

The participant was asked to stand in the anatomical position while the PI took the measurements. The acromion process was located on the left side of the body and marked horizontally. The participant was asked to bend their left arm at a right angle with the palm facing upwards. An anthropometry tape measure (MABIS) was used to measure the distance between the acromion process and the tip of the elbow. The midpoint was marked horizontally. The MUAC was then measured at the midpoint with the participants arm hanging in a relaxed position. The research assistant ensured that the tape was level both anteriorly and posteriorly and not pulled too tightly or too loosely. The reading was recorded on the data collection sheet (Appendix I). A second reading was taken. If the reading differed by 2 mm a third reading was taken and the mean of the 2 closest readings were used. The MUAC measurements were interpreted using the reference ranges for women from the Centre for Disease Control (CDC) as the majority of patients were females (Centre for Disease Control 2007-2010).

3.6.6 Triceps Skinfold Thickness

The TST was measured on the left side of the body by the PI. A vertical line was drawn across the horizontal line on the triceps. The skinfold was picked up on the vertical line 1 cm above the horizontal line mark. The calipers (LANGE) were put at right angles to the mark at an approximate depth of a mid-finger nail. After two seconds the skinfold reading was taken without releasing the skinfold. The skinfold was then released. The measurement was repeated. If there was a difference of 1 mm in the readings, a third measurement was taken and the mean of the 2 closest readings were used. The TST measurements were interpreted using the reference ranges for women from the CDC as the majority of patients were females (Centre for Disease Control 2007-2010).

3.6.7 Measurement of fat free mass using deuterium dilution technique

The loss of fat free body mass mainly from skeletal tissue (as opposed to total body mass) is common in those infected with HIV (Wanke *et al* 2000) (Lazanas *et al* 2003) resulting in a loss of strength and the ability to function independently (Grinspoon *et al* 1999), decreased immune resistance, reduced pulmonary function and increased mortality (Roubenoff *et al* 1997). It is very important to use an advanced body composition technique to measure fat free mass as HIV-infected individuals have been shown to have a lower fat free mass than healthy counterparts even in the early stages of HIV infection, which is not reflected by measurements of body mass (Ott *et al* 1993) (Lazanas *et al* 2003). Deuterium dilution is the gold standard for the assessment of body composition (Camarneiro *et al* 2013). Deuterium is a stable (non-radioactive) isotope of hydrogen, with the symbol ^{2}H. It is given orally as deuterium oxide (D_2O). The methodology used is that found in the IAEA manual and involved the steps outlined as follows (International Atomic Energy Agency 2010).

3.6.7.1 Preparation of the deuterium dose

The preparation of the deuterium dose was done by the PI. The disposable wide mouth screw capped 60 ml plastic dosing bottles (EYDAM Thermo Scientific, Rochester), measuring cylinders (Glassco 50 ml) and 3 ml sterile Pasteur plastic pipettes (EYDAM) used in this process were completely dry. The doses were weighed to 0.001 g using a balance scale (BEL Mark 500) placed on a stable level surface. The scale was calibrated prior to use. The dosing bottle was placed on the weighing platform and the scale was tared. The deuterium oxide (SERCON Ltd UK Lot EB2039) was then poured into the dosing bottle using a measuring cylinder (Glassco 50 ml). The cylinder was not washed out between measuring the doses to avoid dilution of the deuterium. Excess solution was removed using a 3 ml dry sterile pipette until the deuterium solution added to the bottle weighed as close to 30.000 g as possible. During the process of weighing out the deuterium, the lid was replaced on the stock bottle of deuterium oxide to prevent loss by evaporation. The lid was tightly screwed on the dosing bottle which was labelled in indelible marking pen with the participant's number. The date, participant study number, batch number and exact weight of the deuterium solution, were recorded in a book. The deuterium samples were

then stored upright at 4°C in a refrigerator (LG GR389sQF) before use. To avoid cross contamination, deuterium doses were not stored next to saliva samples and were transported in separate containers between the clinic and the laboratory.

3.6.7.2 Preparation of calibration standards for Fourier Transform Infrared Spectroscopy

The calibration standard was prepared according to the IAEA SOP (Appendix N). The calibration or standard solution was prepared of approximately 1g/kg by weighing an accurately measured amount of deuterium oxide (approximately 1g) and diluting it with standard drinking water to the volume of one litre and reweighing the flask. The exact concentration of the calibration standard was then calculated. A second litre of the standard drinking water was used as the zero standard. These solutions were placed in borosilicate bottles with polytetrafluoroethylene (PTFE) lined screw caps and kept in a cool, dark place separate from the deuterium oxide. To check the accuracy of the Fourier Transform Infrared Spectroscopy (FTIR) (FTIR IRPrestige-21 SHIMADZU) over a range of concentrations of deuterium, 100 ml standards were prepared from 100 to 2000 mg/kg deuterium oxide according to the IAEA SOP (Appendix N). All standards were analysed in triplicate and a calibration curve was constructed.

3.6.7.3 Saliva collection

The participants were asked to eat and drink normally the day before the test and not to do any vigorous exercise the previous evening to avoid dehydration and depletion of glycogen stores. They were instructed not to eat or drink for the 1 hour prior to saliva collection in the morning although most fasted overnight. Saliva was collected by the PI.

They placed two dental cotton wool swabs (Henry Schein Dental Cotton Rolls) in their mouth which they sucked on until the swabs were wet with saliva. They were asked to move the swabs around their mouth and to keep their mouth closed during the process. The plunger was removed from a new 20 ml disposable syringe. The swabs were then transferred directly from the participant's mouth into a 20 ml syringe (Healthease Plus LUER SLIP). The plunger was replaced and the saliva from the swabs was syringed into a completely dry 4.5 ml cryovial (NUNC™

CryoTube™ Vials) labeled with the participants study number, date, whether baseline or end study and whether pre or post fasting. The cap was replaced on the cryovial and this process of collection was repeated until 4.5 ml of saliva had been collected. The cap was firmly replaced on the cryovial and the cryovial was placed in a separate zip lock bag away from other saliva samples. The participant then drank the solution containing 30 g of deuterium oxide (SERCON Ltd UK Lot EB2039) from the dosing bottle (EYDAM Thermo Scientific, Rochester) using a straw. Fifty ml of tap water was then placed in the dosing bottle, the container lid was replaced and the bottle was inverted and then thoroughly shaken to ensure that any remaining deuterium was washed from the top and sides of the dosing bottle. The participant then drank this to ensure that no deuterium was remaining in the dosing bottle. A further 50 ml of tap water was placed in the dosing bottle and the procedure repeated. The time the deuterium dose was ingested was then noted in the participants file and they were instructed not to eat or drink anything (including water and chewing gum) for the next four hours. They were given reading material and were seated in a cool place to avoid sweating and were asked not to exercise or walk around. This was to prevent water from leaving the body via transdermal evaporation and rapid breathing as increased insensible water losses would increase the concentration of deuterium in the body and result in an overestimation of body fat. After 4 hours the saliva was resampled using the technique described previously and the post dose sample was placed in a separate zip lock bag. The pre and post dose sample was then placed in one larger zip lock bag. The saliva was kept at room temperature (20 to 28°C) for a maximum of 6 hours before being frozen at –20°C until analysis.

3.6.7.4 Measurement of deuterium concentrations in the saliva samples

The deuterium content of the pre and post fasting samples was measured by the PI using the FTIR (FTIR IRPrestige-21 SHIMADZU) which was situated on a stable, level, independent surface in a well-ventilated, air conditioned, temperature (21°C) and humidity controlled room at DDMRI. The machine was not exposed to vibration from any nearby instruments such as a centrifuge.

The saliva samples were thawed at room temperature and then centrifuged for 10 minutes at 3000 rpm (ALC PK 121 R multispeed refrigerated centrifuge). This was to remove condensation from the caps of the cryovials, to remove air bubbles and to ensure that any solid matter (remains of

food or cotton wool used for sampling) settled at the bottom of the cryovial, leaving a clear liquid above which could be used for FTIR analysis.

The FTIR was prepared for measurement in accordance with the IAEA SOP (Appendix N). After filling the cell, lint free tissue was used to clean the window of the cell. At least two ml of drinking water was syringed through the cell to remove all traces of the previous sample. Folded absorbent paper was then firmly pressed over the exit port of the cell to absorb excess sample and to prevent the entrance of air. The background standard drinking water was then drawn up into a two ml disposable syringe and the cell was filled by attaching the syringe to the cell and firmly pressing the syringe plunger. Excess was removed from the outside of the cell using absorbent paper. The cell was held up to the light to check for air bubbles. More sample was syringed though the cell if there were air bubbles until all the air bubbles had been excluded. The cell was then placed in the FTIR and the absorbance measured at 2300-2900 cm^{-1}. On completion, the cell was removed from the FTIR and the procedure was then repeated with the calibration standard to obtain the spectrum which was used to calibrate the software. The saliva samples were then analyzed using the same technique. A pre-dose (background) saliva sample was run and then the post dose sample was analysed. The resulting FTIR spectrum was then compared to the calibration standard using the Medical Research Council software. A new one ml syringe was used for each sample to avoid cross contamination and the FTIR cell was flushed with drinking water between samples to avoid any memory effect. The background and calibration standards were reanalysed in the middle and end of the batch to check the calibration of the FTIR. On completion of the batch of samples, the cell was thoroughly rinsed with drinking water before storing. During the entire process, care was taken to avoid evaporation by keeping the caps on the cryovials/bottles at all times and only removing them to access the sample/standard.

3.6.7.5 Calculation of body composition

V_D is the volume of distribution.

The dilution space of ^2H (V_D) is 4.1% higher than total body water (TBW) due to the exchange of hydrogen (H) with non-aqueous H in the body.

TBW (kg) = V_D/1.041

Where V_D (kg) = Dose D_2O (mg)/enrichment ^2H in saliva (mg/kg)

The hydration of fat free mass is assumed to be 73.2% in adults:

Fat free mass (kg) = TBW (kg)/0.732

Fat mass is calculated by the difference between body mass and fat free mass

Fat mass (kg) = body mass (kg) – fat free mass (kg)

Results are often expressed as percent body weight

Fat mass (%) = fat mass (kg)/body mass (kg) x 100

Fat free mass (%) = fat free mass (kg)/body mass (kg) x 100

Example calculations are shown in Appendix P. The calculations were done by the PI.

3.6.8 Inulin-type fructan content

3.6.8.1 Calculation of dietary inulin-type fructan content

The inulin-type fructan content per 100 g of food (wet weight) was sourced from publications as there were no food data bases containing this information (Van Loo *et al* 1995) (Campbell *et al* 1997) (Moshfegh *et al* 1999). Where there were a range of values for a food the mean was used (Van Loo *et al* 1995) (Campbell *et al* 1997) (Moshfegh *et al* 1999). Information was obtained directly from the manufacturers for Future Life©, coffee (Nestlé) and bread (Premier foods, Karishma.Jithoo@PremierFMCG.com) (Table 8).

Table 8: Inulin-type fructan content of local foods

Food	Measure	Content per portion	Content per 100 g
Ricoffy Coffee powder	1 teaspoon (2 g)	0.65	32.5
Garlic	½ clove (1 g)	0.125	12.5
Onion	1 heaped tablespoon (50 g)	1.8	3.6
Bread white	1 slice (35 g)	0.61	1.75
Bread brown	1 slice (35 g)	0.58	1.66
Banana	1 large (100 g)	0.5	0.5
Future Life©	1 cup (250 ml)	0.25	0.1
Beetroot grated	1 heaped tablespoon (20 g)	0.072	0.36
Oats	1 cup (250 ml, 90 g)	0.27	0.3

There was considerable difficulty estimating the inulin-type content of both the coffee/chicory blend and the bread as the manufacturers wanted to keep their "special recipes" confidential. The inulin-type fructan content of coffee was calculated by multiplying the grams of coffee (g) by the percent of chicory (32.5%) and then dividing by 100 to establish the chicory content (g). The calculations for the inulin-type fructan content of a slice of bread is found in the table which follows (Table 9).

Table 9: Calculations of the inulin-type fructan content of a slice of bread

	Brown bread	White bread
Wheat flour in batch	175 kg of which 157 kg is white flour and 18 kg is bran	175 kg
Number of loaves per batch	369	380
Wheat flour per loaf (g)	157/369= 0.4254 X 1000 = 426	175/380 = 0.460 X 1000 = 460
Average loaf (slices)	18	18
Average weight of wheat in a slice (g)	426/18 = 24	460/18 = 25.5
Average content of inulin-type fructans in wheat/100 g	2.4	2.4
Inulin content (g) per slice	24 X 2.4/100 = 0.58	25.5 X 2.4/100 = 0.61

Portions were translated into grams using the Medical Research Council Food Quantities Manual (Langenhoven *et al* 1991) and the inulin-type fructan content calculated for individual foods (Table 8). The mean inulin-type fructan content was calculated over the three month period.

3.6.8.1 Contents of supplement

The supplement, an energy drink supplied by Future Life© is known locally as EXCEL (Figure 3).

Figure 3: Study supplement

The inactive and active energy drink were identical in composition except for the 15 g of inulin-type fructans added to the active supplement. They were equivalent in energy, macro and micronutrients (Table 10). The energy value of inulin-type fructans ranges from 0 to 10.5 kJ (2.5 kCal) per gram depending on the extent of the fermentation in the colon (Roberfroid 1999). The supplement with inulin-type fructans therefore may have provided a maximum

of an additional 157 kJ per sachet which was considered a negligible contribution to the total energy intake.

Each step of the manufacture and processing was overseen by the PI. Fifteen grams of inulin-type fructans (Frutafit®) was added to the active supplement during processing – no inulin-type fructans was added to the placebo supplement (Appendix A). The supplement was packed into identical sachets and these sachets were packed into identical boxes (Figure 4).

Figure 4: Boxes of study supplement

Each box contained 32 sachets rather than 30 sachets to allow for those who missed their appointment date. Each box was then weighed to confirm that the box contained the correct number of sachets. If there was a discrepancy in the weight, the box was opened and the sachets recounted. The boxes were then delivered to DDMRI where an independent researcher administrator labelled the boxes with the study number following the randomly generated table produced by the statistician.

Table 10: Nutritional content and ingredients of the EXCEL supplement per day (50 g sachet)

Nutrients	EXCEL Sachet (50 g)
Energy (kJ)	798
Protein (g)	8
Glycemic carbohydrates (g)	30
Of which sugar (g) was	7.5
Total fat (g) of which	4
Fibre (g)	2.6
Vitamin A (retinol) ug	450
Thiamine (mg)	0.6
Riboflavin (mg)	0.55
Niacin (mg)	8
Pantothenic acid (mg)	2.5
Pyridoxine (mg)	0.85
Folic acid (ug)	200
Vitamin B12 (ug)	1.2
Vitamin C (mg)	50
Vitamin D (ug)	7.5
Calcium (mg)	165
Copper (mg)	0.2
Iodine (ug)	75
Iron (mg)	4.5
Magnesium (mg)	28
Manganese (mg)	0.4
Molybdenum (ug)	7.5
Phosphorus (mg)	123
Potassium (mg)	228
Selenium (ug)	28
Zinc (mg)	2.3
Sodium (mg)	125
Ingredients	
Corn, soya, sucrose, maltodextrin, soya milk powder, salt and flavouring	

Half a sachet of the supplement was consumed for the first 8 days so that the dose of inulin-type fructans was increased in a graded increment to avoid side effects such as flatulence (Pedersen *et al* 1997) (Rycroft *et al* 2001), bloating, rumbling, cramps, and liquid stools (Schaafsma and Slavin 2015). As side effects tend to occur at doses greater than 20 g per day with shorter chain inulin-type fructans such as FOS and are less likely to occur when ingested with solids, intolerance to the supplement was not anticipated or found.

3.6.9 Tuberculosis screening

The nursing sister working in the TB Treatment Centre at Lancers Road Clinic was responsible for the collection of the two sputum samples required to diagnose TB. The collection bottles were labeled with the participant's name and clinic number. Participants were instructed not to touch the inside of the bottle with their hands at any time. They were informed about the importance of collecting sputum/phlegm from the lungs and not saliva from the mouth. They were asked to rinse their mouth out with water first so that food or other solid particles would not contaminate the sample. They were instructed to breathe deeply while pressing firmly on the sides of their chest and then to cough deeply several times until they could cough up enough phlegm to spit into the bottle while being careful not to soil the outside of the container. Once the sputum was collected, the participants then screwed the lid on the bottles tightly and returned the bottles to the nursing sister who placed the bottles with the required NHLS forms in a cooler bag (2 – 8°C) for collection by the NHLS within 4 hours. A minimum of 1 ml of sputum was collected for each sample.

Participants who could not produce sputum were sent home with collection bottles and instructed to collect the samples after a very hot shower or bath or else to cover their heads with a towel and inhale steam for 5 minutes from a pot of hot water. If they failed to bring the samples on their return to the clinic, they were nebulized for 15 minutes using normal saline (SABAX Pour Saline 0.9% Adcock Ingram) placed in a disposable mask (Venturi Mask W/2M tubing PN-1125 BESMED Health Business Corp Taiwan) attached to an oxygen cylinder. Sputum was then collected. Failing this the nebulization was repeated a second time. If sputum was not produced they were sent to the Durban Chest Clinic for chest X-rays.

Real time PCR (GeneXpert MTB/RIF), as well as fluorescence microscopy (auramine O stain), for *Mycobacterium tuberculosis* was performed by the NHLS using SOP.

3.6.10 Faecal messenger ribonucleic acid analysis

3.6.10.1 Collection of stool samples

Each participant was issued with a stool collection kit by the PI consisting of gloves (Healthesase Latex examination gloves), polystyrene tray, plastic spoon, stool collection sample bottle labeled with their study number, paper bag, plastic bag and toilet paper. The participant put on the gloves and then passed urine. They placed the polystyrene tray in the toilet bowl and defecated onto it and then transferred the stool to the stool collection bottle using the plastic spoon. Once the bottle was half full, they closed the lid tightly and put it into the paper bag. They then flushed the toilet and washed off the tray under the flushing water. They placed the tray, spoon and gloves in a plastic bag which was disposed of in a human waste container by the PI.

The PI then transferred the stool from the collection bottle into 6 cryovials each containing 1.8 ml (CRYO.S™ Greiner bio-one) labeled with the participants study number, date and whether the sample was baseline or end study. The outside of the cryovials were washed, dried and stored within 6 hours at -75°C in DDMRI prior to mRNA analysis.

3.6.10.2 Analysis of mRNA

The use of digital droplet polymerase chain reaction (ddPCR) from faecal extractions was novel. It was very important to be able to quantify the nucleic acids with accuracy and precision. The relatively new technology of ddPCR enabled the precise quantification of target mRNA in the samples by counting nucleic acid sequences encapsulated in discrete, volumetrically defined, water-in-oil droplet partitions (Pinheiro *et al* 2011). The technique of ddPCR has a much lower level of uncertainty for quantification of specific RNA target sequences than that of the traditional chamber based digital PCR (cdPCR).

The mRNA markers included GAPDH, PIGR, SOD2, IL8, IL1B and S100A8. As GAPDH is a housekeeping gene found in intact cells shedded in the faeces and the amount is relatively constant in a cell, the amount of GAPDH extracted therefore gives an idea of the number of cells being shedded. High levels of GAPDH therefore reflect increased levels of cell shedding which is thought to occur in HIV infection. This study isolated genetic inflammatory markers. For example rather than measuring the actual faecal calprotectin levels, the study identified and quantified the presence of the gene (S100A8) responsible for the production of faecal calprotectin. This is a more sensitive indicator since if faecal calprotectin was not detected this could have been due to degradation whereas if the gene could not be detected it was unlikely to have been present. Markers of mRNA were determined at baseline and study end as a measure of changes in inflammation or damage to the cells lining the GIT. The mRNA analysis was conducted by the laboratory of Professor Mark Manary, Washington University School of Medicine, St Louis, United States of America. Samples were shipped frozen to the laboratory. Confirmation was received that samples arrived in a frozen state and were immediately transferred to a -75° freezer until analysis.

An aliquot of frozen faeces (approximately 200 mg) was transferred to a 2 ml tube containing 150 mg of 425-600 µM glass acid washed beads (Sigma) and seven to ten 2.3 mm zirconium/silica beads (Research Products International, Corp.). To this, 1 ml of EasyMAG lysis buffer was added and the mixture was "bead-beated" with a FastPrep-24 tissue homogenizer (MP Biomedicals) for two consecutive 45 second runs then centrifuged at 13 200 rpm for 10 minutes. Clarified lysate (approximately 1 ml) was increased to a final volume of 2.2 ml with additional lysis buffer, and used to isolate total nucleic acids using the Specific A Protocol for the NucliSENS easyMag instrument (bioMérieux, Boxtel, The Netherlands), following the manufacturer's instructions (Dundas et al 2008) (Loens et al 2007). The total human mRNA in the samples was measured using GAPDH, which was then used to normalize results for SOD2, IL8, IL1B, S100A8, SOD2 and PIGR expression assays (Bennett Jr et al 2010). Multiplex PCR of 20 µl were prepared using 7.76 µl total nucleic acids (20 ng/µl or less), 10µl droplet digital PCR Supermix for Probes (BioRad), 0.08 µl SuperScript III Reverse Transcriptase (200 U/µl, Invitrogen), 0.16 µl recombinant ribonuclease inhibitor (40 U/µl, Invitrogen), 1 µl 20 by TaqMan Gene Expression Assay (Applied Biosystems, Inc) labeled with VIC, a proprietary green fluorescent dye (Life

Technologies, Grand Island, NY) or FAM (6-carboxyflourescein). Duplicates were run on all samples. Droplets were generated according to the manufacturer guidelines with the QX100 Droplet generator (BioRad) before cycling in a C1000 Touch thermal cycler (BioRad) at 50°C (30:00), 95°C (10:00), 40 cycles of 94°C (0:30) followed by 60°C (1:00), 98°C (10:00). Plates were held at 4°C between amplification and droplet reading. Data from the QX100 Droplet Reader was analyzed with the QuantaSoft software (Kosice, Slovakia). Fluorescent droplets were deemed positive by manually set thresholds based on results from the negative control wells containing RNase free water instead of RNA.

3.6.11 Haemoglobin measurements

Anaemia is commonly found in HIV-infected individuals and results in increased mortality independent of CD4 counts and viral load (Fangman and Scadden 2005). As one in four female South Africans suffer from anaemia (Shisana *et al* 2013) screening for iron deficiency anaemia was essential.

Haemoglobin was analysed using the HemoCue© Hb201 by the research assistant. The reaction in the microcuvette is a modified azidemethemoglobin reaction. The erythrocytes are haemolyzed to release the Hb which is converted to methemoglobin and then combined with azide to form azidemethemoglobin. Transmittance is measured in the analyser and the absorbance and Hb level calculated. The absorbance is directly proportional to the Hb concentration.

The puncture site from the blood sampling was used to draw up approximately 10 ul of blood into the microcuvette cavity (HemoCue© Hb201) by capillary action. The microcuvette was held opposite the filling end without touching the optical eye. The outside of the microcuvette was wiped with a clean lint-free tissue without touching the open end and checked for air bubbles. If air bubbles were present the sample was discarded and a new microcuvette was used to collect another sample. Within ten minutes the filled microcuvette was placed in the cuvette holder of the analyser (HemoCue© Hb201) and the holder was closed. The reading was recorded after 15 to 60 seconds. The microcuvette was then removed from the holder and discarded into a sharps

container. If the Hb level was below 10 g/dl the test was repeated on another machine to confirm the reading.

Although recent recommendations by the National Institute of Health, Division of AIDS (NIH DAIDS) for Grading the Severity of Adult and Pediatric Adverse Events are available (U.S. Department of Health and Human Services), the classification recommended by the World Health Organisation (WHO) (World Health Organization 2014) was used so that the results could be compared to those of the South African National Health and Examination Survey (SANHANES-1) (Shisana *et al* 2013). The definition of mild anaemia was 11 – 11.9 g/dl, moderate anaemia 8 – 10.9 g/dl and severe anaemia <8 g/dl. When reviewing the HIV studies in the discussion section, the Hb was reclassified according to the criteria used by each study to enable comparisons.

3.6.12 CD4 count

3.6.12.1 Collection of samples

Four ml of whole blood was drawn into an ethylenediaminetetraacetic acid (EDTA) containing endotoxin free tube (BD vacutainer K2E 7.2 mg ref 368861) by venipuncture. After being vigorously shaken 6 to 8 times, the blood samples were maintained at room temperature until being collected within 6 hours by the NHLS.

3.6.12.2 Measurement of peripheral CD4 count

Flow cytometry, an immunofluorescence analysis, is the gold standard for the measurement of CD4 T lymphocytes[34]. Cells passing through a laser beam scatter light differently in relation to differences in cell size and granularity. These cells also emit fluorescence after being stained with a specific monoclonal antibody which binds to CD4 T helper cells. The CD4 lymphocytes can then be identified (gated) and the percentage of the total lymphocytes calculated. A single platform approach using multi colour analysis was used (as opposed to the 2 colour methodology) which

[34] Laboratory Guidelines for enumerating CD4 T Lymphocytes in the context of HIV/AIDS, 2007. Accessed from http://www.who.int/hiv/amds/ LaboratoryGuideEnumeratingCD4TLymphocytes.pdf.

enables the absolute CD4 count to be automatically calculated rather than calculated from the CD4 lymphocyte percentage obtained from flow cytometry and the differential lymphocyte count estimated from the haematology analyzer.

The samples were analysed by the SANAS accredited Durban NHLS (149 Prince Street, Durban 4001) by flow cytometry (BD FACSCalibur) using BD Multiset CD3/CD4/CD8/CD45 reagents (Becton Dickinson TriTEST immunofluorescence reagent San Jose, CA). The analysis was done according to SOP laid out in the manufacturers insert within 24 to 48 hours after collection.[35]

Fifty microliters of EDTA anticoagulated blood was added directly to each of the two labeled TruCount tubes (tube A MultiTEST CD3/CD8/CD45/CD4 reagent 20 ul, tube b MultiTEST CD3/CD16+CD56/CD45/CD19 reagent 20 ul). The tubes were then incubated with fluorochrome-labeled antibody reagent for 15 minutes in the dark. Four hundred and fifty millilitres of 1XFACSTM Lysing Solution was then added and the cells incubated for a further 15 minutes in the dark. The samples were then acquired and analysed. A wash step was not needed. The absolute CD4 count was automatically calculated using the following formula:

# of events in region X # of beads per pellet = absolute count (cells/ul) of subset population
# of events in absolute sample volume (ul) count bead region.

3.6.13 C-Reactive protein

3.6.13.1 Collection of samples

Four ml of whole blood was drawn into a serum separator tube (BD vacutainer SST II advance ref 367995 5 ml) by venipuncture. After being vigorously shaken 6 to 8 times, the blood samples were maintained at room temperature and then centrifuged (2000 rpm, 10 minutes, 21°C, ALC PK 121 R multispeed refrigerated centrifuge) within 6 hours at DDMRI by the PI. The microbiological safety cabinet class II (validated on the 4/10/2012 by an independent laboratory)

[35] http://www.bdbiosciences.com/ds/europe/tds/23-5351.pdf.

was thoroughly wiped down with a 70% alcohol solution before use. The serum was pipetted using a 3 ml sterile endotoxin free Pasteur pipette (Ratiolab Pasteur-Plast Pipetten 3 ml Makro, graduiert 150 mm steril) into endotoxin free 1.8 ml cryovials (Greiner bio-one Cryo.s™ sterile). New pipettes were used for each sample. The samples were then stored at -75°C (ultra low temperature New Brunswick Scientific Freezer) in DDMRI until analysis.

3.6.13.2 Assessment of C-reactive protein

C-reactive protein is an acute-phase protein used as a non-specific marker of infections (particularly bacterial) and inflammation (Epstein *et al* 1999). HIV disease progression has been shown to be related to levels of CRP independent of both CD4 count and HIV RNA level (Lau *et al* 2006). Raised levels of CRP in HIV-infected adults was found to be both a predictor of mortality (Ledwaba *et al* 2012) as well as of opportunistic disease (Rodger *et al* 2009) (Nixon and Landay 2010).

High-sensitivity CRP was analysed using a serum quantitative latex immunoturbidimetric assay. Anti-CRP polyclonal antibody (rabbit) is adsorbed to latex particles. When this is exposed to CRP, an antigen-antibody reaction occurs which results in agglutination. This is detected as an absorbance change (572 nm) with the rate of change being proportional to the quantity of CRP in the sample.

The samples were analysed by the SANAS accredited Lancet Laboratories (102 Lancet Medical Centre, 74 Lorne Street, Durban). The serum samples were processed following SOP according to the manufacturer's instructions for the kit CRP vario 6K26-30 using the Abbott Architect ci8200.[36] Internal quality control samples were run at the beginning of each eight hour shift.

To verify the results, 10 samples were sent to AMPATH, an independent SANAS accredited laboratory (Chelmsford Medical Centre 3, St Augustine's Hospital, Durban). The serum samples were processed with the Roche Cobus C501 using a particle enhanced immunoturbidimetry assay

[36] http://www.ilexmedical.com/files/PDF/CRPVARIO_ARC_CHEM.pdf

with CRPL3 as the reagent. As there were no major anomalies, the results from Lancet were accepted as being accurate.

3.6.14 Albumin

The serum was collected and processed as for CRP.

Albumin levels <38g/l have been associated with increased mortality and incident TB in HIV-infected adults (Sudfeld *et al* 2013). Each 1 g/l decrease in serum albumin has been associated with a 13% increase in the risk of progressing to a CD4 count <200 cells/mm^3, after adjustment for set point plasma viral load (Graham *et al* 2007). Serum albumin may be useful for clinical monitoring as it is a strong independent predictor of mortality in HIV-infected women (Feldman *et al* 2003a).

The Albumin BCP assay is based on the principle that bromcresol purple reacts specifically to human albumin, producing a coloured complex that is detectable as 604 nm and that is directly proportional to the albumin concentration of the solution.

The samples were analysed by the SANAS accredited Lancet Laboratories (102 Lancet Medical Centre, 74 Lorne Street, Durban). The serum samples were processed according to the manufacturer's instructions for the 7D54 Albumin BCP Reagent kit using the Abbott Architect ci8200.[37] Internal quality control samples were run at the beginning of each 8 hour shift.
To verify the results, 10 samples were sent to AMPATH, an independent SANAS accredited laboratory (Chelmsford Medical Centre 3, St Augustine's Hospital, Durban). The serum samples were processed using the Roche Cobus C501 via a colourimetric test using Albumin P reagent. As there were no major anomalies, the results from Lancet were accepted as being accurate.

[37] https://www.abbottdiagnostics.com/en-us/products/ARCHITECT-ci4100.html#documentation.

3.6.15 Lipopolysaccharide assay

Levels of LPS increase as a consequence of disruption to the GIT mucosa (Nixon and Landay 2010). Levels of plasma LPS have been shown to correlate to systemic immune activation and therefore changes in LPS concentrations are often used as a direct index of changes in microbial translocation (Douek 2007) (Brenchley *et al* 2006a) (Novati *et al* 2015). According to Marchetti (2011), LPS is a strong predictor of HIV disease progression which is independent of CD4+ cell count in early HIV infection and suggested that LPS may be useful as a biomarker for HIV monitoring and evaluation in clinical trials (Marchetti *et al* 2011).

The most frequently used method of LPS detection is the chromogenic limulus amebocyte lysate (LAL) assay (Ishihata *et al* 2013). The principle is that the aqueous extract (LAL) from the amobocytes of the horseshoe crab (*Limulus polyphemus*) coagulates in the presence of bacterial endotoxin/LPS. When endotoxin is present in a sample containing LAL, a proenzyme is activated which liberates p-nitroaniline from a colourless synthetic substrate (Ac-lle-Glu-Ala-Arg-pNA) which in turn releases a yellow colour. The reaction is stopped with a stop agent and the absorbance is detected spectrophotometrically at 405-410 nm. As the correlation between the endotoxin concentration and the absorbance is linear in the 0.1-1.0 EU/ml range, the concentration of endotoxin is calculated from the absorbance values of solutions containing known amounts of endotoxin standards. The LAL therefore is a quantitative test for gram negative bacterial endotoxin.

At the time the blood samples were drawn the participants displayed no signs or symptoms of acute infection, gastrointestinal disease, opportunistic infections or overt signs of bacterermia, cirrhosis or end stage liver disease which could have influenced the results. No patients were taking heparin which also interferes with the analysis. All patients were fasting to prevent the assay being affected by the turbidity of the lipids.

The Lonza LAL QCL-1000 kit was used by the PI with the assistance of Dr Brodie Daniels.

Careful techniques were used to prevent microbial or endotoxin contamination. All procedures were carried out in a microbiological safety cabinet class II which was thoroughly cleaned between each use with a 70% alcohol solution. Endotoxin free cryovials, individually wrapped endotoxin free pipettes, endotoxin free pipette tips and endotoxin free glass test tubes were used.

The E. coli endotoxin standard was reconstituted by adding 1.0 ml of LAL Reagent Water warmed to room temperature and vortexed vigorously for 15 minutes to detach any endotoxin attached to the glass. The chromogenic substrate was reconstituted by adding 6.5 ml of LAL Reagent Water and covered with tin foil to protect from light.

Prior to running the assay, effective inactivation of the samples is essential as blood contains a number of proteins such as proteases that interfere with the assay (Dawson 2005). Some proteases neutralize endotoxin, others deactivate the LAL enzyme cascade[38] while others activate the cascade (Ketchum and Novitsky 2000) (Warren *et al* 1985) (Warren *et al* 1986) (Roth *et al* 1990). As proteins are denatured by heat, heat inactivation is considered the most appropriate method when analyzing endotoxin in blood in combination with sample dilution (Dawson 2005). An initial range of heat activation treatments and dilutions were pretested. The one recommended by Lonza (Dawson 2005) did not allow the recovery of endotoxin [39] nor did the method recommended by Roth *et al* (1990).[40] The optimal recovery of endotoxin was obtained by following the methodology of Pearson *et al* (1985) where the sample was diluted tenfold, vortexed for 10 seconds, covered with Parafilm and then heated for 10 minutes at 70°C in a heating bath and then vortexed again for ten seconds. The samples were then allowed to cool.

The standards were prepared during the heat inactivation. One ml of LAL reagent water was added to the lyophilized standard and vortexed gently for ten minutes. The standards were vortexed gently between dilutions so as not to introduce bubbles. This was then serially diluted into

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[38] The LAL assay is dependent on the proper activation of the cascade of serine proteases that comprises the lysate.

[39] The samples were diluted either twofold or fourfold and then 1 ml of sample was added to an endotoxin free glass test tube which was covered with parafilm. The bottom half of the test tube was then immersed in boiling water for 2 minutes. The sample was cooled and the assay commenced.

[40] The plasma was diluted fourfold with 0.15 M NaCl which was followed by a 30 minute heat treatment at 60°C prior to the assay.

standards (0.5. 0.25, 0.125, 0.063, 0.032, 0.016) in pre labelled tubes. The Chromogenic Substrate vial from the provided kit had 7.5 ml of LAL reagent water added to it. After being resuspended in water the substrate was vortexed and equilibrated to 37°C in an incubator. The LAL was reconstituted and used immediately due to its instability. All unused reconstituted LAL reagent was disposed of. The LAL lysate vial had 3.6 ml of LAL reagent water added to it and was vortexed gently prior to immediate use.

Each sample was run in duplicate with an additional sample blank i.e. the well contained nothing other than 50 µl of diluted, heat inactivated plasma sample. A pipette (BioPette) was used to pipette 50 µl of sample into each of the wells. After both the samples and standards were loaded, the plate was equilibrated to 37°C in the microplate reader for 5 minutes. The multi-channel pipette (BioPette) was then used to add 50 µl reconstituted LAL to the first two wells of each sample and the standards at an even, regular pace. The plate was then incubated at 37°C for 12 minutes. A 100 µl of reconstituted Chromogenic Substrate was added to the first two wells of each sample and standards. This was incubated for 7 min at 37°C. A stop solution (sodium dodecyl sulfate (SDS) solution) was added in 50 µl aliquots to the first two wells of each sample and standards. The plate was read at 405 nm immediately.

The standard curves were calculated by averaging the concentrations of the duplicates for each standard concentration, and graph absorbances versus concentration on an XY graph. A trend line was added via linear regression. The boxes to show the equation and the r-value were selected on the graph. An r-value > 0.96 was acceptable, and the negative control had an absorbance < 0.10.

Unfortunately the endotoxin spikes were not recovered in the expected amounts from the spiked samples and the variation between the duplicates was not acceptable. Two independent researchers repeated the assay twice in our laboratory and experienced similar issues.

Natasha Samsunder (Head of the Centre for the AIDS Programme of Research in South Africa) who was trained in the technique of LPS assay by Daniel Douek (Chief of the Human Immunology

SectionVaccine Research Center)[41] from the National Institute of Health was consulted. She informed us that in spite of the manufacturer's protocol recommending the use of endotoxin free glass tubes, endotoxin free polystyrene tubes were essential as the endotoxin attaches to the glass which in turn interferes with the reading. Wendy Burgers (Senior Researcher in the Division of Medical Virology and an associate member of the Institute of Infectious Diseases and Molecular Medicine, University of Cape Town) and Tracey Muller (University of Cape Town, Institute of Infectious Disease and Molecular Medicine, Research Officer) were also consulted were also consulted and they confirmed that polystyrene not glass test tubes should be **used. They also** recommended that the standards should be vortexed vigorously **rather than** gently for at least 10 minutes. Daniel Doueks heat inactivation protocol of 80°C for ten minutes was also recommended.

The LPS assay was then repeated with the following modifications: heat inactivation at 80°C for 15 minutes; 5ml BD Falcon tubes (BD 352054) endotoxin free polystyrene test tubes were used and the standards were vortexed vigorously. Unfortunately this did not resolve the issues around recovering the spiked samples or the extent of variation.

Wendy Burger, Natasha Samsunder and Daniel Douek were then approached to see if they would be willing to run the samples for the study. All declined as none of their laboratories were currently running the assay nor was there any intention to run the assay in the foreseeable future.

Although the technique of measuring LPS was selected on the advice of Professor Gregor Reid (Director of the Canadian Research and Development Centre for Probiotics), it is well accepted in the scientific literature that LPS is extremely difficult to measure (Redd *et al* 2009b) (Lederman *et al* 2011a) (Sandler *et al* 2011). These assay difficulties were experienced despite having been assisted by Dr Brodie Daniel who has extensive experience in the measurement of LPS in non-biological samples. It was therefore decided that the inactivation process was proving too difficult to warrant continuation of the assay. In view of the fact that our study was already measuring a

[41] Who has published extensively in the area of HIV and LPS and whose laboratory is considered to have mastered the technique.

second marker of microbial translocation (sCD14) the research team decided to discontinue LPS measurements and only report on one marker instead of two.

3.6.16 Soluble CD14

Lipopolysaccharide binds to CD14 (glycoprotein) which exists as both membrane bound and in a soluble form – CD14 then transfers the LPS to TLR4/MD-2 complex in the plasma membrane which in turn triggers the release of pro-inflammatory cytokines and type I IFN (Płóciennikowska *et al* 2015). Soluble CD14 therefore is used as a marker of LPS stimulated monocyte or macrophage activation (Landmann *et al* 1996) (Hiki *et al* 1998).

The method of detection was a quantitative sandwich enzyme immunoassay. The microplate was pre-coated with a monoclonal antibody specific for sCD14. After the standards/samples are pipetted into the well the sCD14 is bound by immobilized antibody. The unbound substances are then washed away and an enzyme-linked polyclonal antibody specific for sCD14 is pipetted into the wells. After washing to remove unbound reagent, a substrate solution is added to the wells and the colour develops proportionally to the amount of sCD14. After the development is stopped the intensity of the colour is measured using a microplate reader.

A Quantikine Human sCD14 Immunoassay enzyme-linked immunosorbent assay (ELISA) was performed by the PI with the assistance of Dr Brodie Daniels.

All reagents were brought to room temperature before use. Twenty ml of Wash Buffer Concentrate was diluted with distilled water to prepare 500 ml of Wash Buffer. The Calibrator Diluent RD5P concentrate was diluted with distilled water to a volume of 100 ml. The sCD14 standard was reconstituted with 5 ml of Calibrator Diluent RD5P to produce a stock solution of 16 000 pg/ml. This was left to stand for at least 15 minutes being agitated gently prior to making dilutions. Five hundred microliters of Calibrator Diluent RD5P was pipetted into each tube. A series of six dilutions was produced with the Calibrator Diluent RD5P as the zero standard (0 pg/ml) and the 16 000 pg/ml being the highest standard.

Ten microliters of sample was diluted with 1990 microliters of distilled water. A 100 ul of Assay Diluent RD1W was added to each well before adding 100 ul of either standard, control or sample to each the well. The plate was then covered with the adhesive strip and incubated at room temperature for 3 hours. Each well was washed four times using the Wash Buffer and the liquid was completely removed between each wash step by blotting on clean paper towels. Two hundred microliters of sCD14 conjugate was then added to each well. The plate was covered with a new adhesive strip and incubated for a further hour. The wells were then washed as before. The colour reagents (substrate solution) were mixed in equal volume not more than 15 minutes before use and protected from light. Two hundred microliters of the Substrate Solution was then added to each well which was then incubated at room temperature for 30 minutes and protected from the light. Fifty microliters of Stop Solution was added to each well and the plate tapped gently to ensure uniform mixing. The optical density was determined immediately at 450 nm with the wavelength correction being set at 570 nm to correct for optical imperfections in the plate.

The standard curve for the 3 microwell plates was created by plotting the mean absorbance for each standard on the y-axis against the concentration of the x-axis and a best fit curve drawn through the points on the graph (R2 = 0.9923, R2 = 0.9923, R2 = 0.9923).[42] The concentration read from the standard curve was multiplied by the dilution factor of 200.

3.7 TRAINING

The research assistant was a qualified phlebotomist and HIV counsellor. As she had participated in numerous HIV-related trials while in the employment of the Medical Research Council, she was extensively trained and experienced in the process of taking a detailed informed consent, the importance of precise and consistent data collection and had completed a Good Clinical Practice course.

[42] R-squared is a statistical measure of how close the data are to the fitted regression line. It is also known as the coefficient of determination, or the coefficient of multiple determination for multiple regression. 0% indicates that the model explains none of the variability of the response data around its mean.

The clinician had previously participated extensively in a number of HIV related trials for the Medical Research Council and had vast experience in the treatment of HIV-infected individuals.

The PI was a registered dietitian and lecturer at the University of KZN whose teaching portfolio included the accurate determination of body composition and lecturing HIV/AIDS.

The research assistant and the clinician attended a six hour training course conducted by the PI. This was to familiarize them with the reasons for conducting the trial, the trial design, what was expected of them and to revisit the importance of informed consent and accurate reliable measuring and recording.

3.8 PILOT STUDIES

Two pilot studies were conducted. The one tested the tolerability, the other the feasibility of the methodology.

3.8.1 Tolerability of inulin-type fructans

It is well accepted that an excessive intake of inulin-type fructans can result in gastrointestinal side effects such as diarrhoea, abdominal cramping, bloating and borborygmus (Schaafsma and Slavin 2015) with flatulence being the most commonly reported side effect (Dahl *et al* 2014). To ensure that the dose of inulin-type fructans was tolerable, ten black African females of similar demographic characteristics who were not attending the Lancers Road Clinic were enrolled for a period of three weeks. In a double blind fashion, five received the EXCEL supplement containing 7.5 g of inulin-type fructans for one week and then the supplement containing 15 g of inulin-type fructans for a further 2 weeks. The remaining five received the EXCEL supplement without inulin-type fructans. At the end of the three week period, there was no difference in gastrointestinal symptoms between the two groups according to the GSRS scale and the Bristol stool chart confirming the acceptability of the dose.

3.8.2 Testing methodology

Ten participants were recruited from the clinic for the pilot study. The purpose was to test the feasibility of the methodology, the flow of data collection, clarify any misunderstandings and to ensure that all members of the research team were competent in their roles. The exact methodology as outlined was followed. As no changes to the methodology were needed, these participants were included as part of the main study.

3.9 DATA CAPTURING, REDUCTION OF BIAS AND STATISTICAL ANALYSIS

To minimize errors associated with data capturing, measurements such as weight, height, MUAC and TST were repeated twice. A third measurement was taken if the initial measurements were not similar enough and the 2 closest readings were averaged. Duplicate samples were run for the saliva deuterium measurements and mRNA analyses. The albumin and CRP readings were confirmed by a sub sample analysed by an independent laboratory. The food frequency and GSRS were checked with the previous visit and queried if there were any major changes to ensure that there was an actual change and not a misunderstanding. A translator was used if there was the slightest doubt that the participant did not speak or understand English adequately.

Bias was reduced by conducting the pilot studies. Further reduction in bias was ensured by only using study personnel with adequate training and experience. The research assistant and clinician in HIV trials had extensive experience working in previous HIV trials conducted by the Medical Research Council. The same people were responsible for the same measurements throughout the study and the same equipment was used. The albumin and CRP were analysed using one kit in one run by one dedicated technician. This was not possible for the CD4 count as this was analysed by the NHLS. The PI was present at all times to deal with queries and difficulties.

The data was double entered by the PI into the computer program Excel. The two data bases were compared to ensure that there were no input errors. The PI checked the data for outliers and discrepancies. The cleaned database was then exported into Statistical Analysis Software (SAS) version 9.2.

Quantitative variables were examined for departure from normality using the skewness statistic and its standard error. Normally distributed quantitative variables were described using means, standard deviations (SD) and ranges. Non-normally distributed continuous variables were described using medians and inter quartile ranges (IQR). Between-group differences were compared using independent sample t-tests and paired t-tests used for within-group changes. Where the data was non-normally distributed the Wilcoxon rank sum test was used. Categorical variables were described using frequency and relative frequency tables. Comparisons of categorical dependent variables between groups was achieved using the McNemar's chi square tests for paired groups. Where the assumption of the chi square test of large cell frequencies had been violated the Fisher Exact Test was used. The Spearmans rank correlation coefficient was used for nonparametric data to assess the relationship between two variables whether continuous or discrete. A $p < 0.05$ indicates a significant difference between the inulin-type fructans and the control group.

3.10 THE ROLE OF RESEARCH TEAM

3.10.1 Doctoral Candidate

As the PI, I designed the study and the questionnaires, obtained ethics approval and approached the eThekwini Municipality Health Unit for permission to conduct the trial at Lancers Road Clinic. I presented to the Lancers Road Clinic staff on the benefits of the trial and how it would be conducted.

I secured funding from the University of KZN; the IAEA and the National Research Foundation.

I was on site daily and oversaw all aspects of the trial; collected both saliva and stool; measured the anthropometry; and administered both the food frequency questionnaire and the GSRS. I centrifuged, aliquoted and stored all the plasma and serum samples in preparation for analysis.

The deuterium dilution assays were done by the PI with training and oversight provided by Helen Mulol, who has a MSc in instrumental methods of chemical analysis.

The deuterium dilution assays were done by the PI with training and oversight provided by Helen Mulol, who has a MSc in instrumental methods of chemical analysis.

The LPS and sCD14 assays were done by the PI with the assistance of a post-doc fellow, Dr Brodie Daniels.

I was responsible for all the data input and statistical analyses which was overseen by Prof Louise Kuhn, Professor of Epidemiology, Gertrude H. Sergievsky Center and Department of Epidemiology, Columbia University.

3.10.2 Other team members

The clinician performed the routine clinical examination at the start and end of the trial and treated any participants with medical issues or concerns during the trial.

The research assistant, who was also a phlebotomist and HIV counsellor, recruited and obtained informed consent after ensuring that the enrolment criteria were met. She drew all the blood samples, labelled them and ensured the blood was sent through to the NHLS for CD4 count analysis. She performed the Hb test. She was responsible for continued subject participation and follow up phone calls. Where necessary she also functioned as a translator.

Dr Narainsamy Pillay from Lancet Laboratories oversaw the analysis of hs-CRP and serum albumin.

Faecal mRNA was measured by Prof Mark Manary, Department of Pediatrics, Washington University School of Medicine, St Louis.

3.11 ETHICS

Permission to conduct the study at Lancers Road Clinic was obtained from the eThekwini Municipality Health Unit in December 2012 (Appendix B). Ethics approval was granted by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BFC 145/010) in February 2013 (Appendix C). The trial was registered with the Pan African Clinical Trials Registry in 26/11/2011.

A detailed informed consent process was followed with the study being explained thoroughly to the participant in their home language by the research assistant. The research assistant had been previously trained by the Medical Research Council in the technique of obtaining informed consent for HIV trials. The participant was asked to explain what they thought the trial was about and what their commitment would be. Only if they demonstrated clear understanding were they asked to sign the informed consent.

The participant's identity was protected by the allocation of a study number which was used for all laboratory and data analysis.

The data base is password protected and can only be accessed by the PI. The participant's files are stored in a secure locked cabinet and will be destroyed after a 5 year period.

CHAPTER 4: RESULTS

4.1 DEMOGRAPHICS

Of the 100 adults enrolled, 50 were randomised to receive inulin-type fructans and 50 to the control group (Figure 5). Sixteen were withdrawn from the study. See figure below for reasons for withdrawal.

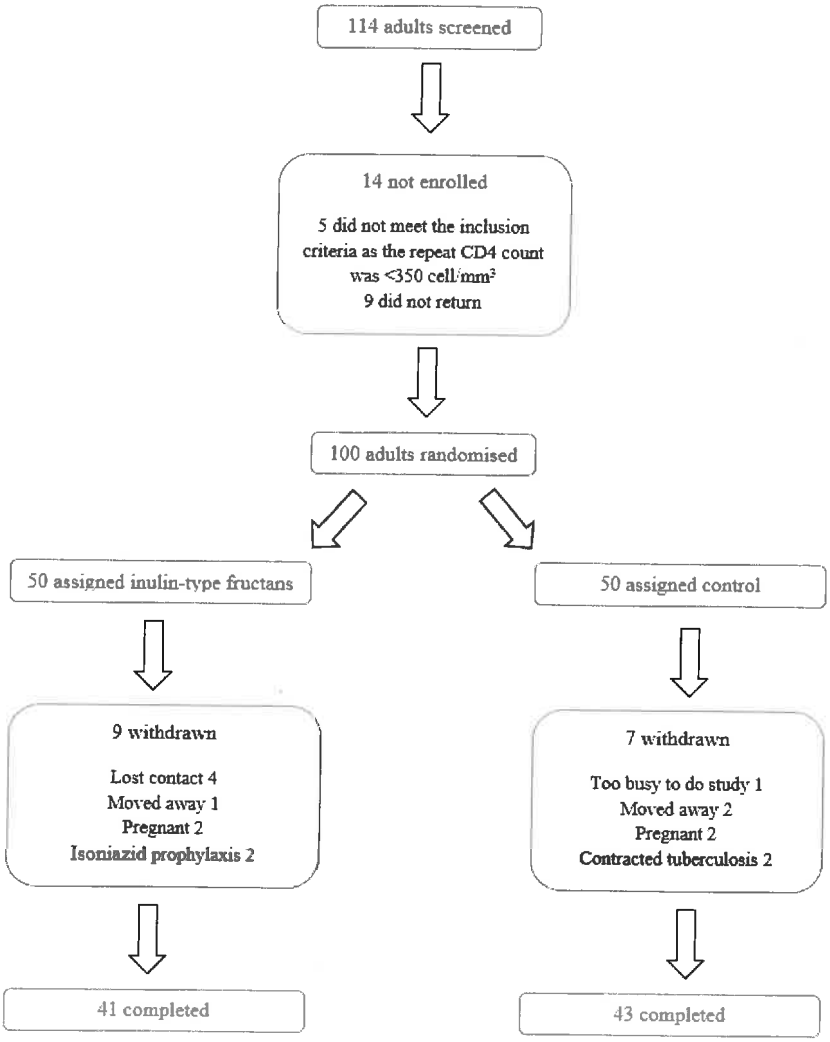

Figure 5: Overview of participant inclusion

Eighty four subjects (84.0%) completed the study. Of these, 41 (48.8%) received 15 g inulin-type fructans daily. There were no significant differences, apart from age, between those who completed and those who did not (Table 11). The non-completers were on average approximately four years younger (p = 0.01).

Table 11: Comparison of participant demographics and clinical characteristics of non-completers and completers

	Non completers n (%)	**Completers** n (%)	***p-value**
Product Code			
Inulin-type fructans	9 (55.6)	41 (48.8)	0.60
Control	7 (44.4)	43 (51.2)	0.65
Karnofsky score			
Score of 90	0 (0)	3 (3.6)	0.42
Score of 100	16 (100)	81 (96.4)	1.0
Sex			
Male	1 (5.6)	7 (8.3)	0.69
Female	15 (94.4)	77 (91.7)	1.0
	Mean (±SD) [min, max]	**Mean (±SD) [min, max]**	
Age (years) (median, IQR)	31.0 (±5.6) [18.9, 40.2]	35.2 (±9.3) [19.3, 60.2]	0.01
Height (cm)	162.4 (5.1) [154.6, 171.6]	160.0 (6.2) [147.0, 172.8]	0.16
Weight (kg)	68.6 (20.2) [39.5, 127.7]	75.2 (15.9) [41.8, 128.3]	0.15
BMI (kg/m^2)	26.1 (±7.8) [14.3, 47.3]	29.5 (±6.4) [15.7, 51.9]	0.065
MUAC (cm)	30.8 (±4.8) [27.2, 43.5]	33.0 (±4.8) [20.5, 46.2]	0.1
TST (mm)	19.7 (8.1) [5.5, 35.0]	22.0 (8.0) [3.0, 40.0]	0.30
HB (g/dl)	11.7 (±1.5) [8.2, 13.8]	12.0 (±1.6) [7.7, 15.3]	0.41
CD4$^+$ (cells/mm^3)	504.4 (±121.2) [354.0, 798.0]	542.5 (±144.8) [350.0, 948.0]	0.32
Total GI score	2.7 (±2.8) [0, 9.0]	2.9 (±3.5) [0, 15.0]	0.85

* the p value was calculated using the students t-test to compare means and the chi-squared test to compare proportions. When an important assumption of the Chi-Squared Test had been violated, the Fisher Exact Test was used. Where the data was non-normally distributed the Wilcoxon rank sum test was used. A $p < 0.05$ indicates a significant difference between the inulin-type fructans and control group.

In the group of 84 participants who completed the study, the majority were female (77/84, 91.7%), with a 100% Karnofsky score (81/84, 96.4%) and a mean CD4 count of 542.5 (±144.8) cells/mm^3. When comparing the participants demographics and clinical characteristics at baseline no significant differences were found for any of the variables (Table 12).

Table 12: Participant demographics and clinical characteristics at baseline

	Total (84) n (%)	**Inulin-type fructans (41)** n (%)	**Control (43)** n (%)	**p-value***
Karnofsky score				
90	3 (3.6)	2 (4.9)	1 (2.3)	0.53
100	81 (96.4)	39 (95.1)	42 (97.7)	0.61
Sex				
Male	7 (8.3)	4 (9.8)	3 (7.0)	0.64
Female	77 (91.7)	37 (90.2)	40 (93.0)	0.71
GI severity score total**				
0	24 (28.6)	13 (31.7)	11 (25.6)	
1 (mild)	15 (17.9)	8 (19.5)	7 (16.3)	
2 (moderate)	10 (11.9)	5 (12.2)	5 (11.6)	0.88
3 (severe)	11 (13.1)	4 (9.8)	7 (16.3)	
4 (very severe)	24 (28.6)	11 (26.8)	13 (30.2)	
	Mean (±SD) [min, max]	**Mean (±SD) [min, max]**	**Mean (±SD) [min, max]**	
Age (years)	35.2 (±9.3) [19.3, 60.2]	37.1 (±10.6) [20.3, 60.2]	33.4 (±7.7) [19.3, 52.1]	0.08
Height (cm	164.0 (±6.2) [147.0, 172.8]	160.6 (±7.2) [147.0, 172.8]	158.5 (±5.1) [148.6, 168.6]	0.42
Weight (kg)	75.2 (±15.9) [41.8, 128.3]	73.0 (±13.4) [46.9, 110.9]	77.3 (±17.9) [41.8, 128.3]	0.22
BMI (kg/m^2)	29.5 (±6.4) [15.7, 51.9]	28.5 (±5.8) [18.1, 43.3]	30.4 (±7.0) [15.7, 51.9]	0.17
MUAC (cm)	33.0 (±4.8) [20.5, 46.2]	32.5 (±4.2) [25.1, 40.0]	33.5 (±5.4) [20.5, 46.2]	0.37
TST (mm)	22.0 (±8.0) [3.0, 40.0]	21.3 (±8.1) [3.0, 38.0]	22.6 (±8.0) [6.0, 40.0]	0.46
Fat free mass (kg)	40.1 (±10.6) [18.4, 67.7]	40.3 (±10.1) [18.4, 67.7]	40.1 (±11.2) [19.1, 61.6]	0.93
Fat free mass (%)	55.1 (±18.7) [15.4, 97.2]	55.6 (±15.8) [17.8, 92.6]	54.6 (±21.2) [15.4, 97.2]	0.83
Fat mass (kg)	36.5 (±21.2) [1.5, 108.5]	34.2 (±16.5) [4.3, 85.1)	38.7 (±24.8) [1.5, 108.5]	0.35
Fat mass (%)	44.9 (±18.7) [2.8, 84.6)	44.4 (±15.8) [7.4, 82.2]	45.4 (±21.2) [2.8, 84.6]	0.81
Albumin (g/dl)	36.2 (±3.8) [28.0, 46.0]	35.8 (±3.0) [28.0, 43.0]	36.6 (±4.4) [28.0, 46.0]	0.31
Hb (g/dl)	12.0 (±1.6) [7.7, 15.3]	11.7 (±1.7) [7.7, 14.2]	12.3 (±1.5) [9.4, 15.3]	0.12
CRP (mg/l)	5.5 (±7.2) [0.2, 36.9]	5.3 (±7.3) [0.3, 36.9]	5.6 (±7.3) [0.2, 36.0]	0.89
CD4 (cells/mm^3)	542.5 (±144.8) [350.0, 948.0]	519.9 (±142.6) [350.0, 948.0]	564.2 (±145.2) [369.0, 825.0]	0.16
CD4 > 500 (cells/mm^3)	44 (52.4) n (%)	18 (43.9) n (%)	26 (60.5) n (%)	0.13
sCD14 (pg/ml)	1.5887 (±.6083) [0.1098, 2.7448]	1.6104 (±0.5842) [0.6443, 2.7188]	1.5665 (± 0.5897) [0.1098, 2.7448]	0.76
Total GI score	2.9 (±3.5) [0, 15.0]	2.6 (±3.3) [0, 13.0]	3.2 (±3.7) [0, 15.0]	0.48

* the p value was calculated using the students t-test to compare means and the chi-squared test to compare proportions. When an important assumption of the Chi-Squared Test had been violated the Fisher Exact Test was used. A $p < 0.05$ indicates a significant difference between the inulin-type fructans and control group.

** This reflects the distribution of the total GI score. For example 15 participants had a total GI score of 1 out of a maximum of 4 meaning their GI symptoms were mild and 24 participants experienced very severe symptoms.

The mean daily dietary intake of inulin-type fructans was 4.14 g (±1.51) at baseline and 3.76 (±1.66) at study end. There was no significant difference in baseline or end dietary inulin-type fructans intake within each group nor was there a significant difference in the mean change from baseline to end when comparing the two groups (Table 13).

Table 13: Mean daily inulin-type fructans intake at baseline and study end

		Inulin-type fructans (41) Mean (±SD) [min, max]	**Control (43)** Mean (±SD) [min, max]	***p-value**
Dietary inulin-type fructans intake (g)	Baseline	4.22 (±1.53) [1.53, 8.87]	4.07 (±1.49) [0.77, 6.74]	0.64
	Week 12	3.69 (±1.76) [1.10, 9.90]	3.84 (±1.59) [1.40, 8.30]	0.63
	Change	0.7 (±1.7) [-3.2, 5.5]	0.3 (±1.5) [-2.4, 3.9]	0.33

* the p value was calculated using the students t-test to compare means and the chi-squared test to compare proportions. A $p < 0.05$ indicates a significant difference between the inulin-type fructans and control group.

The largest source of inulin-type fructans was from both white and brown bread (2.4 g), onions (1.2 g), coffee/chicory mix (0.39 g) and bananas (0.15 g) (Figure 6). Miniscule amounts were supplied by beetroot (0.02 g), oats (0.02 g) and garlic (0.01 g).

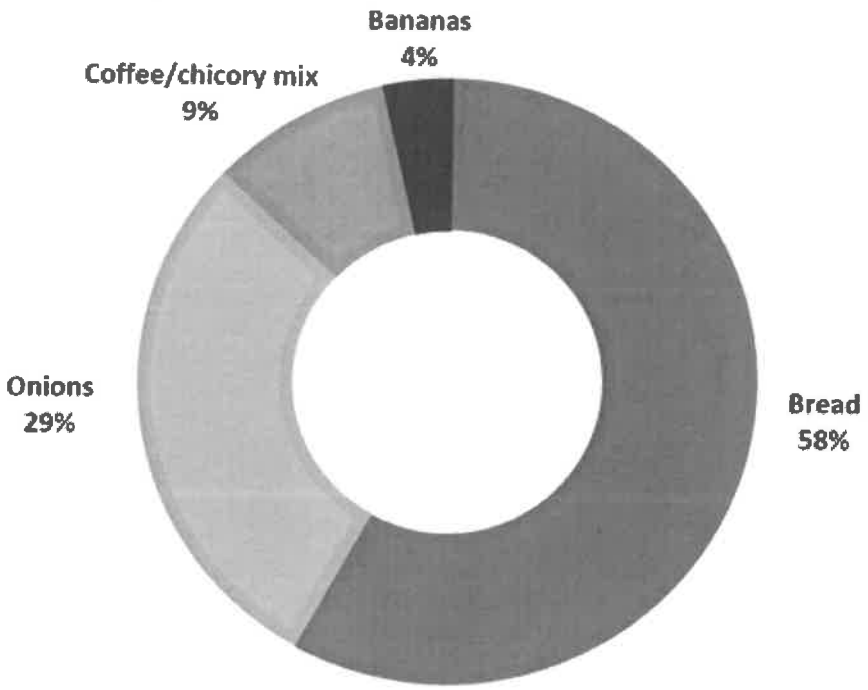

Figure 6: Contribution of food sources to inulin-type fructan intake

Compliance was measured monthly by counting returned sachets. There was no significant difference in the sachets of product consumed by either of the two groups ($p = 0.85$). Compliance was high with a maximum of only 6 of the 84 participants (7.1%) being non-compliant per month. Those who missed one or more days of consuming the supplement per week were deemed noncompliant. No participant was non-compliant for more than one month.

4.2 MICROBIAL TRANSLOCATION

4.2.1 Lipopolysaccharide

Unfortunately due to difficulties performing the assay no results were available for LPS.

4.2.2 Soluble CD14

There was no significant difference in sCD14 levels at baseline between the inulin-type fructans and the control (p = 0.76) or at study end (p = 0.99) (Table 14). Neither was there a significant change between levels from baseline to study end (p = 0.38).

Table 14: Soluble CD14 results at baseline and study end

		Inulin-type fructans (n = 37) Mean (±SD) [min, max]	**Control** (n = 38) Mean (±SD) [min, max]	***p-value**
Soluble CD14 (µg/ml)	Baseline	1.61 (±0.58) [0.64, 2.72]	1.57 (± 0.59) [0.11, 2.74]	0.76
	Week 12	1.62 (±0.62) [0.48, 2.89	1.62 (±0.59) [0.79, 2.79]	0.99
	Change	0.01 (±0.27) [-0.66, 0.64]	-0.06 (±0.37) [-1.56, 0.73]	0.38

* the p value was calculated using the students t-test to compare means and the chi-squared test to compare proportions. A p <0.05 would indicate a significant difference between the inulin-type fructans and control group.

4.3 INFLAMMATION OF THE GASTROINTESTINAL TRACT

Sixty four paired stool samples were available for analysis. Initially a random subset of 41 (41/64) paired stool samples were analysed for mRNA corresponding to GAPDH. GAPDH is a housekeeping gene/transcript that is present in every cell in reasonably consistent known amounts and, although GAPDH is not a direct marker of inflammation, increased levels of GAPDH in the faeces indicates increased cell shedding which is a consequence of inflammation. A cut off value of ≥25 copies indicating the presence of inflammation was based on the recommendations of Professor Mark Manary (Washington University School of Medicine) whose vast experience has demonstrated that a GAPDH of ≥25 copies enables better detection of the lower expression targets of inflammation. As very few samples (4/41, 10%) contained levels of GAPDH ≥25 copies, the decision was made, due to financial constraints, not to attempt to isolate GAPDH in the outstanding samples (23/64). Essentially the low recovery of GAPDH indicated an absence of overt inflammation in the study population. The decision was made however to investigate despite the very low levels of GAPDH to determine whether supplementation with inulin-type fructans reduced the concentration of these markers. It was decided that any samples with at least 1.5 copies of GAPDH would be used in the analysis of inflammatory markers. Twenty one (21/41, 51.3%) contained GAPDH at levels ≥1.5. The very low levels of GAPDH necessitated the choice of higher mRNA expression markers of inflammation all of which were normalized to GAPDH[43]. Currently there are no normal or standard reference ranges for any of these inflammatory markers.

Out of these 21 samples, 21 contained the gene for PIGR an Fc receptor which is a protein found on the cell surface of specific immune system cells such as NK killer cells, macrophages and neutrophils. The Fc receptor binds to immunoglobulin attached to infected cells/invading pathogens in turn stimulating antibody-mediated phagocytosis/cell-mediated cytotoxicity. Seventeen contained the gene encoding for IL8, an important mediator of the innate immune response, which induces both chemotaxis and phagocytosis and is often associated with inflammation. Fourteen expressed the IL1β coding gene, a cytokine which is an important mediator of the inflammatory response and the increased production of which leads to a variety of

[43] Similarly to the BMI being used to interpret weight in relation to height for a more appropriate assessment so the inflammatory markers are interpreted in correlation with the concentrations of GAPDH.

auto inflammatory syndromes. The S100A8 gene, which encodes for faecal calprotectin a marker of inflammation, was expressed by fourteen. Fourteen contained the gene encoding for SOD2 an enzyme which catalyses the partitioning of the superoxide radical released during the acute inflammatory response into either oxygen or hydrogen peroxide.

For samples with a level of GAPDH $\geq$1.5 copies, there were no significant differences ($p \leq 0.05$) between either group from baseline to study end for all 6 markers. Furthermore there were no significant differences between changes in the markers between baseline to study end (Table 15). However as evidenced from the direction of change arrows in Table 15, a downward trend in the inflammatory markers can be seen with those supplemented with inulin-type fructans.

Table 15: Inflammatory markers baseline to study end of those with a GAPDH ≥1.5

Test		Inulin-type fructans 15 g/day Mean (±SD) [min, max]	Control Mean (±SD) [min, max]	*p-value
PIGR		n=10	n=11	
	Baseline	4.5446 (3.5382) [0.7227,12.5556]	3.9296 (3.2363) [0.8147,11.6878]	0.6820
	Week 12	3.0665 (2.2868) [0.1475,7.2808]	2.5989 (2.9172) [0.3069, 9.3044]	0.6894
	Change ⬇	-1.4781 (3.5319) [-9.8730,3.5241]	⬇ -1.3307 (5.1636) [-10.3702,8.4897]	0.9406
IL8		n=9	n=8	
	Baseline	0.8032 (1.1261) [0.0730, 3.5156]	0.6665 (0.9395) [0.0322, 2.7801]	0.7911
	Week 12	0.5002 (0.5760) [0, 1.6228]	1.4118 (1.4459) [0, 3.6221]	0.1295
	Change ⬇	-0.3031 (1.4367) [-3.5156,1.4892]	⬆ 0.7452 (1.1363) [-0.2263,2.3410]	0.1191
IL1β		n=7	n=7	
	Baseline	0.7272 (1.3149) [0, 3.5751]	0.4288 (0.5136) [0, 1.4127]	0.5917
	Week 12	0.1795 (0.1410) [0, 0.3617]	1.1589 (1.8913) [0, 5.0293]	0.2203
	Change ⬇	-0.5477 (1.3713) [-3.4876,0.3131]	⬆ 0.7301 (1.8177) [-0.6998,4.2457]	0.1634
S100A8		n=7	n=7	
	Baseline	0.5369 (0.9134) [0, 2.5241	0.3939 (0.5213) [0.0185, 1.5030]	0.7252
	Week 12	0.2111 (0.1512) **[0.0183, 0.4481]**	0.5898 (0.6870) **[0.0320, 1.7296]**	0.2000
	Change ⬇	-0.3258 (0.9117) [-2.2697, 0.4023]	⬆ 0.1960 (0.5367) [-0.3053,1.1802]	0.2164
SOD2		n=7	n=7	
	Baseline	0.6463 (1.5062) [0, 4.0595]	0.5023 (0.5631) [0, 1.5030]	0.8189
	Week 12	0.2308 (0.2052) [0, 0.5700]	1.3848 (2.2656) [0, 6.2150]	0.2273
	Change ⬇	-0.4156 (1.6190) [-4.0595, 0.5700]	⬆ 0.8825 (2.0206) [-0.4752,5.1641]	0.2094

* the p value was calculated using the students t-test to compare means. A $p < 0.05$ indicates a significant difference between the inulin-type fructans and control group.

4.4 NUTRITIONAL ASSESSMENT

4.4.1 Body composition

At baseline the mean BMI was 29.5 kg/m^2 (±6.4) (pre obese) (Table 12). Two (2.4%) were malnourished, only 21 (25.0%) were an appropriate body weight in relation to height and 39 (46.4%) were obese (Table 16) (WHO 1998).

Table 16: Nutritional status classified according to body mass index (kg/m^2)

BMI (kg/m^2)	**Number** n (%)	**Interpretation**
<16	1 (1.2)	Severe Malnutrition
16 – <17	0 (0.0)	Moderate malnutrition
17 – <18.5	1 (1.2)	Mild Malnutrition
18.5– <25	21 (25.0)	Normal weight
25 – <30	22 (26.2)	Pre-Obese
30 – <35	22 (26.2)	Obese Class I
35 – 40	14 (16.7)	Obese Class II
>40	3 (3.5)	Obese Class III
Total	84 (100.0)	

There were no significant differences in either anthropometry/body composition at baseline to study end between the inulin-type fructans and control group nor was there a significant change from baseline to study end (Table 17). The males had a significantly lower mean BMI, TST, fat mass and a significantly higher mean fat free mass than the females at baseline and study end although there was no significant difference in the mean change from baseline to study end (Table 18). At baseline the mean fat free mass index was 18.7 kg/m^2 for males and 15.5 kg/m^2 for females and the mean fat mass index was 5.83 kg/m^2 for males and 15.0 kg/m^2 for females.

Table 17: Anthropometry/body composition parameters in the inulin-type fructans and control group at baseline and study end

		Inulin-type fructans Mean (±SD) [min, max] n = 41	**Control** Mean (±SD) [min, max] n = 43	***p-value**
Weight (kg)	Baseline	73.0 (±13.4) [46.9, 110.9]	77.3 (±17.9) [41.8, 128.3]	0.22
	Week 12	74.2 (±13.2) [49.3, 112.9]	78.1 (±17.9) [41.6, 122.6]	0.26
	Change	1.2 (±1.9) [-1.9, 6.5]	0.7 (±2.0) [-5.7, 5.4]	0.32
BMI (kg/m^2)	Baseline	28.5 (±5.8) [18.1, 43.3]	30.4 (±7.0) [15.7, 51.9]	0.17
	Week 12	28.9 (±5.7) [19.2, 44.1]	30.7 (±6.9) [15.7, 49.6]	0.20
	Change	0.4 (±0.7) [-0.8, 2.6]	0.3 (±0.8) [-2.3, 2.0]	0.31
MUAC (cm)	Baseline	32.5 (±4.2) [25.1, 40.0]	33.5 (±5.4) [20.5, 46.2]	0.37
	Week 12	33.0 (±4.6) [25.7, 49.7]	33.3 (±5.1) [20.6, 42.6]	0.75
	Change	0.4 (±1.7) [-1.5, 10.0]	-0.2 (±0.9) [-3.7, 1.6]	0.06
TST (mm)	Baseline	21.3 (±8.1) [3.0, 38.0]	22.6 (±8.0) [6.0, 40.0]	0.46
	Week 12	22.9 (±8.3) [3.0, 40.5]	24.1 (±8.5) [6.0, 42.5]	0.49
	Change	1.5 (±3.2) [-7.5, 8.0]	1.5 (±4.5) [-13.0, 14.5]	0.96

		Inulin-type fructans Mean (±SD) [min, max] n = 36	**Control** Mean (±SD) [min, max] n = 39	***p-value**
Fat free mass (kg)	Baseline	40.3 (±10.1) [18.4, 67.7]	40.5 (±11.4) [19.1, 61.6]	0.93
	Week 12	39.5 (±10.0) [18.1, 66.1]	40.3 (±10.9) [18.8, 60.3]	0.73
	Change	-0.8 (±2.3) [-6.7, 5.4]	-0.2 (±2.6) [-6.8, 6.8]	0.29
Fat free mass (%)	Baseline	55.6 (±15.8) [17.8, 92.6]	55.1 (±18.7) [15.4, 97.2]	0.83
	Week 12	53.8 (±15.2) [16.8, 88.6]	53.8 (±17.7) [16.7, 92.0]	0.68
	Change	-1.8 (±3.8) [-10.1, 6.8]	-1.0 (±4.9) [-17.6, 12.1]	0.40
Fat mass (kg)	Baseline	34.2 (±16.5) [4.3, 85.1]	38.7 (±24.8) [1.5, 108.5]	0.35
	Week 12	36.0 (±16.7) [6.7, 90.2]	39.5 (±24.1) [4.2, 98.7]	0.46
	Change	1.8 (±3.4) [-5.4, 9.6]	0.8 (±3.8) [-9.8, 9.9]	0.24
Fat mass (%)	Baseline	44.4 (±15.8) [7.4, 82.2]	44.0 (±21.2) [2.8, 84.6]	0.81
	Week 12	46.2 (±15.2) [11.4, 83.2]	46.3 (±20.0) [8.0, 82.5]	0.98
	Change	1.8 (±3.8) [-6.8, 10.1]	0.9 (±4.9) [-12.1, 17.6]	0.36

* the p value was calculated using the students t-test to compare means and the chi-squared test to compare proportions. When an important assumption of the Chi-Squared Test had been violated the Fisher Exact Test was used. A $p < 0.05$ indicates a significant difference between the inulin-type fructans and control group.

Table 18: Anthropometry/body composition characteristics of males and females from baseline to study end

		Males Total Mean (±SD) [min, max] n = 7	**Females Total** Mean (±SD) [min, max] n = 77	***p-value**			**Males Total** Mean (±SD) [min, max] n = 7	**Females Total** Mean (±SD) [min, max] n = 68	***p-value**
Weight (kg)	Baseline	68.6 (±10.9) [53.9, 84.9]	75.8 (±16.3) [41.8, 128.3]	0.25	Fat free mass (kg)	Baseline	51.8 (±14.3) [22.7, 67.7]	39.9 (±9.5) [18.4, 60.8]	0.002
	Week 12	69.4 (±10.0) [57.1, 85.0]	76.8 (±16.1) [41.6, 122.6]	0.24		Week 12	51.1 (±13.8) [22.5, 66.1]	38.6 (±9.3) [18.1, 59.0]	0.002
	Change	-0.84 (±1.38) [-3.20, 1.00]	-0.94 (±2.02) [-6.50, 5.70]	0.9		Change	-0.01 (±1.6) [-2.7, 1.8]	0.5 (±2.4) [-6.8, 6.8]	0.61
BMI (kg/m^2)	Baseline	24.2 (±4.3) [18.1, 29.1]	30.0 (±6.4) [15.7, 51.9]	0.02	Fat free mass (%)	Baseline	74.9 (±16.3) [42.2, 92.6]	53.0 (±17.8) [15.4, 97.2]	0.004
	Week 12	24.4 (±4.1) [19.2, 29.1]	30.3 (±6.3) [15.7, 49.6]	0.02		Week 12	73.0 (±16.3) [39.5, 88.6]	51.8(±16.8) [16.7, 92.0]	0.003
	Change	-0.26 (±0.51) [-1.10, 0.40]	-0.36 (±0.80) [-2.60, 2.30]	0.74		Change	11.2 (±24.6) [-0.50, 66.8]	1.8 (±4.6) [-12.1, 17.6]	0.33
MUAC (cm)	Baseline	30.0 (±3.2) [25.1, 34.5]	33.3 (±4.9) [20.5, 46.2]	0.08	Fat mass (kg)	Baseline	16.7 (±8.8) [4.3, 31.1]	38.6 (±21.1) [1.5, 108.5]	0.01
	Week 12	30.3 (±2.9) [25.7, 34.5]	33.4 (±4.9) [20.6, 49.7]	0.99		Week 12	18.3 (±9.3) [6.7, 34.6]	39.8 (±20.6) [4.2, 98.7]	0.01
	Change	-0.3 (±0.5) [-0.70, 0.5]	-0.11 (±1.5) [-10.0, 3.7]	0.73		Change	-1.6(±1.2) [-3.5, 0.1]	-1.3 (±3.8) [-9.9, 9.8]	0.8
TST (mm)	Baseline	10.5 (±5.6) [3.0, 17.5]	23.1 (±7.4) [6.0, 40.0]	≤0.00	Fat mass (%)	Baseline	25.1 (±16.3) [7.4, 57.8]	47.0 (±17.8) [2.8, 84.6]	0.03
	Week 12	10.9 (±5.9) [3.0, 18.0]	24.7 (±7.6) [6.0, 42.5]	≤0.00		Week 12	27.0 (±16.3) [11.4, 60.5]	48.2 (±16.8) [8.0, 83.2]	0.02
	Change	-0.4 (±1.4) [-2.5, 2.0]	-1.8 (±4.5) [-19.5, 13.0]	0.39		Change	0.2 (±4.0) [-4.8, 6.8]	-1.5 (±4.5) [-17.6, 12.1]	0.39

* the p value was calculated using the students t-test to compare means and the chi-squared test to compare proportions. When an important assumption of the Chi-Squared Test had been violated the Fisher Exact Test was used. A $p < 0.05$ indicates a significant difference between the inulin-type fructans and control group.

The baseline mean MUAC was 33.0 cm (±4.8) (Table 12) and 27 (32.5%) exceeded the 75th percentile (Table 19).

Table 19: Classification of mid upper arm circumference and triceps skinfold thickness at baseline and study end

Percentile	MUAC start n (%)	MUAC end n (%)	TST start n (%)	TST end n (%)
<5th	5 (6.0)	4 (4.9)	9 (10.7)	5 (6.0)
<10th	2 (2.4)	1 (1.2)	5 (6.0)	5 (6.0)
<15th	1 (1.2)	3 (3.7)	3 (3.6)	3 (3.6)
<25th	6 (7.1)	4 (4.9)	7 (8.3)	6 (7.1)
< 50th	17 (20.2)	18 (22.0)	19 (22.6)	18 (21.4)
< 75th	25 (29.8)	24 (29.3)	23 (27.4)	25 (29.8)
< 85th	14 (16.7)	15 (18.3)	11 (13.1)	7 (8.3)
< 90th	5 (6.0)	8 (9.8)	3 (3.6)	6 (7.1)
< 95th	8 (10.7)	6 (6.1)	2 (2.4)	5 (6.0)
> 95th	0 (0.00)	0 (0.00)	2 (2.4)	4 (4.8)
Total	83 (100.0)	83 (100.0)	84 (100.0)	84 (100.0)

4.4.2 Nutritional Biochemistry

4.4.2.1 Albumin

The mean albumin levels at baseline (36.2 ±3.8 g/l) were on the borderline low range of normal (35 – 50 g/l) (Table 12). Mildly depleted albumin levels (25 to <35 g/l) were present in 27 (32.1%), the remainder had levels within the normal range. There was no significant change (p = 0.41) in levels from baseline to study end (Table 20). Using the Spearman Rank correlation test there was a weak positive correlation between the CD4 count and the albumin levels at baseline (rho (ρ) = +0.175; p = 0.114) and the end of the study (rho (ρ) = +0.218 p = 0.0531) although this was not significant. There was a weak negative correlation using the Spearman Rank Correlation test between the levels of albumin and CRP at baseline (rho (ρ)

= -0.204; p = 0.64) and at the end (rho (ρ) = -0.146; p = 0.187) although this was not significant.

Table 20: Nutritional biochemical parameters at baseline and study end

		Inulin-type fructans Mean (±SD) [min, max]	**Control** Mean (±SD) [min, max]	***p-value**
Albumin (g/l)	Baseline	35.8 (±3.0) [28.0, 43.0]	36.6 (±4.4) [28.0, 46.0]	0.31
	Week 12	36.3 (±4.1) [26.0, 44.0]	36.5 (±3.7) [28.0, 45.0]	0.78
	Change	0.6 (±3.0) [-11.0, 6.0]	-0.1 (±3.7) [-7.0, 9.0]	0.41
Hemoglobin (g/dl)	Baseline	11.7 (±1.7) [7.7, 14.2]	12.3 (±1.5) [9.4, 15.3]	0.12
	Week 12	12.2 (±2.6) [8.5, 15.7]	12.7 (±1.3) [9.9, 15.3]	0.26
	Change	0.4 (±2.6) [-10.5, 5.8]	0.4 (±1.1) [-2.0, 3.5]	0.96

* the p value was calculated using the students t-test to compare means and the chi-squared test to compare proportions. A p <0.05 indicates a significant difference between the inulin-type fructans and control group.

4.4.2.2 Haemoglobin

The mean Hb levels at baseline (12.0 ±1.6 g/dl) (Table 12) were on the minimum range of normal (12.1 – 15.1 g/dl). Haemoglobin levels <12 g/dl were found in 38 (42.8%) at baseline and 30 (35.7%) at the end. Of these 8 (22.2%) at baseline and 6 (16.7%) at the end had levels below 10 g/dl. Despite receiving the appropriate treatment to correct the iron deficiency anaemia there was no significant difference (p = 0.96) in mean Hb from baseline to study end (Table 20). Mild anaemia was present in 18 (21.1%) at baseline and 21 (25.0%) at study end, moderate anaemia in 17 (20.2%) at baseline and 8 (9.5%) at study end and severe anaemia in 1 (1.2%) at baseline and none at the study end. Using the Wilcoxon rank sum test there was no significant correlation between CRP and Hb levels at baseline (p = 0.596) and at study end (p = 0.932) or in mean CD4 count by anaemia status at baseline (p = 0.542) and at study end (p = 0.590).

4.5 IMMUNE STATUS

The mean baseline CD4 was 542.5 (±144.8) cells/mm^3 (Table 12). Of these 44 (52.4%) had a count of >500 cells/mm^3. There was no significant change in CD4 count from baseline to study end between either groups (p = 0.56) (Table 21).

Table 21: Immune and inflammatory biochemical parameters at baseline and study end

		Inulin-type fructans Mean (±SD) [min, max]	**Control** Mean (±SD) [min, max]	***p-value**
Total CD4 (cells/mm^3)	Baseline	519.9 (±142.6) [350.0, 948.0]	564.2 (±145.2) [369.0, 825.0]	0.16
	Week 12	503.2 (±169.5) [216.0, 958.0]	529.9 (±170.3) [253.0, 966.0)	0.48
	Change	-14.5(±186.9) [-571.0, 443.0)	-39.0 (±189.0) [-430.0, 409.0]	0.56
CRP (mg/dl)	Baseline	5.3 (±7.3) [0.3, 36.9]	5.6 (±7.3) [0.2, 36.0]	0.89
	Week 12	6.8 (±12.7) [0.2, 74.6]	5.0 (±6.1) [0.5, 31.5]	0.42
	Change	1.5 (±9.3) [-23.6, 37.7]	-0.5 (±7.8) [-33.6, 27.6]	0.28

* the p value was calculated using the students t-test to compare means and the chi-squared test to compare proportions. A p <0.05 indicates a significant difference between the inulin-type fructans and control group.

4.6 INFLAMMATORY STATUS

The mean CRP compared to normal reference values was high (5.5 ±7.2) mg/dl (Table 12). There was no significant change (p = 0.28) in levels from baseline to study end between either group (Table 21).

4.7 MORBIDITY

4.7.1 Infections

There was no significant difference in the number of courses of antibiotics prescribed during the three month period for either the inulin-type fructans or control group (p = 0.41) (Table 22).

Table 22: Frequency of antibiotic use

	Inulin-type fructans n (%)	Control n (%)	* p-value
None	33 (80.5)	36 (83.7)	0.39
One course	7 (17.1)	4 (9.3)	0.40
Two courses	1 (2.4)	3 (7.0)	0.41

* the p value was calculated using the students t-test to compare means and the chi-squared test to compare proportions. A p <0.05 indicates a significant difference between the inulin-type fructans and control group.

4.7.2 Gastrointestinal symptoms

In the total study population, the mean gastrointestinal score significantly decreased from 2.9 (±3.5) to 0.7 (±1.1) from baseline to study end (p < 0.0001) and significantly more participants switched from symptoms at the start to no symptoms at the end (30, 35.7%) compared to those who switched from no symptoms at start to symptoms at end (7, 8.3%) (p = 0.0002) (McNemar's test). Although the GI score decreased at each visit, no significant difference was found between the groups (Table 23) (Table 24).

Table 23: Gastrointestinal score from baseline to end study

		Inulin-type fructans (n = 41) Mean (±SD) [min, max]	**Control (n = 43)** Mean (±SD) [min, max]	***p-value**
Gastrointestinal symptoms	Visit 1 (baseline)	2.6 (±3.3) [0, 13]	3.2 (±3.7) [0, 15]	0.47
	Visit 2	1.3 (±1.8) [8, 8]	1.8 (±2.5) [0, 14]	0.32
	Visit 3	0.8 (±1.1) [0, 6]	1.4 (±2.3) [0, 12]	0.17
	Visit 4 (end study)	0.7 (±1.2) [0, 6]	1.1 (±1.9) [0, 8]	0.29
	Change (baseline to end)	2.2 (±3.1) [-3, 13]	1.8 (±3.5) [-5, 13]	0.38

* the p value was calculated using the students t-test to compare means and the chi-squared test to compare proportions. A $p < 0.05$ indicates a significant difference between the inulin-type fructans and control group.

Table 24: Gastrointestinal symptoms from baseline to study end

	Baseline (84)			Visit 2 (84)			Visit 3 (84)			End (84)		
	Active	Control	p-value	Active	Control	p-value	Active	Control	p-value	Active	Control	p-value
Diarrhoea												
0	37 (90.2)	38 (88.4)	0.68	36 (87.8)	38 (88.4)	1.00	36 (87.8)	43 (100)	0.06	38 (92.7)	40 (93.0)	0.22
1	2 (4.9)	3 (7.0)		4 (9.8)	4 (9.3)		3 (7.3)	0 (0)		1 (2.4)	3 (7.0)	
2	1 (2.4)	0 (0)	1.00	1 (2.4)	1 (2.3)	1.00	2 (4.9)	0 (0)	0.02	2 (4.9)	0 (0)	0.36
3	1 (2.4)	2 (4.7)		0 (0)	0 (0)		0 (0)	0 (0)		0 (0)	0 (0)	
Increased stools												
0	41 (100)	40 (93.0)	0.09	37 (90.2)	40 (93.0)	0.65	38 (92.7)	42 (97.7)	0.28	39 (95.1)	43 (100)	0.14
1	0 (0)	3 (7.0)		4 (9.8)	3 (7.0)		3 (7.3)	1 (2.3)		2 (4.9)	0 (0)	
2	0 (0)	0 (0)	0.24	0 (0)	0 (0)	0.71	0 (0)	0 (0)	0.35	0 (0)	0 (0)	0.24
3	0 (0)	0 (0)		0 (0)	0 (0)		0 (0)	0 (0)		0 (0)	0 (0)	
Increased urgency												
0	39 (95.1)	41 (95.4)	1.00	39 (95.1)	43 (100)	0.14	41 (100)	43 (100)		41 (100)	43 (100)	
1	1 (2.4)	1 (2.3)		2 (4.9)	0 (0)		0 (0)	0 (0)	----	0 (0)	0 (0)	----
2	1 (2.4)	1 (2.3)	1.00	0 (0)	0 (0)	0.24	0 (0)	0 (0)		0 (0)	0 (0)	
3	0 (0)	0 (0)		0 (0)	0 (0)		0 (0)	0 (0)		0 (0)	0 (0)	
Decreased stools												
0	35 (85.4)	31 (72.1)	0.33	39 (95.1)	36 (83.7)	0.22	39 (95.1)	36 (83.7)	0.34	40 (97.6)	40 (93.0)	0.33
1	5 (12.2)	10 (23.3)		1 (2.4)	5 (11.6)		2 (4.9)	5 (11.6)		1 (2.4)	3 (7.0)	
2	0 (0)	0 (0)	0.40	0 (0)	0 (0)	0.31	0 (0)	1 (2.3)	0.30	0 (0)	0 (0)	0.62
3	1 (2.4)	2 (4.7)		1 (2.4)	2 (4.7)		0 (0)	1 (2.3)		0 (0)	0 (0)	
Constipation												
0	24 (58.5)	27 (62.8)	0.16	31 (75.6)	33 (76.7)	0.52	35 (85.4)	30 (71.4)	0.34	35 (85.4)	33 (78.6)	0.69
1	9 (22.0)	14 (32.6)		8 (19.5)	10 (23.3)		6 (14.6)	10 (23.8)		5 (12.2)	8 (19.1)	
2	6 (14.6)	1 (2.3)	0.14	1 (2.4)	0 (0)	0.69	0 (0)	1 (2.4)	0.27	0 (0)	0 (0)	0.77
3	2 (4.9)	1 (2.3)		1 (2.4)	0 (0)		0 (0)	1 (2.4)		1 (2.4)	1 (2.4)	
Incomplete evacuation												
0	27 (65.9)	29 (67.4)	0.32	38 (92.7)	32 (74.4)	0.03	39 (95.1)	37 (86.1)	0.26	37 (90.2)	38 (88.4)	0.78
1	12 (29.3)	8 (18.6)		1 (2.4)	10 (23.3)		2 (4.9)	4 (9.3)		4 (9.8)	5 (11.6)	
2	1 (2.4)	5 (11.6)	0.30	1 (2.4)	1 (2.3)	0.01	0 (0)	2 (4.7)	0.44	0 (0)	0 (0)	1.0
3	1 (2.4)	1 (2.3)		1 (2.4)	0 (0)		0 (0)	0 (0)		0 (0)	0 (0)	

* the p value was calculated using the chi-squared test to compare proportions. A $p < 0.05$ would indicate a significant difference between the inulin-type fructans and control group

Table 24: Gastrointestinal symptoms from baseline to study end continued

	Baseline (84)			Visit 2 (84)			Visit 3 (84)			End (84)		
	Active	Control	p-value	Active	Control	p-value	Active	Control	p-value	Active	Control	p-value
Nausea and vomiting												
0	34 (82.9)	34 79.1)	0.65	39 (95.1)	38 (88.4)	0.54	41 (100)	40 (93.0)	0.23	41 (100)	41 (95.4)	0.38
1	5 (12.2)	7 (16.3)		2 (4.9)	3 (7.0)		0 (0)	2 (4.7)		0 (0)	1 (2.3)	
2	2 (4.9)	1 (2.3)	0.82	0 (0)	1 (2.3)	1.0	0 (0)	0 (0)	0.49	0 (0)	1 (2.3)	1.0
3	0 (0)	1 (2.3)		0 (0)	1 (2.3)		0 (0)	1 (2.3)		0 (0)	0 (0)	
Burping												
0	41 (100)	43 (100)	----	41 (100)	43 (100)	----	41 (100)	43 (100)	----	41 (100)	43 (100)	----
1	0 (0)	0 (0)		0 (0)	0 (0)		0 (0)	0 (0)		0 (0)	0 (0)	
2	0 (0)	0 (0)		0 (0)	0 (0)		0 (0)	0 (0)		0 (0)	0 (0)	
3	0 (0)	0 (0)		0 (0)	0 (0)		0 (0)	0 (0)		0 (0)	0 (0)	
Flatulence												
0	37 (90.2)	38 88.4)	0.35	35 (85.4)	41 (95.4)	0.12	37 (90.2)	40 (93.0)	0.65	41 (100)	40 (93.0)	0.09
1	4 (9.8)	3 (7.0)		6 (14.6)	2 (4.7)		4 (9.8)	3 (7.0)		0 (0)	3 (7.0)	
2	0 (0)	2 (4.7)	0.55	0 (0)	0 (0)	0.15	0 (0)	0 (0)	0.71	0 (0)	0 (0)	0.24
3	0 (0)	0 (0)		0 (0)	0 (0)		0 (0)	0 (0)		0 (0)	0 (0)	
Stomach pain												
0	37 (90.2)	40 93.0)	0.59	40 (97.6)	41 (95.4)	0.59	39 (95.1)	41 (95.4)	0.96	40 (97.6)	39 (90.7)	0.18
1	3 (7.3)	3 (7.0)		1 (2.4)	2 (4.7)		2 (4.9)	2 (4.7)		1 (2.4)	4 (9.3)	
2	1 (2.4)	0 (0)	0.83	0 (0)	0 (0)	1.0	0 (0)	0 (0)	1.0	0 (0)	0 (0)	0.36
3	0 (0)	0 (0)		0 (0)	0 (0)		0 (0)	0 (0)		0 (0)	0 (0)	
Stomach rumbling												
0	38 (92.7)	38 88.4)	0.73	41 (100)	40 (93.0)	0.23	39 (95.1)	40 (93.0)	0.68	39 (95.1)	41 (95.4)	0.96
1	2 (4.9)	4 (9.3)		0 (0)	2 (4.7)		2 (4.9)	3 (7.0)		2 (4.9)	2 (4.7)	
2	1 (2.4)	1 (2.3)	0.84	0 (0)	1 (2.3)	0.49	0 (0)	0 (0)	1.0	0 (0)	0 (0)	1.0
3	0 (0)	0 (0)		0 (0)	0 (0)		0 (0)	0 (0)		0 (0)	0 (0)	
Bloating												
0	34 (82.9)	34 (791)	0.73	38 (92.7)	39 (90.7)	0.54	40 (97.6)	41 (95.4)	0.40	41 (100)	39 (90.7)	0.14
1	5 (12.2)	5 (11.6)		3 (7.3)	2 (4.7)		1 (2.4)	0 (0)		0 (0)	1 (2.3)	
2	2 (4.8)	4 (9.3)	0.84	0 (0)	1 (2.3)	1.0	0 (0)	1 (2.3)	1.0	0 (0)	3 (7.0)	0.18
3	0 (0)	0 (0)		0 (0)	1 (2.3)		0 (0)	1 (2.3)		0 (0)	0 (0)	

* the p value was calculated using the chi-squared test to compare proportions. A $p < 0.05$ would indicate a significant difference between the inulin-type fructans and control group.

Table 24: Gastrointestinal symptoms from baseline to study end continued

	Baseline (84)			Visit 2 (84)			Visit 3 (84)			End (84)		
	Active	Control	p-value	Active	Control	p-value	Active	Control	p-value	Active	Control	p-value
Heartburn												
0	34 (82.9)	35 (81.4)	0.42	37 (90.2)	37 (86.1)	0.38	39 (95.1)	37 (86.1)	0.16	39 (95.1)	41 (95.4)	0.96
1	5 (12.2)	3 (7.0)		4 (9.8)	4 (9.3)		2 (4.9)	6 (14.0)		2 (4.9)	2 (4.7)	
2	2 (4.9)	5 (11.6)	0.49	0 (0)	2 (4.7)	0.63	0 (0)	0 (0)	0.27	0 (0)	0 (0)	1.0
3	0 (0)	0 (0)		0 (0)	0 (0)		0 (0)	0 (0)		0 (0)	0 (0)	
Acid/reflux												
0	37 (90.2)	36 (83.7)	0.67	38 (92.7)	41 (95.4)	0.61	38 (92.7)	41 (95.4)	0.61	40 (97.6)	43 (100)	0.30
1	3 (7.3)	5 (11.6)		3 (7.3)	2 (4.7)		3 (7.3)	2 (4.7)		1 (2.4)	0 (0)	
2	1 (2.4)	2 (4.7)	0.78	0 (0)	0 (0)	0.67	0 (0)	0 (0)	0.67	0 (0)	0 (0)	0.49
3	0 (0)	0 (0)		0 (0)	0 (0)		0 (0)	0 (0)		0 (0)	0 (0)	

* the p value was calculated using the chi-squared test to compare proportions. A $p < 0.05$ would indicate a significant difference between the inulin-type fructans and control group.

The most commonly reported gastrointestinal complaint at baseline was constipation (33, 39.3%) and feelings of incomplete evacuation (28, 33.3%) (Figure 7) (Table 24). Diarrhoea, reported by only 9 (10.7%) at baseline did not significantly change by study end (6, 7.1%) ($p = 0.22$). None reported chronic diarrhoea.

Inulin-type fructans have a known potential to increase gastrointestinal discomfort, particularly flatulence, at higher doses. The daily dose of 15 g was well tolerated as only 12 reported a mild increase, and 6 a moderate increase in flatulence which was not severe enough to alter the GSRS. None felt that the increase in flatulence warranted withdrawal from the study.

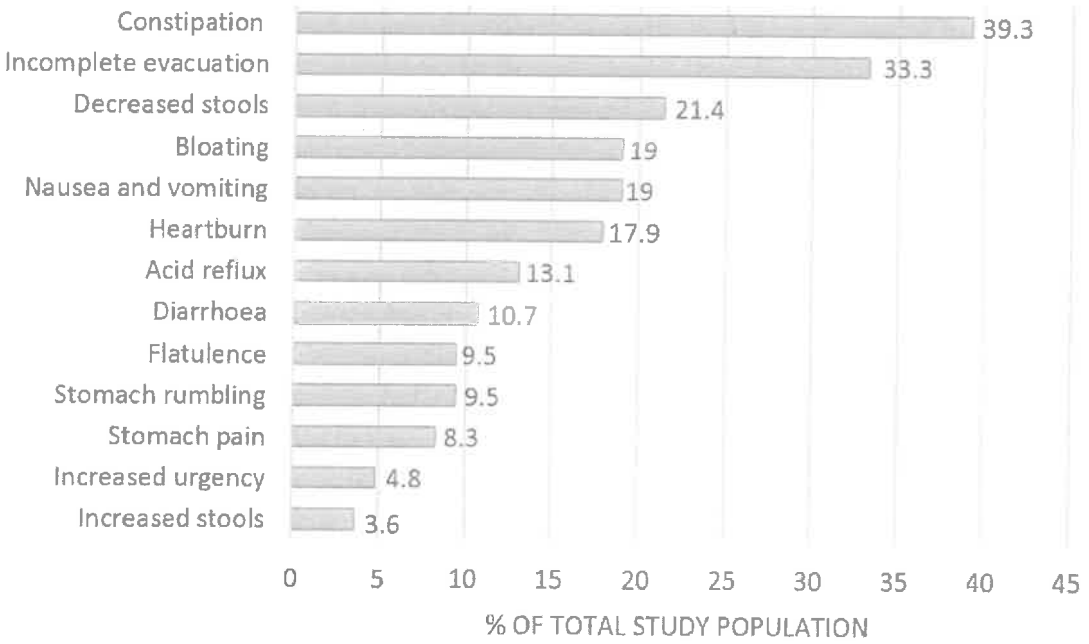

Figure 7: Prevalence of gastrointestinal symptoms at baseline

CHAPTER 5: DISCUSSION

The study population was composed of asymptomatic HIV-infected primarily overweight/obese adults, the majority of whom were female. Most completed the study and appeared compliant.

5.1 INTAKE OF INULIN-TYPE FRUCTANS

This was the first study to document the daily inulin-type fructan intake in an African population. Although inulin-type fructans are classified as a soluble dietary fibre (Kabeerdoss *et al* 2011), their content in food has not been well researched and they are not included in nutritional composition databases. Estimating the daily intake of the population therefore was based on data obtained from research studies. Their intake may be higher as a large variety of plants contain inulin-type fructans as they are plant storage carbohydrates, therefore indigenous foods such as the local varieties of wild spinach and amadumbes[44], which are not currently analysed, may provide a rich source. Previously, when plant foods as opposed to animal foods were predominantly eaten, the mean daily intake was estimated as 25 to 32 grams per day (Roberfroid 1993). With the increased intake of animal and refined plant foods, the daily consumption in most European countries has decreased to 3 to 11 g and 1 to 4 g in the United States, with the most common sources being wheat, onions, garlic and bananas (Van Loo *et al* 1995) (Moshfegh *et al* 1999). The mean daily intake in this study of 4.14 g was similar to that of the European countries and slightly higher than the United States. Similarly the most common sources were wheat (bread), onions and bananas although unusually a significant contribution was from chicory as the affordable local coffees, popular amongst the African population, comprise of added chicory.

No dietary reference intake (DRI) exists for inulin-type fructans. According to Kolida *et al* (2007), the bifidogenic effect based on human studies occurs at between 4 to 40 g of inulin-type fructans per day. The degree of the bifidogenic effect however appears dependent on the concentration of faecal *Bifidobacteria* at the start of the supplementation (Roberfroid 2001) therefore to prescriptively refer to a bifidogenic dose is scientifically incorrect because of the variation in the

[44] Rhizome of the *Colocasia esculenta* plant.

faecal microbiome composition between individuals (Roberfroid 2001). In the absence of a DRI it seemed logical to use the potentially bifidogenic dose and therefore conclude that the intake of the study participants was on the borderline of that required for a bifidogenic effect. Recommendations to increase inulin-type fructan intake to bifidogenic levels is however challenging as most of the population was above the upper range of ideal body weight as determined by BMI, therefore encouraging the increased intake of bread and bananas may be inappropriate.

5.2 MICROBIAL TRANSLOCATION

5.2.1 Lipopolysaccharide

Access to the human GIT is limited inhibiting the investigation of microbial translocation. As obtaining biopsies is difficult, unpleasant and costly, measures of microbial translocation are substituted for direct assays. As a direct marker of microbial translocation was needed, and as LPS is the most commonly used marker in the literature (Novati *et al* 2015), the assay was repeated a number of times in an attempt to determine plasma LPS levels. The assay is known to be complex and difficult to perform, and although it has a high sensitivity there is considerable inter-assay variability (Cohen and McConnell 1984). Authors of publications where the LPS levels were not what was expected, usually highlighted the unpredictableness of the assay as the reason (Sandler *et al* 2011). The most difficult and time consuming part was to inactivate those factors found in plasma which inhibit or enhance the assay thereby interfering with the recovery of endotoxin. Despite testing a variety of approved methods to inactivate the plasma, LPS was not recovered in the expected amounts from the spiked samples in this study and the assay was therefore abandoned.

5.2.2 Soluble CD14

Since sCD14 is a measure of LPS stimulated monocyte or macrophage activation, and thus is an indirect marker of microbial translocation, it was expected that the levels would decrease across the study as a consequence of the inulin-type fructans reducing the permeability of the GIT. There

was no significant difference between the levels of sCD14 in the groups at baseline or at study end nor was there a significant difference in changes from baseline to study end between the groups.

These results do not agree with the only other prebiotic study which evaluated sCD14. The COPA trial reported that supplementation with 15 g of scGOS/lcFOS/pAOS for a period of 3 months significantly reduced sCD14 (Gori *et al* 2011). Supplementation with a probiotic/prebiotic mixture (Synbiotic 2000W[45]) for 4 weeks (Schunter *et al* 2012) or with *Bacillus coagulans* for 3 months (Yang *et al* 2014) had no significant impact on sCD14. No studies have investigated *Bifidobacteria*.

Although the mean sCD14 (1.61 μg/ml) was similar to that found by Pérez-Santiago *et al* (1.70 μg/ml) it was lower than that of other African (2.1 – 2.47 μg/ml) (Redd *et al* 2009a) (Cassol *et al* 2010) (Canipe *et al* 2014) and international studies (2.1 – 4.0 μg/ml) (Brenchley *et al* 2006b) (Ancuta *et al* 2008) (Sun *et al* 2010) (Rajasuriar et al 2010) (Sandler *et al* 2011) (Marchetti *et al* 2011) (Merlini *et al* 2011) (Schunter *et al* 2012) (Vesterbacka *et al* 2013) and was instead similar to healthy controls (1.61 – 1.96 μg/ml) (Brenchley *et al* 2006b) (Cassol *et al* 2010) (Sun *et al* 2010) (Rajasuriar *et al* 2010) (Merlini *et al* 2011) (Sandler *et al* 2011).

When compared to the results of other studies, the sCD14 levels appeared to be normal suggesting that there was no increase in bacterial translocation in this study population. This was supported by the lack of evidence of GIT inflammation as determined by the mRNA inflammatory markers. The evidence therefore does not support the current hypothesis that increased GIT permeability in all stages of HIV infection results in raised LPS and sCD14 levels which in turn promotes the release of pro-inflammatory mediators driving the progression to AIDS.

[45] Synbiotic 2000W contained 4 strains of probiotic bacteria (10^{10} each) *Pediococcus pentosaceus* 5–33:3, *Leuconostoc mesenteroides* 32–77:1, *L. paracasei subsp paracasei* 19 and *L. plantarum* 2362 plus 2.5 g of each of the following four nondigestible, fermentable dietary fibers: betaglucan, inulin; pectin and resistant starch.

5.3 INTESTINAL INFLAMMATION

This was the first study to use droplet digital PCR to analyse stool mRNA levels as markers of gastrointestinal inflammation in adults with HIV. Inflammation results in the increased shedding of enterocytes resulting in temporary gaps in the epithelial barrier and increased intestinal permeability. High recovery of GAPDH was expected as this would reflect increased levels of cell shedding. The very low recovery of GAPDH and the stool mRNA inflammatory markers suggested that the GIT in asymptomatic HIV-infected adults was not overtly damaged or inflamed. The current hypothesis is that increased GIT permeability results in increased microbial translocation which drives the chronic immune stimulation (Gori *et al* 2008b) (Sandler and Douek 2012). There was no evidence of GIT inflammation or evidence of microbial translocation (sCD14) therefore our results do not support the current beliefs.

Because of the inaccessibility of the GIT and associated ethical issues, most of the evidence supporting the hypothesis of increased microbial translocation, such as early disruption of the integrity of the epithelial barrier and the deposition of microbial products in the lamina propria and distal lymph nodes, has been based on the simian immunodeficiency virus model (Estes *et al* 2010). Although in HIV there is evidence of structural abnormalities of the GIT mucosa and increased intestinal permeability, an in-depth review of the studies which traditionally are used to support the hypothesis of disruption of the epithelial barrier do not conclude that intestinal inflammation/impaired permeability is present in *asymptomatic* HIV-infected individuals. A review of these key studies follow.

The earliest study by Kotler *et al* (1984) in HIV-infected malnourished men found that only those with diarrhoea has significantly increased permeability in comparison to those without diarrhoea and healthy controls (Kotler *et al* 1984) (Table 25). Similarly Gillin *et al* (1985) demonstrated that absorption was normal in the asymptomatic HIV group and that malabsorption only occurred in those with diarrhoea (Gillin *et al* 1985). Stockman *et al* (1988) showed there was no difference in the absorption of asymptomatic HIV-infected individuals versus controls but those with diarrhoea had a significantly decreased absorption (Stockmann *et al* 1998). Kapembwa *et al* (1991) concluded that altered small-intestinal permeability was only associated with advanced

HIV infection (Kapembwa *et al* 1991) in Caucasian and African adults but not in those who were asymptomatic. However Lim *et al* (1993) concluded that abnormal permeability and reduced intestinal absorption capacity is common in HIV, occurs at all stages of HIV disease, especially in the presence of diarrhoea (Lim *et al* 1993). Keating *et al* (1995) reported that malabsorption did not occur in asymptomatic, well males but only in those with symptoms (Keating *et al* 1995). Bjarnason *et al* (1996) found a normal intestinal absorption/permeability in well HIV-infected adults which only became impaired on progression to AIDS (Bjarnason *et al* 1996). Sharpstone *et al* (1999) demonstrated that intestinal permeability was significantly increased in all with HIV except those who were asymptomatic and concluded that those with HIV and no AIDS defining illness had a reasonably normal intestinal absorptive capacity (Sharpstone *et al* 1999). Stein *et al* (1997) concluded that the excretion of urinary butyrate between controls, asymptomatic HIV-infected adults and adults with AIDS were comparable but that the levels were significantly higher only in the AIDS group with weight loss (Stein *et al* 1997). Pernet *et al* (1999) found that impaired intestinal absorption was only present in those with advanced disease when diarrhoea and other GI symptoms were present (Pernet *et al* 1999).

Table 25: Intestinal permeability in HIV-infected adults

Authors	Investigation	Asymptomatic	Symptomatic
Kotler *et al* (1984)	D-xylose	Normal	Increased
Gillin *et al* (1985)	C-glycerol tripalmitin	Normal	Increased
Stockman *et al* (1988)	Lactulose mannitol flux	Normal	Increased
Kapembwa *et al* (1991)	Lactulose mannitol flux	Normal	Increased
Lim *et al* (1993)	D-xylose, lactulose, L-rhamnose and 3-O-methyl-D-glucose	Abnormal	Increased
Keating *et al* (1995)	D-xylose, lactulose, L-rhamnose and 3-O-methyl-D-glucose	Normal	Increased
Bjarnason *et al* (1996)	111-indium leukocytes	Normal	Increased
Sharpstone *et al* (1999)	D-xylose, lactulose, L-rhamnose and 3-O-methyl-D-glucose	Normal	Increased
Stein *et al* (1997)	Urinary butyrate	Normal	Increased
Pernet *et al* (1999)	Lactulose mannitol flux and absorption of D-Xylose	Normal	Increased
Crenn *et al* (2009)	Citrulline concentrations	Normal	Increased
Gori *et al* (2008)	Faecal calprotectin	Increased	Not known

Crenn *et al* (2009) concluded that asymptomatic HIV-infected adults in all stages of severity had an absorptive capacity comparable to healthy controls unlike those with diarrhoea (Crenn *et al* 2009). The only study to find raised faecal calprotectin in well, asymptomatic HIV-infected adults was the COPA trial (Gori *et al* 2008a) where 50% had raised faecal calprotectin and of these, one third had very high levels. They also reported sCD14 levels (9.9 ug/l) far in excess of those reported by other studies (<4 ug/l). The study was recently criticized for not using faecal or blood analysis to exclude other causes of inflammation (Mantegazza *et al* 2014). The overall conclusion

from these key studies supported the findings of this study which concluded that intestinal permeability/absorption was normal in asymptomatic HIV-infected individuals and that the lack of recovery of GAPDH and inflammatory faecal markers was an appropriate finding. Although the use of mRNA as a faecal marker of inflammation in HIV-infected adults is novel these results correlate with the current literature.

5.4 NUTRITIONAL STATUS

5.4.1 Body composition as determined by advanced methods

Supplementation with inulin-type fructans did not significantly impact body composition. As there was no significant difference in the anthropometry between the start and end of the study, the nutritional supplement used as the vehicle to deliver the inulin-type fructans did not impact on body composition and therefore was not a confounding factor. It was expected that the fat free mass would improve as a consequence of a reduction in the incidence of diarrhoea and infections resulting from both the local and systemic immune enhancing properties of the inulin-type fructans.

There are only a few studies in South Africa which have used advanced methods of body composition analysis, such as deuterium dilution, doubly labelled water and Dual-Energy X-ray Absorptiometry (DXA) to determine body composition in HIV-infected ARV naive adults (Table 26).

Table 26: South African studies measuring body composition using advanced analysis techniques in HIV-infected ARV naive adults

Study group	Breastfeeding mothers (2001 – 2002) (Papathakis *et al* 2005)	Breast or formula feeding mothers (2006 – 2008) (Kindra *et al* 2012)	Current study using data for females only (2013)	Non lactating females (Wrottesley *et al* 2014)
Area	Rural KZN	Urban KZN (Durban)	Urban KZN (Durban)	Soweto
Technique	Deuterium dilution	Deuterium dilution	Deuterium dilution	DXA
Study number	17	173	77	74
Age (years)	25.7	26.2	35.2	33.0
CD4 (cells/mm^3)	631	423	542.5	420
Fat Free Mass (kg)	40.2	45.9	39.9	39.7
Fat Free Mass (%)	60.8	-	53.0	-
Fat Free Mass Index (kg/m^2)	15.7	18.51	15.5	15.9
Fat Mass (kg)	26.6	20.4	38.6	25.3
Fat Mass (%)	39.2	31.2	47.0	39.5
Fat Mass Index (kg/m^2)	10.5	8.2	15.0	10.1
BMI	26.2	26.5	30.0	26.5
MUAC	29.6	27.9	33.3	-
TST	19.0	15.96	23.1	-

HIV-infected ARV naive black South African breastfeeding mothers living in a rural area of KZN during 2001 to 2002 (Papathakis *et al* 2005) had a similar fat free mass index[46] (15.7 kg/m^2) as that of a recent study which included 74 black African HIV-infected ARV naive females in Soweto, South Africa (15.9 kg/m^2) (Wrottesley *et al* 2014). These results are similar to the fat free mass index of (15.5 kg/m^2) found in our study and are in agreement with the findings of earlier international studies which concluded that the fat free mass of asymptomatic HIV-infected adults

[46] The NHANES recommendations to use the fat free mass index and fat mass index when interpreting the fat free and fat mass was published after the majority of these studies therefore these indices were calculated from the data obtained from the published studies and not from the raw data.

is lower than that of their healthy adult counterparts (18.03 kg/m^2) (Simon and Gorbach 1984) (Ott *et al* 1993) (Wanke *et al* 2000) (Lazanas *et al* 2003) (Delpierre *et al* 2007) (Dillon *et al* 2015). Lower fat free mass in HIV has been correlated with low bone mineral density (Cotter *et al* 2013) which is common in HIV (Bolland *et al* 2007) (Grijsen *et al* 2013). As a low fat mass and a low BMI has been associated with lower bone mineral density (Bolland *et al* 2007) (Carr *et al* 2001) the effects of a lower fat free mass in this study population may be countered by their high body fat content and high BMI.

The study by Kindra *et al* (2012) in Durban, KZN however found that black South African HIV-infected ARV naive mothers, who were either breast or formula feeding, had a fat free mass index (18.5 kg/m^2) comparable to that of healthy black Americans (Kindra *et al* 2012). This is in agreement with some studies who suggest that the loss of weight during the course of HIV infection is mainly fat (Spor *et al* 2011) (Sharifuzzaman 2014). Interestingly the fat free mass (17.05 kg/m^2) of 20 young HIV-*uninfected* black African females in Khayelitsha, an urban informal settlement in Cape Town, South Africa was lower than that of American blacks (18.03 kg/m^2) and resembled that of white Americans (16.19 kg/m^2) (Dugas *et al* 2009) therefore the reference ranges for white Americans may be more appropriate for comparison. Regardless of the reference range used, the fat free mass index was substantially lower in our study population compared to HIV-uninfected populations.

Although our study population had a similar fat free mass when compared to the majority of the other South African studies, our group was substantially fatter as represented by the higher fat mass index (15.0 kg/m^2), BMI (30.1 kg/m^2) and fat mass percent (46.2 %) than that of Papathakis *et al* (10.5 kg/m^2: 26.2 kg/m^2; 39.2%), Kindra *et al* (8.2 kg/m^2: 26.5 kg/m^2; 31.2%) and Wrottesley *et al* (10.1 kg/m^2: 26.5 kg/m^2; 39.5%) (Papathakis *et al* 2005) (Kindra *et al* 2012) (Wrottesley *et al* 2014) (Table 26). When compared to a healthy American black population, the other South African study groups were below the norm for fat mass index (12.09 kg/m^2) and fat mass percent (40.1%) however our study group exceeded the norm.[47]

[47] A discussion of the implications of the high body fat levels/obesity follows later.

Although there is no South African research specifically investigating the gender difference in body composition in HIV-infected ARV naive adults, data using the technique of bioelectrical impedance (Nell *et al* 2015) supports a significant difference with males having a higher fat free and lower fat mass as was found in this study.

5.4.2 Body composition determined using anthropometry

Only a quarter of the study participants were an appropriate weight for height, a quarter were overweight and almost half were obese. The Transition and Health During Urbanisation of South Africans (THUSA) cross sectional study (1996 to 1998) of 1040 healthy adult black women (HIV status unknown) in the North West Province, South Africa, reported that 39.9% were a normal weight, 25.2% were overweight and 28.6% were obese (Kruger *et al* 2002) (Figure 8).

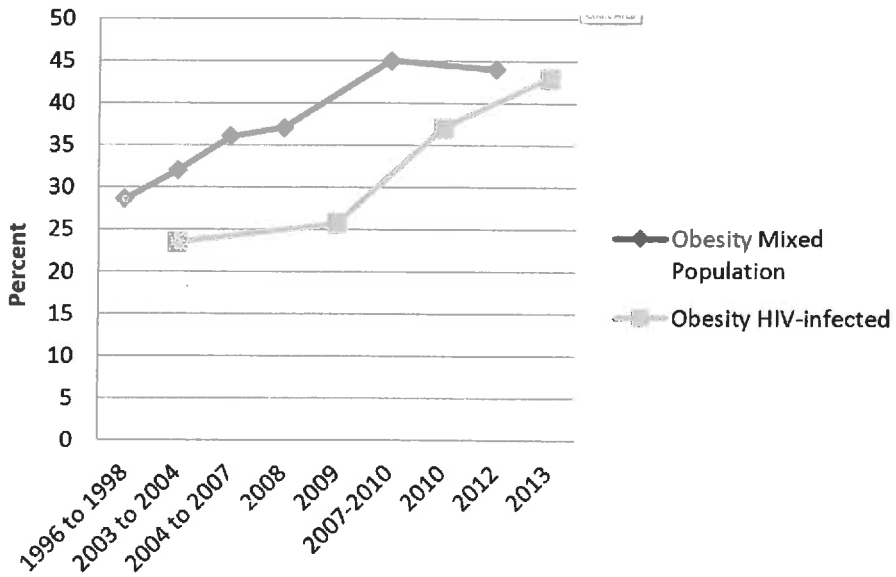

Figure 7: The increasing prevalence of obesity in both HIV-infected and in mixed populations in South Africa

Of the 5495 HIV negative women (2004 to 2007) enrolled in the Methods for Improving Reproductive Health in Africa (MIRA) trial, 32% were overweight and 36% were obese (Wand and Ramjee 2013). Results from the National Income Dynamics Study (NIDS) (2008) conducted by the Southern Africa Labour and Development Research Unit (SALDRU) concluded that 26% of black African women (HIV status unknown) were overweight and 37% were obese (Averett *et*

al 2014). Data (2007 to 2010) from a longitudinal cohort survey of 3836 black African adults (HIV status unknown) reported that 45% were obese and there was no difference in the prevalence of obesity between urban and rural dwellers (Wu *et al* 2015). The baseline data figures (2012) from the SANHANES-1 for female South African adults (HIV status unknown) living in KZN reported a mean BMI of 29.5 kg/m^2 of which 25.2% were overweight and 44.0% were obese (Shisana *et al* 2013). The incidence of obesity in adults whose HIV status was unknown in South Africa has risen dramatically since 1996 (Figure 8).

Data reporting weight trends amongst those infected with HIV is sparse (Crum-Cianflone *et al* 2010). A large population-based survey in rural Umkhanyakude in KZN between 2003–2004, reported that of the 383 HIV-infected ARV naive women, 53.5% to 68.2% were overweight and 23.5% to 27.3% were obese depending on age category (Bärnighausen *et al* 2008) (Figure 8). Three hundred HIV-infected black South African adults (either ARV naive or on ART) living in either an informal semi-urban settlement (France) or in a rural area (Sweetwaters) in KZN were assessed as part of a household based cross-sectional study in 2009 (Oketch *et al* 2011). Thirty two percent were a normal weight, 23.3 % were overweight and 25.7 % were obese. Although this population was similar to the current study, the levels of obesity were almost 20% lower in 2009. The study in Soweto using DXA (2010) reported figures for overweight of 28% and for obesity of 37% (Wrottesley *et al* 2014) (Figure 8).

Most of the studies have been done on females. When looking at gender based differences it was obvious that the overweight/obese individuals in the study population were female. This conclusion was supported by a study conducted in the Cape, South Africa from 2012 to 2013 (Nell *et al* 2015) where significant differences in the anthropometry between males and females was found. The study which included 102 HIV-infected females and 44 males who were similar in age to this study population (30 years) concluded that the females were predominantly overweight. The BMI was found to be slightly higher in the current study (males 23.7 vs 21.5 kg/m^2; females 30.1 vs 29.3 kg/m^2).

Although international studies have reported a high prevalence of both overweight (24% to 37%) and obesity (9 to 20%) in HIV-infected ARV naive individuals (Crum-Cianflone *et al* 2010) (Tate

et al 2012), the prevalence of obesity in South Africa appeared to be more than double. These results challenged the long standing belief that involuntary weight loss/wasting is an integral part of the disease process prior to the initiation of ART. It has been proposed that the prevalence of obesity in those infected with HIV currently mirrors that of the general population of the country where they are resident (Crum-Cianflone *et al* 2010) (Tate *et al* 2012). As the baseline data from the SANHANES-1 report (Shisana *et al* 2013) was very similar to that of this current study, the study population was not only representative but confirmed that the patterns of overweight/obesity amongst HIV-infected individuals currently follows the general population trends.

Obesity and related co-morbidities, already a significant public health problem in South Africa (Wand and Ramjee 2013) (Averett *et al* 2014), seems to have extended to those who are HIV-infected. It is well accepted that excess body fat results in adverse health consequences in the non HIV-infected population (Flegal *et al* 2005) however the consequences where HIV has been superimposed, has not been investigated extensively (Crum-Cianflone *et al* 2010). Forty two black urban female South Africans initiating ART (stavudine) who were followed over a 2 year period, showed that those who had a higher BMI and skinfold thickness and those with higher insulin levels, were more likely to experience lipodystrophy (George *et al* 2009). As weight gain has been shown to commonly occur during ART, the prevalence of obesity is likely to increase particularly as ART is now being initiated in South Africa at a CD4 count of <500 cells/mm^3 making many more eligible for therapy. A United States Military HIV Natural History Study (1985–2004) claimed that 62% of the 1682 adult males gained weight while on ART (Crum-Cianflone *et al* 2010). Thirty South African black females showed significant increases in BMI, fat mass and body fat percent within six months of ART initiation (Esposito *et al* 2008). As both ART and obesity are independently related to increased rates of cardiovascular disease, hyperlipidemia, hypertension and insulin resistance (Dubé 2000) (Friis-Møller *et al* 2003) (Warriner *et al* 2014), and obesity in HIV is associated with a greater likelihood of multimorbidity, it is feasible to assume that ART in combination with obesity will lower life expectancy further (Kim *et al* 2012).

The high levels of overweight and obesity in this study population conforms with the general population trends in KZN dispelling the theory that involuntary weight loss is inevitable prior to ART. As obesity and associated co morbidities lowers life expectancy, and further weight is likely

to be gained on ART initiation, it is important that guidelines such as The South African National Guidelines on Nutrition For People Living With HIV, AIDS, TB and other Chronic Debilitating Conditions address the issue (Department of Health South Africa 2007). Currently these guidelines focus on the prevention and treatment of weight loss and wasting in HIV and totally disregard the increasing prevalence of obesity and the deleterious impact on the quality of life and health of those HIV-infected ARV naive individuals.

5.4.3 Albumin

There was no significant difference in mean serum albumin levels before (35.8 g/l) and after supplementation (36.3 g/l) with inulin-type fructans. The serum albumin levels were expected to increase, regardless of whether albumin was being used as a marker of the acute phase response or a marker of nutritional status. If inulin-type fructans had reduced microbial translocation, the chronic inflammatory response should have been dampened thereby reducing the acute phase response with a resultant increase in albumin levels. A decrease in GIT infections from inulin-type fructans displacing pathogens and improving immunity, was expected to protect fat free mass thereby improving malnutrition and serum albumin concentrations.

These results concur with those of Gori *et al* (2011) and Wolf *et al* (1998) who found no change in serum albumin levels in HIV-infected adults after supplementation with the synthetic prebiotic combination of scGOS/lcFOS/pAOS (Gori *et al* 2011) or after supplementation with the probiotic *L. reuteri* (Wolf *et al* 1998).

This study, in addition to finding no effect of inulin-type fructans on albumin, found surprisingly low albumin levels and a higher prevalence of hypoalbuminemia. The borderline low (36.2 g/l) mean albumin levels, although similar to those found in Rwanda, were approximately 5 g below those described by other South African studies while the prevalence of hypoalbuminemia was four fold higher (Table 27). The THUSA study reported median albumin levels of 40.9 g/l for 216 asymptomatic HIV-infected adults (Oosthuizen *et al* 2006). The median albumin of 249 HIV-infected ARV naive women from two formal settlements (Phahameng and Botchabela) and two informal settlements (Joe Slovo and Namibia) in Mangaung, South Africa was 41.3 g/dl (Hattingh

et al 2009). An observational cohort study in Rwanda, the Rwandan Women's Interassociation Study and Assessment (RWISA) found that the mean albumin levels of 710 HIV-infected women was 36 g/l (Dusingize *et al* 2012). Graham *et al* (2007) found that in Mombasa (Kenya) 79 HIV-infected ARV naive women had a mean albumin of 38.5 g/l (Graham *et al* 2007). When compared internationally, the mean serum albumin for women was 37.4 g/litre and for men was 40.2 g/l in 495 HIV-infected ARV naive adults attending the Royal Free Hospital in London (Shah *et al* 2007).

Table 27: Serum albumin levels and percent hypoalbuminemia of HIV-infected ARV naive adults

Location	Study	Number	Albumin (g/l)	Hypoalbuminemia(%)
South Africa	Current study, Durban	84	36.2	32
South Africa	THUSA study, Western Province (Oosthuizen *et al* 2006)	216	40.9	-
South Africa	Mangaung (Hattingh *et al* 2009)	249	41.3	8
Rwanda	RWISA (Dusingize *et al* 2012)	710	36	-
Kenya	Mombasa (Graham *et al* 2007)	79	38.5	16
Britain	Royal Free Hospital, London (Shah *et al* 2007).	495	37.4 – 40.2	

There is no clear explanation why the adults in our study experienced lower mean serum albumin levels and a higher prevalence of hypoalbuminemia than those found in comparable populations living in other regions in South Africa.

The mean serum albumin levels, while not correlated to CD4 count, were lower than those found in similar populations and the prevalence of hypoalbuminemia (32%) was substantially higher. Only 8% in Mangaung (Hattingh *et al* 2009) and 16% in Mombassa (Graham *et al* 2007) were hypoalbuminemic. Chong *et al* (2015) found that none of the 75 HIV-infected adults in East London, London were hypoalbuminic (Chong *et al* 2015). The high prevalence of hypoalbumineia had serious health implications.

Hypoalbuminemia appears to be a strong predictor of mortality in HIV (Feldman *et al* 2000) (Feldman *et al* 2003a) (Mehta *et al* 2006). Pre ART albumin levels of <35 g/l in a study on 2145 HIV-infected adults in Tanzania was associated with a 4.52 times greater risk of death at the initiation of ART (Sudfeld *et al* 2013). Furthermore they concluded that albumin levels <38 g/l (lower range of normal) at the initiation of ART was associated with a greater likelihood of negative outcomes and increased mortality (Sudfeld *et al* 2013). Sixty seven percent of our study population had albumin levels <38 g/l placing them at an increased risk of mortality. The United States Women's Interagency HIV Study (WIHS), which included 2056 HIV-infected women at various stages of the disease, showed that the three year mortality for those with albumin levels <35 g/l was 48% versus 11% in those with levels >42 g/l and that the relative hazard of death was five times greater regardless of CD4 count (Feldman *et al* 2000). Only 7% of our study group had albumin levels >42 g/l.

Hypoalbuminemia is also associated with increased progression to AIDS (Mehta *et al* 2006) and may be a valuable independent prognostic marker of disease progression especially for asymptomatic women with little or no evidence of immunosuppression (Olawumi and Olatunji 2006) (Shah *et al* 2007). Graham *et al* (2007) found that for every 1 g/l decrease in serum albumin, there was a 13% increase in the risk of progression to CD4 counts <200 cells/mm^3 and that a drop in serum albumin of 10% was associated with 3.5 times the risk of progession (Graham *et al* 2007).

Hypoalbuminemia is also linked to enhanced progression to renal failure, heart failure and atherosclerotic disease in the HIV-infected. The WIHS found that for each 0.5 g/l decrease in serum albumin concentration, there was a corresponding faster annual decline in the estimated glomerular filtration rate of 0.56 ml/min as well as a 1.71 fold greater risk of a rapid decline in kidney function in a 2 year follow up of 908 HIV-infected women (Lang *et al* 2014). They concluded that independent of the stage of HIV disease, BMI or albuminuria, a decline in kidney function was strongly associated with hypoalbuminemia (Lang *et al* 2014). The United States Department of Veterans Affairs HIV Clinical Case Registry analysis concluded that serum albumin levels of <25 g/l were a strong predictor of heart failure and atherosclerotic disease (Lang *et al* 2013).

Although asymptomatic and not wasted, a third of the current study population was therefore at an increased risk of disease progression, renal failure, cardiac failure and death. The reasons for the high prevalence of hypoalbuminemia was unclear.

The causes of hypoalbumenia are not well determined (Olawumi and Olatunji 2006) (Lang *et al* 2013) but are probably multifactorial (Fuhrman *et al* 2004) (Shah *et al* 2007). Potential causes include laboratory error, gender, malnutrition, anorexia, dietary factors, mucosal gut damage, malabsorption, the HIV infection itself, the acute phase response, chronic inflammation, late pregnancy, trauma as well as liver and renal dysfunction (Lang *et al* 2013).[48]

The possiblity of laboratory error had already been reduced as the original samples were processed using a dedicated technician on a dedicated machine and all the samples were analysed in one run using the same kit in a SANAS credited laboratory. A subsample was sent to an independent SANAS accredited laboratory to verify the results. As there were no major differences in the readings, laboratory error was eliminated as a factor.

Women are more likely to be hypoalbuminemic (Shah *et al* 2007), and as the study population was predominantly female, it could be argued that gender played a role. However the prevalence of hypoalbuminemia was much lower in both the Mangaung (Hattingh *et al* 2009) and Mombasa (Graham *et al* 2007) studies which only included women. Therefore gender was unlikely to be a major factor.

In HIV it is difficult to distinguish whether the low serum albumin levels are a consquence of preexisting malnutrition or the HIV infection itself (Graham *et al* 2007). The RWISA study investigated whether albumin was a reliable nutritional index in 187 HIV-infected women (CD4 >350 cells/mm^3) and controls (Dusingize *et al* 2012). They concluded that albumin should not be used to interpret nutritional status for either those HIV-infected or HIV-uninfected. According to the Food and Agriculture Organization of the United Nations (FAO) BMI is a useful indicator of nutritional status (FAO 2003). Body mass index is often used to classify malnutrition in

[48] As the study's target population included only adults who were asymptomatic and not pregnant and therefore not suffering from trauma, liver and renal dysfunction these will not be included in the discussion.

publications investigating the nutritional state of HIV-infected individuals (De Socio *et al* 2011). Based on BMI, it is very unlikely that malnutrition contributed to the prevelance of hypoalbuminemia in this study, as only two (2%) met the classifcation of under nutrition. Most were overweight/obese ruling out anorexia as a cause. A low protein intake may result in hypoalbuminemia although the impact of other factors such as the acute phase response appears more influential (Nicholson *et al* 2000). A recent cross sectional study on 135 black African adults in KZN, reported a dietary protein intake of 60 to 75 g per day – this exceeded the DRI[49] (Kolahdooz *et al* 2013). A study in the Eastern Cape including 128 black African women reported a daily protein intake of approximately 52 g (May *et al* 2014). The THUSA study concluded that the mean protein intakes of black South African adults were adequate (MacIntyre *et al* 2002). Although the dietary protein intake was not measured in this study, based on these other local studies it is reasonable to assume that the intake was adequate and did not contribute. If malnutrition as a consequence of living in a resource poor country was a factor, then the other South African studies which measured albumin in HIV-infected adults should have found a similar mean albumin level and prevalence of hypoalbuminemia.

Although the intake of dietary protein may have beeen adequate, reduced protein absorption and enteropathy with associated protein loss, may be a result of cellular apoptosis in the GALT which is thought to be present from early on in the course of HIV (Centlivre *et al* 2007) (Graham *et al* 2007). There were no overt signs of malabsorption however, as most study participants had normal stools or were constipated and diarrhoea was uncommon. The lack of significant concentrations of GAPDH and stool inflammatory markers suggested that in this population, increased GIT inflammation and permeability was not present and therefore protein absorption was unlikely to be impeded.

The most probable cause was the effect of the HIV infection itself (Graham *et al* 2007) (Shah *et al* 2007) on the acute phase response and on the state of chronic inflammation (Fuhrman *et al* 2004) as evidenced by the high mean CRP levels although no significant correlation was found between the levels of CRP and albumin in this study. Negative acute phase proteins, such as

[49] DRI for protein is 46 to 56 g per day.

albumin, decrease acutely during infections (Fuhrman *et al* 2004). The mediators of inflammation[50] signficantly impact the serum albumin levels by altering the normal hepatic protein metabolism and inducing capillary leak with fluid relocating into the extravascular body compartment so there is less synthesis and greater dilution of albumin (Fuhrman *et al* 2004). Serum albumin levels are an indicator of an inflammatory process (Fuhrman *et al* 2004) rather than a marker of malnutrition.

The high prevalence of hypoalbuminemia puts this apparently well study population at a high risk of morbidity and mortality and although the causes were not investigated, the most probable was the HIV infection itself and the consequent impact on the acute phase response and ongoing chronic inflammation.

5.4.4 Anaemia

Supplementation with inulin-type fructans did not significantly improve Hb levels. It was expected that the reduced inflammation of the GIT as a consequence of inulin-type fructan supplementation and the consequent suppression of the chronic state of inflammation would improve the absorption of dietary iron and reduce the anaemia associated with inflammation. No other studies have investigated the impact of inulin-type fructans on Hb levels in HIV. The initial study to investigate the safety of probiotics in HIV showed no change in Hb (14.7 g/dl) after 3 weeks of supplementation with *L.reuteri* (Wolf *et al* 1998).

In this asymptomatic HIV-infected population nearly one in two were mildly anaemic. The varying definitions of anaemia have made comparisons between studies difficult.[51] The SANHANES-1 survey of KZN females (HIV status unknown) found the prevalence of anaemia to be 33% (Shisana *et al* 2013) which was 10% lower than this study although the mean Hb was similar (Table 28). As the prevelance was not much higher in this study population, the anaemia could feasibly be a result of a deficient iron intake rather than being a consequence of HIV.

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[50] These include cytokines, hematopoietic factors, prostaglandins, thromboxanes, and complement.

[51] The current study results were redefined for each study to enable comparisons.

Table 28: Comparison of the prevalence of iron deficiency anaemia in the SANHANES-1 survey (mixed population) to the current study (HIV-infected)

	SANHANES-1	Current study
Hb (g/dl) (mean)	12.4	12.0
Total anaemia (%)	33	42
Mild anaemia (%)	18	21
Moderate anaemia (%)	14.4	20.2
Severe anaemia (%)	0.8	1.2

A low vitamin D status has also been implicated in anaemia related to HIV (Mehta *et al* 2010). In the past, the high exposure of South African individuals to sunlight across the year was thought to exclude the likilihood of a vitamin D deficiency. However high risk populations in South Africa, such as those who are dark skinned, have been documented as having low serum levels of vitamin D (Pettifor *et al* 1996) (Lips 2010). Apart from nutritional causes, anaemia may also be a consequence of the HIV virus. The HIV virus can result in the upregulation of hepcidin[52] and cytokines which results in bone marrow macrophages sequestering iron (Chelucci *et al* 1995), there is impaired erythropoiesis (Costantini *et al* 2009), a deficiency of glucose-6-phosphate dehydrogenase[53] (Serpa *et al* 2010) as well as the reduced survival of red blood cells (Adias *et al* 2005). Although co-trimoxazole may cause anaemia (Samuel *et al* 2014) this was not a contributing factor as it was an exclusion criteria.

There are very few South African studies investigating the Hb levels of HIV-infected ARV naive adults. Most included populations with CD4 counts of <200 cells/mm^3 and each used different criteria for the definition of anaemia (Table 29).

[52] Hepcidin is an important regulator of iron homeostasis and when levels are raised such as in inflammation or infections a resulting decrease in serum iron levels occurs potentially resulting in the anaemia of inflammation.

[53] A deficiency of glucose-6-phosphate dehydrogenase causes haemolysis of red blood cells.

Table 29: Comparison of the prevalence of mild iron deficiency anaemia in HIV-infected populations

	African				International		
	Bloem-fontein	Johannes-burg	Cape Town	Zimbabwe	Euro SIDA	London	Brazil
Study number	248	10 259	814	898	5706	495	701
Mean CD4 (cells/mm^3)	Not stated	< 200	104	121	201	<200	180 <200 521 ≥ 200
Mean Hb (g/dl)	13.5	-	11.3	10.8	-	11.2	14.4 males 12.6 females
Total anaemia (%)	18	26	71	79	58	42 males 55 females	38
Total anaemia (%) current study*	38	10	46	Criteria not stated	48	38	48
Difference in prevalence (%)	20	-16	-25	-	-10	-17	10

*The percent anaemia was recalculated for each study using that particular study criteria for comparison purposes

A descriptive study conducted in 2000, which included 248 black African HIV-infected ARV naive women from Bloemfontein, South Africa found that 9% were below 11.7 g/dl in the age group of 25 to 34 years and 9% in the 35 to 44 year olds – the total prevalence therefore was 18% which was 20% lower than that found in this study (38%) (Walsh *et al* 2010) (Table 29). The prevalence of anaemia in HIV-infected South Africans therefore appears to have increased over the last thirteen years even though some staple foods have been fortified with iron since 2003 (Steyn *et al* 2009) (Appendix R). The absorption of dietary iron from food, supplements and food fortification however might be reduced in HIV (Chelucci *et al* 1995). A prospective study (2004 to 2009) of 10 259 HIV-infected adults (CD4 <200 cells/mm^3) just prior to ART initiation in Johannesburg, South Africa described the prevalence of anaemia (Hb <10 g/dl) as 26% (Takuva *et al* 2013) compared to 10% in this study. A study conducted in the Gugulethu township, Cape Town from 2002 to 2006 which included 814 HIV-infected adults (mean CD4 104 cells/mm^3) prior to ART initiation found the incidence of anaemia was 71%. An investigation of 40 health care facilities in Zimbabwe found an extremely high prevalence of anaemia of 79% in 898 adults

(mean CD4 121 cells/mm^3) eligible for ART (Takarinda *et al* 2015). It was not clear what criteria had been used to define anaemia.

The EuroSIDA study[54] which included 5706 pre-ART HIV-infected adults found the prevalence of mild anaemia (males Hb 8–14 g/dl, females Hb 8–12 g/dl) to be 58% which was 10% higher than this study (Mocroft *et al* 1999). The prevalence of total anaemia was 55% in females and 42% in males in the study by Shah *et al* (2007) which included 495 HIV-infected pre-ART adults at the Royal Free Hospital, London (Shah *et al* 2007) compared to 38% in this study. Although the prevalence of anaemia appeared to be lower in South Africa, the study populations were disimilar in that the CD4 counts were low in the other studies as adults prior to the initiation of ART were being investigated. A study investigating a reasonably similar population to this study was done by De Santis *et al* (2009) who reviewed the data of 701 consecutive HIV-infected adult outpatients in Brazil. They found the total prevalence to be 38% which was 10% lower than this study and that the likelihood was higher in those with lower CD4 counts (De Santis *et al* 2011). No relationship was found between Hb and CD4 count in this study. It is difficult to draw conclusions because of the differing definitions of anaemia but more importantly, most studies have not investigated asymptomatic HIV-infected populations. Regardless however, the prevalence of anaemia is high.

Anaemia is a strong independent risk factor for disease progression, mortality and a loss of quality of life independent of both viral load and CD4 count (Volberding 2002) (Belperio and Rhew 2004) (O'Brien *et al* 2005) (Shah *et al* 2007) (McHardy *et al* 2013) (Takuva *et al* 2013). Even mild anaemia (Hb 8–14 g/dl men, Hb 8–12 g/dl women) was shown to have a relative hazard of disease progression of 2.2 when compared to those without anaemia (Lundgren and Mocroft 2003). According to Shah, it might be useful to consider the albumin levels in relation to the Hb levels to identify high risk individuals (Shah *et al* 2007). Although no significant correlation was found, two thirds (18/27, 67%) of those with hypoalbuminemia in this study were also anaemic (Hb <12 g/dl) further raising their risk of progression to AIDS.

[54] A prospective European study of 70 HIV treatments centers.

Considering the impact of even mild anaemia on the health of the HIV-infected individual, it is important that the prevalence is further investigated in asymptomatic HIV-infected adults in South Africa to determine whether a similar occurance would currently be found in other areas. Even if it is established that the prevalence is high however, the action that needs to be taken to correct this is unclear.

The treatment of anaemia in the presence of HIV is complex. Although mild anaemia is associated with poor outcomes in HIV, paradoxically iron supplementation may promote an increase in viral load and disease progression (Clark and Semba 2001) (Olsen *et al* 2004). Iron deficiency anaemia may be a beneficial response to infection in that during inflammation iron is sequestered and withheld from the invading pathogens in what is thought to be a defensive strategy (Zarychanski and Houston 2008). Iron supplementation therefore may promote replication of the HIV virus. Iron overload in general has been shown to increase the severity and frequency of infections (Martí-Carvajal and Solà 2007) and has been associated with chronic infectious diseases in Sub-Saharan Africa (Kerkhoff and Lawn 2015). Iron deficiency anaemia (unrelated to HIV) is common in South Africa and the deleterious consequences are well established (Lawrie *et al* 2008) (Visser and Herselman 2013) including the weakening of the immune system (Cunningham-Rundles *et al* 2005) which potentially offers the advantage to the HIV virus. Paradoxially the HIV virus may be offered a growth advantage both by supplementation and by withholding supplementation. The conundrum remains unresolved as to date there are no large clinical trials investigating the outcome of iron supplementation in those infected with HIV who live in countries where the prevalence of iron deficiency anaemia is high in the general population.

Currently there are no international or South African guidelines recommending the type and amounts of iron supplemention required to treat iron deficiency anaemia in HIV. The Standard Treatment Guidelines and Essential Medicines List (2014) South Africa[55] state that non-pregnant females with an Hb of <12g/dl and males with an Hb of <13g/dl are classified as anaemic and should be treated with ferrous sulphate compound orally[56] after eliminating and treating the other

[55] http://www.kznhealth.gov.za/pharmacy/edlphc2014.pdf. Accessed November 2015.

[56] Iron (Gulf ferrous sulphate FeSO4 170 mg), manganese sulphate 2.5 mg and copper sulphate 2.5 mg three times per day and folic acid (5 mg) taken once per day at a separate time from the iron.

causes of the anaemia. Forty six percent (39/84) of this study population were eligible for supplementation, and had this been available, they would have been issued supplements by the clinic staff despite the controversy surrounding the use. The supply of iron supplements at Lancers Road Clinic was severely limited however during the study period (2013) so only those with an Hb of <8g/dl received supplementation. The shortage continued after the completion of data collection (2013) with iron being completely unavailable in the clinic from November 2014. Currently (October 2015) a limited supply of iron supplements were available which were reserved for the treatment of symptomatic pregnant females only. The danger therefore of inappropriate iron supplementation in the HIV-infected, before safe guidelines based on clinical research have been established, seems unlikely.

Appropriate supplementation in those with an Hb of <8g/dl did not significantly alter the prevalence of moderate anaemia in this study population. The reasons were not investigated but could have been due to compliance as the side effects of the supplementation can be unpleasant, or could be a result of the other factors related to HIV (Baker 2003) (Opie 2012).

This apparently well, asymptomatic population of HIV-infected individuals was at an increased risk of morbidity, progression and death due to the high prevalence of hypoalbuminemia and iron deficiency anaemia. The high prevalence of both overweight and obesity further increased their risk of morbidity and mortality. Ironically overnutrition in the form of an excess intake of energy needed to be addressed while improving undernutrition in the form of micronutrient deficiencies such as iron.

5.5 IMMUNE STATUS

This was the first study to investigate the impact of inulin-type fructans as a sole intervention on peripheral CD4 counts. The CD4 count was expected to increase as a result of reduced translocation decreasing the chronic immune activation. The CD4 count remained unchanged from baseline to end study in both groups. This was similar to the results of Gori *et al* (2011) who found that supplementation with a synthetic prebiotic mix (scGOS/lcFOS/pAOS) did not significantly improve CD4 counts (Gori *et al* 2011). Similarly Schunter *et al* (2012) found no

significant effect on CD4 count when supplementing with probiotics[57] and a dietary fibre mix. Although the fibre mixture contained 2.5 g of inulin-type fructans (Schunter *et al* 2012), the dose was insufficient to have resulted in a bifidogenic effect. Agave fructans however in combination with *B. lactis* plus *L. rhamnosus* HN001 resulted in a significant increase in CD4 count (González-Hernández *et al* 2012) as did supplementation with *B. bifidum* and *Streptococcus thermophilus* (Trois *et al* 2008). A combination of both inulin-type fructans and *Bifidobacteria* may synergistically increase CD4 counts, but inulin-type fructans as a sole intervention, or as part of a prebiotic cocktail, have not shown a significant effect.

The impact of other probiotics on CD4 count has been conflicting. Supplementation with *L. rhamnosus Fiti* (Irvine *et al* 2010) or a combination of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 (Anukam *et al* 2008) resulted in significant increases in CD4 counts in some studies but not in others (Hummelen *et al* 2011). While supplementation with *L. rhamnosus* CAN-1 (Hemsworth *et al* 2012) or with the yeast *Saccharomyces boulardii* (Villar-García *et al* 2015) did not significantly change CD4 counts, supplementation with *Bacillus coagulans* showed a significantly improved CD4 count (Yang *et al* 2014). It appears that the impact on CD4 count is dependent on the type of probiotic.

It could be argued that peripheral CD4 counts are not a good marker of immune status. It is important when using peripheral CD4 counts to assess the effect of an intervention on immunological status, to understand that the CD4 count is influenced by factors other than the HIV virus. Within an individual, there can be considerable variability which in turn could interfere with the interpretation of the change. Van Rood *et al* (1991) demonstrated in 47 healthy adults that 70% of the variance in CD4 count over 12 months was due to subject variability (Van Rood *et al* 1991). Hughes *et al* (1994) reported in a study on 1020 asymptomatic ARV naive adults, that over a 2 year period, the coefficient of variation for an individual averaged at least 25% especially for those with lower CD4 counts (Hughes *et al* 1994). Translated into practice this meant that 6% of the individuals had either half or double the CD4 count at the end of a 2 month period (Hughes *et al* 1994). One hundred and sixty one adults, stable on effective ART over a 6.4 year study

[57] These did not include *Bifidobacteria*.

period, displayed a clinically relevant variation in CD4 count of approximately 17 to 20% (Gordon *et al* 2015). Apart from the within subject variability, other factors influence the CD4 count including age, exercise, smoking, race, infection, inter laboratory variability/using different laboratory supplies (Sax *et al* 1995), the diurnal variation in immunological activities (Kaplan *et al* 1976) and seasonal rhythms[58] (Van Rood *et al* 1991) although most of these factors were accounted for during the study.[59]

In addition, the peripheral CD4 count is not necessarily representative of the total body CD4 count as most of the CD4 T cells are found in the lymphatic tissue, particularly that of the GIT rather than in the blood (Schneider *et al* 1994) (Mowat and Viney 1997) (Brenchley *et al* 2004). The most extensive depletion of CD4 T cells occurs in the GALT at all stages of HIV disease (Brenchley *et al* 2004) – the depletion can occur early on in the disease when the CD4 counts are still reasonable. The lymphoid tissue is a major replication site and reservoir of the HIV virus, particularly as the highest concentrations of CCR5 cells which express the co receptor for HIV, are found in the GIT (Mehandru *et al* 2004) (Douek 2007) (Novati *et al* 2015). Although monitoring the lymphatic or GALT CD4 T cell count, where the impact of the inulin-type fructans was most likely to occur, would have been the most accurate method to measure change post intervention, lymphatic biopsies are difficult to obtain and therefore the "crude" measurement of peripheral CD4 count was used.

5.6 INFLAMMATORY STATUS

This was the first study to measure the impact of inulin-type fructans on CRP levels as the other prebiotic HIV interventions excluded this parameter. Our study showed that inulin-type fructan supplementation did not significantly lower the CRP levels. It was expected that inulin-type fructans would decrease intestinal permeability thereby reducing the translocation of microbes and LPS. This in turn would reduce the chronic stimulation of the immune system and inflammation

[58] Known as the month of date sampling effect.

[59] In this study, obviously the age, race were constant - exercise, smoking was not specifically queried – they were screened for infections monthly- the CD4 bloods were sent to the same laboratory and the bloods samples were always drawn in the morning.

as reflected by lowered CRP levels. Previous studies using supplementation with probiotics including *P. pentosaceus, L. plantarum, L. mesenteroides* and *L. paracasei* (Schunter *et al* 2012), *B. coagulans* (Yang *et al* 2014) and *S. boulardii* (Villar-García *et al* 2015) also did not report significantly lower CRP levels.

Raised levels of CRP are an important predictor of inflammation in HIV, even in those with CD4 counts >500 cells/mm^3 (Tien *et al* 2010). There are however varying definitions in the literature as to what constitutes high. Some studies regard high as being above the normal laboratory range (5 mg/l) and others as being above 10 mg/l. The mean of this study group was high (>5 mg/l) with 12% being above 10 mg/l.

Most South African studies have reported CRP levels pre-ART rather than in asymptomatic HIV-infected adults. Data collected during 1995 to 1996 from 132 HIV-infected ARV naive adults (44% with CD4 ≤200 cells/mm^3) living in Cape Town, South Africa, found that the CRP levels were high (>10 mg/l) in 37% (Visser *et al* 2003) possibly because of the low CD4 counts. In a nested case-control study within the longitudinal South African Phidisa Study II (2004), the median pre-ART CRP levels for the 187 participants who died after ART initiation were 11.3 mg/l versus 3.6 mg/l for the 359 survivors (Ledwaba *et al* 2012). They concluded that high pre-ART levels of CRP was strongly associated with early mortality after commencing ART (Ledwaba *et al* 2012) which put 12% of this study at high risk. Although a study including 606 HIV-infected ARV naive Tanzanian women found that approximately 16% had high CRP levels (<10 mg/l) within three to eleven months postpartum (Drain *et al* 2007) the median levels (1.95 mg/l) were much lower than that found in this study. Drain *et al* (2007) concluded that high CRP levels were associated with an increased risk of death independent of anthropometry, CD4 count and viral load (Drain *et al* 2007).

Some international trials investigated CRP levels in asymptomatic HIV-infected adults which was more representative of this study group. The study of Fat Redistribution and Metabolic Change (FRAM) in HIV infection investigated the relationship between CRP and all-cause mortality in 922 HIV-infected adults living in California (Tien *et al* 2010). The 36% with a CD4 count of >500 cells/mm^3 and a CRP level >3 mg/l had a 2.7-fold higher adjusted odds of death when compared

to those whose CRP was <1 mg/l (Tien *et al* 2010). When compared to this study, a similar percent (39%) with a CD4 count >500 cells/mm^3 had CRP levels >3 mg/l, reinforcing that this apparently well population was at particularly high risk. The SMART trial investigated 255 HIV-infected ARV naive adults with a CD4 >600 cells/mm^3 and concluded that those in the highest quartile of CRP had twice the risk of death than those in the lowest quartile (Kuller *et al* 2008). Raised CRP levels are associated with all-cause mortality even after adjustment for viral load and CD4 count (Feldman *et al* 2003b) (Kuller *et al* 2008) (Tien *et al* 2010).

Raised CRP levels also increase the risk of AIDS events, the immune reconstitution inflammatory syndrome (IRIS), acute myocardial events and opportunistic disease. A nested case-control study using samples from 63 HIV-infected and 126 healthy controls enrolled in the Flexible Initial Retrovirus Suppression Therapies (FIRST) trial, reported that those with pre-ART CRP levels >2.1 mg/l had an increased risk of AIDS events and IRIS (Boulware *et al* 2011). This translated to 37% of the current study population. The study on Tanzanian post-partum females found that high CRP levels were associated with increased progression (2.26 relative risk) to AIDS (Drain *et al* 2007). The United States Partners HealthCare System (1997 to 2006) who investigated 487 HIV-infected adults and 69 870 controls (Triant *et al* 2009) concluded that those with high CRP levels (>5 mg/l) had an odds ratio that was increased more than 4 fold for the risk of an acute myocardial infarction. The SMART trial concluded that there was a 3.5 higher odds ratio of opportunistic disease in those with levels >5 mg/l (Rodger *et al* 2009).

The high CRP levels amongst our study participants was probably in part explained by the high prevalence of obesity. Weight, BMI, waist circumference, hip circumference, and waist-hip ratio are positively significantly correlated to CRP (Park *et al* 2005). C-reactive protein is an important inflammatory marker in obesity and tends to be raised in the presence of excessive adiposity (de Ferranti and Mozaffarian 2008) (Nishide *et al* 2015). Addressing obesity in this population of HIV-infected ARV naive adults therefore is important to reduce the consequences of inflammation. In South Africa, this would require a paradigm shift in policy as the emphasis locally for so long has been on preventing wasting and protecting fat free mass. The South African guidelines briefly refer to obesity as being undesirable when on ART but primarily focus on preventing weight loss and the importance of regaining the lost weight (Department of Health

South Africa 2007). These guidelines only refer briefly to obesity as being non desirable in those on ART although the Nutritional Guidelines for HIV-infected Adults and Children in Southern African (2008) are clear that overweight asymptomatic adults need to lose weight sensibly (Spencer *et al* 2008).

Based on CRP levels, approximately a third of the asymptomatic adults in this study were at a high risk of death, progression to AIDS, acute myocardial events, opportunistic infections and IRIS. These adults look deceptively well and in the overloaded clinic system would not be identified as being at high risk and in need of additional care. C-reactive protein levels are relatively inexpensive, and although available locally, are seldom if ever measured at clinic level. Perhaps CRP should be accessed routinely in the HIV-infected ARV naive adults, particularly those pre-ART, to identify those at high risk? The cost may prove inhibitive however on a large scale basis.

5.7 MORBIDITY

5.7.1 Infections

As inulin-type fructans exhibit immune modulatory actions independent of the bifidogenic effect (Drain *et al* 2007) (Franco-Robles and López 2015) it was expected that those supplementing with inulin-type fructans would experience less infections as measured by the frequency of antibiotic use. The use of antibiotics did not decrease in those supplementing with inulin-type fructans. The lack of effect could be due to the small number of infections experienced by the group as a whole, making statistical significance unlikely or because inulin-type fructans simply did not have a discernible influence on susceptibility to infections.

5.7.2 Gastrointestinal symptoms

According to the literature, HIV-infected individuals without AIDS (Elfstrand and Florén 2010) commonly experience diarrhoea (Lubeck *et al* 1993) (Anukam *et al* 2008) (Kelly *et al* 2009) (Wilson *et al* 2013) which can have considerable clinical implications (Siddiqui *et al* 2007) (Dikman *et al* 2015) including a significant deterioration in quality of life (Siddiqui *et al* 2007).

Supplementation with inulin-type fructans has been shown to beneficially modulate the microbiome, increase resistance to gastrointestinal pathogens (Schaafsma and Slavin 2015) and improve gastrointestinal symptoms. Supplementation therefore was intended to reduce the frequent episodes of diarrhoea thereby preventing weight loss and protecting or improving fat free mass. Despite half (48%) having a CD4 count of <500 cells/mm^3, less than 10% reported diarrhoea and none suffered from chronic diarrhoea. On the contrary approximately a third suffered from constipation and feelings of inadequate evacuation. After having transferred the stools into cryovials, the PI had first hand knowledge that the prevelance of diarrhoea was very low and constipation was high. The low prevalence of diarrhoea supported the findings of the stool mRNA analysis where no discernable GIT inflammation was detected as evidenced by the low recovery of GAPDH and faecal inflammatory markers. The findings of this study do not support the current belief that diarrhoea is an inevitable and common consequence of HIV infection which significantly impacts quality of life.

There was a significant improvement in gastrointestinal symptoms in both groups. This included a reduction in the prevalence of constipation, an increased number of stools passed weekly and improved feelings of incomplete evacuation. It was expected that the group receiving inulin-type fructans only would experience a resolution of gastrointestinal symptoms as these soluble dietary fibres are well known to promote the saccharolytic activities of the microbiome resulting in increased stool bulking, stool frequency and softer stools (Schaafsma and Slavin 2015). As both groups improved, the nutritional supplement itself may have exerted the effect. The primary ingredients however were refined maize meal and soy flour, neither of which is known to alleviate constipation. The fibre content (2.6 g per 50 g) was low in comparison to that found in equivalent amounts of high fibre breakfast cereals (5 to 15 g per 50 g) and therefore could not be credited with the effect. Those with constipation at baseline however did receive dietary counselling and were advised to drink more water and other fluids and to increase their intake of high fibre fruit and vegetables, particularly those which were culturally acceptable and affordable, such as cabbage and beans.

The reduction in nausea, vomiting and bloating was unexpected particularly as inulin-type fructans increase bloating, rumbling, cramps and liquid stools due to the production of SCFA and lactate (Schaafsma and Slavin 2015). The production of SCFA however is more likely to occur at doses of ≥20 g per day and with FOS rather than with the longer chain inulin-type fructans which are more slowly digested (Schaafsma and Slavin 2015). There was no increase in undesirable gastrointestinal effects other than mild flatulence which was not of sufficient consequence to alter the GSRS. For the purposes of this study, inulin-type fructans at a single dose of 15 g per day, given in combination with a supplement drink did not have any serious adverse events associated with their use and resulted only in a tolerable increase in flatulence and no other gastrointestinal symptoms.

5.8 STRENGTHS OF THE STUDY

5.8.1 The use of an advanced technique (deuterium dilution) to measure body composition to ensure an accurate determination of fat free mass.

5.8.2 The use of stool mRNA markers of inflammation. This offers an advantage as faecal calprotectin for example may have been present in the stool but could have been destroyed before or during analysis. The determination of the presence of the gene encoding faecal calprotectin (S100A8) would confirm that the inflammatory substances were being produced even if not detectable in the stool.

5.8.3 The technique of ddPCR had a much lower level of uncertainty for quantification of specific RNA target sequences than that of the traditional cdPCR and was therefore a superior technique.

5.8.4 The clinician and research assistant had extensive experience in participating in HIV trials and were invaluable assets in maintaining a high standard of data collection.

5.9 STUDY LIMITATIONS

5.9.1 Simple, affordable routine screening for pregnancy at each visit would have been appropriate. Pregnancy was an exclusion criteria and the high number who fell pregnant during the study was not anticipated. Testing was only done if suspicion was aroused.

5.9.2 Financial constraints prevented a more inclusive analysis of markers of inflammation and permeability as many of these were not routinely tested in South Africa and the cost of analysis overseas was prohibitive.

5.9.3 The lack of local expertise for tests such as the analysis of LPS.

5.10 SUGGESTIONS FOR FURTHER RESEARCH

As a consequence of this study, several research gaps became apparent. To respond to these gaps in knowledge the following research studies are suggested:

5.10.1 A cross sectional study sampling all provinces in South Africa to determine the prevalence of overweight/obesity, hypoalbuminemia, inflammation and iron deficiency anaemia in asymptomatic HIV-infected ARV naive adults.

5.10.2 A RCT to determine the impact of weight loss on fat free mass and CRP levels in asymptomatic HIV-infected ARV naive adults in South Africa.

5.10.3 A RCT investigating the consequences of iron supplementation to treat mild anaemia in the HIV-infected in South Africa.

5.10.4 A RCT to establish whether gastrointestinal inflammation is present using faecal mRNA inflammatory markers, and if so the effect of inulin-type supplementation, in HIV-infected ARV naive adults with diarrhoea.[60]

5.10.5 The impact of inulin-type fructan supplementation in symptomatic HIV-infected ARV naïve individuals.

5.10.6 The impact of inulin-type fructan supplementation in HIV-infected individuals on ARV.

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[60] Based on the levels of the inflammatory marker Il8 in our study, it has been calculated that in order to perform a sufficiently powered RCT a sample size of 34 per group would be necessary.

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

Inulin-type fructan supplementation at a single dose of 15 g per day, given in combination with a supplement drink, was not associated with any serious adverse events and resulted only in a tolerable increase in flatulence with no other accompanying gastrointestinal symptoms.

This was the first study to document the daily dietary inulin-type fructan intake in an African population. Their daily dietary intake was borderline low for that required for a bifidogenic effect and resembled that of European countries. The major dietary sources were similar to those of other countries and included wheat, onions, bananas and chicory. Recommendations for increasing their intake was problematic as encouraging an increased intake of bread and bananas seems counter-productive due to the high prevalence of overweight/obesity.

Nutritional status, as reflected by body composition, haemoglobin and serum albumin, was not altered by supplementation with inulin-type fructans. It was proposed that the use of inulin-type fructans to manipulate the microbiome would reduce both the incidence of intestinal infections/diarrhoea and the chronic immune stimulation and thereby protect and improve fat free mass. The lack of impact on fat free mass could be attributed to the unexpectedly low incidence of both infections and diarrhoea. This study confirmed that the fat free mass of asymptomatic HIV-infected adults was below that of their healthy American black counterparts and was similar to that found by other South African studies on HIV-infected ARV naive adults.

The very high prevalence of both overweight and obesity in this HIV-infected population was characteristic of the typical healthy black African adult found in KZN. This confirmed the international suspicion that the body weight of HIV-infected adults was representative of the country's general population and that wasting was no longer an inevitable scenario in the progression to AIDS. The prevalence of overweight/obesity in asymptomatic HIV-infected adults in South Africa needs further investigation, as the deleterious health implications of obesity, inducing a state of inflammation, in combination with the long term complications of ART,

compounds the risk for this population. The South African National Guidelines on Nutrition For People Living With HIV, AIDS, TB and other Chronic Debilitating Conditions[61] urgently requires revision as these recommendations are directed towards the prevention and treatment of wasting and ignore the "elephant in the room" challenge of overweight/obesity.

Approximately one in two suffered from mild iron deficiency anaemia which is a strong independent risk factor for disease progression, mortality and a decreased quality of life. Research is needed to investigate the prevalence in the asymptomatic individual as the available data primarily focused on the symptomatic sufferer pre ART. Although the HIV infection itself results in anaemia, the anaemia may also have been a consequence of a nutritional iron deficit as the prevalence of iron deficiency anaemia is high in the general population in South Africa. The treatment is unclear as both withholding supplementation and providing supplementation could offer the virus a growth advantage and clear guidelines on iron supplementation for those HIV infected are not available either locally and internationally. The impact of iron supplementation on the course of the HIV virus needs urgent investigation as according to the South African treatment guidelines, many with HIV are eligible for the supplementation offered by the clinics. The risk of inappropriate supplementation however was unlikely as the clinic experienced serious shortfalls in the supply of iron and currently only those who are pregnant and severely iron deficient may receive treatment. The reasons for the poor supply of iron supplements needs to be investigated and corrected.

The prevalence of hypoalbuminemia was very high in comparison with other HIV-infected populations both in South Africa and internationally, and consequently a third of the study population was at a high risk of morbidity and the risk of progression to AIDS and death. As both low serum albumin and low serum iron levels are deleterious, the routine testing of both could be useful to identify the particularly high risk individual. Although the causes were not investigated, the hypoalbuminemia was unlikely to be a consequence of an inadequate dietary intake, malnutrition or malabsorption from a leaking gut but rather a reflection of the acute phase response as the CRP levels were higher than that of other HIV-infected populations.

[61] www.sahealthinfo.co.za/nutrition/sanational.htm.

Supplementation with inulin-type fructans did not improve the immune status (CD4 count) or the inflammatory response (CRP). In general, the CD4 count is very labile and influenced by a number of factors and is not representative of the CD4 T cell population in the GALT where the action of the inulin-type fructans was anticipated, perhaps explaining the lack of outcome. The raised CRP levels exposed one in three to a high risk of death, progression, acute myocardial events and opportunistic infections. Obesity probably contributed to the raised CRP levels reinforcing the importance of dealing with this concern at the national level.

This apparently well asymptomatic group of HIV-infected adults were at a very high risk of progression to AIDS, morbidity and mortality as a consequence of iron deficiency anaemia, hypoalbuminemia and inflammation which was further complicated by the high prevalence of obesity. The focus of HIV treatment at clinic level in South Africa was mainly directed toward those needing ART and very little support, if any, was offered to the ARV naive. It is suggested that guidelines and training at the clinic level should be provided to enable identification of these high risk individuals who look deceptively healthy. Identification could be achieved by the annual or biannual monitoring of serum Hb, albumin and CRP levels.

Inulin-type supplementation did not improve morbidity as measured by the incidence of infections and diarrhoea. Contrary to expectations based on current scientific literature, the incidence of infections and the prevalence of diarrhoea was very low. The high prevalence of constipation concomitant with the low prevalence of diarrhoea does not support the current belief that significant permeability and inflammation of the GIT is present from early on in the disease process. In addition, there was no evidence of increased cell shedding or inflammation as demonstrated by the novel use of stool mRNA analysis. These results therefore do not support the widely held belief that there is increased intestinal permeability and inflammation of the GIT from early on in the infection permitting microbial translocation which in turn stimulates the chronic immune system activation driving the progression to AIDS. As the rationale behind this study was that supplementation with inulin-type fructans offered a local protective effect on the GIT through manipulation of the microbiome thereby reducing microbial translocation and the chronic immune stimulation, the absence of detectable GIT permeability and inflammation would explain why supplementation appeared ineffective in this study.

6.2 RECOMMENDATIONS

6.2.1 The South African National Guidelines on Nutrition For People Living With HIV, AIDS, TB and other Chronic Debilitating Conditions should be revised to address the management of overweight and obesity in HIV.

6.2.2 There needs to be a clear treatment policy regarding anaemia and iron supplementation in asymptomatic HIV-infected adults.

6.2.3 Clinic staff need to be educated of the high risk of progression that otherwise well asymptomatic HIV-infected adults face and how to identify these individuals.

6.2.4 Routine screening of albumin, Hb and CRP should be considered in order to identify high risk individuals.

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APPENDICES

APPENDIX A: Specifications for Frutafit®

Frutafit® HD

version 2010.01/a (December 2010)

Product Data Sheet

Description
Frutafit® HD is a native inulin/oligofructose. It is a natural powdered food ingredient extracted from chicory roots. Frutafit® HD can be applied in a wide range of food products.

Inulin from chicory is a polydisperse mixture of linear fructose polymers with mostly a terminal glucose unit, coupled by means of β(2-1) bonds. The number of units (degree of polymerization) can vary between 2 and 60.

Specification
(Method of analysis available on request)

Physical aspects		
Dry matter content		95-99%
Composition on dry matter		
Carbohydrates		≥ 99.5 %
Inulin		≥ 90 %
Fructose, glucose, sucrose		≤ 10 %
Average chain length		8-13 monomers
Ash		≤ 0.2%
Heavy metals	Pb, As	each ≤ 0.1 mg/kg
	Cd, Hg	each ≤ 0.01 mg/kg
Microbiology		
Aerobic plate count (30°C)		≤ 1000 CFU/gram
Aerobic plate count (55°C)		≤ 1000 CFU/gram
Moulds		≤ 20 CFU/gram
Yeasts		≤ 20 CFU/gram
Bacillus cereus		≤100 CFU/gram
Listeria monocytogenes		absent/25 grams
Enterobacteriaceae		absent/gram
Staphylococcus aureus		absent/gram
Salmonella		absent/400 grams

Nutritional information
All values are averages expressed per 100 grams Frutafit® HD.

Carbohydrates:	97 grams
- digestible (sugars)	7 grams
- non-digestible (dietary fiber, inulin)	90 grams
Proteins	0 gram
Fats	0 gram
Dietary fibers	90 grams
Moisture	3 grams
Minerals:	
- Sodium	40 mg
- Calcium	11.5 mg
- Potassium	7.5 mg
- Iron	0.4 mg
- Other minerals	negligible
Vitamins	negligible
Cholesterol	absent
Gluten	absent
Lactose	absent
Folate	absent
Insecticides, pesticides	absent
Enzymatic activity	absent
Color, flavor, preservatives	absent
Caloric value	1.2 kcal/gram[1]
Glycaemic response	14[2]

[1] Calculated value based on 1 kcal/gram pure inulin. Please check local legislation and adapt if necessary.
[2] The effect on the blood glucose level of 25 gram carbohydrate coming from Frutafit® HD is compared with the effect on blood glucose level of 25 gram glucose (control=100).

SENSUS

Other information

Additional product characteristics

Appearance	: fine white powder
Dispersability	: good
Wettability	: good
pH	: 4.5 – 7.0
Taste	: neutral, slightly sweet
Sweetness	: 10% (sweetness of a 10 w% sucrose solution is 100%)
Tapped density	: 700 ± 100 gram/liter

(These data are indicative and only meant to provide additional information.)

Packaging
20 kg (44.09 lbs), white multi layer paper bag with colored PE inner liner.

Labeling
In the ingredient list inulin/oligofructose can be declared as an ingredient, not an additive. The product can be labeled as inulin, oligofructose, fructo-oligosaccharide (FOS), polyfructose or as dietary fiber, chicory root fiber or vegetable fiber.

Safety
GRAS status. FDA notice no. GRN 000118.

Storage
The product should be stored under dry conditions in the original unopened bag.

Shelf life
The product can be stored for at least 5 years after production date, if stored in original sealed bags under dry conditions. Production date and best before date are printed on each individual bag.

Non GMO
For the production of this product, Sensus only uses raw materials from officially approved chicory varieties. None of these varieties fall under the scope on GMO directive 2001/18/EC. Therefore no labeling as a GMO derived ingredient is needed for application of this product according to directive 2001/18/EC and regulation (EC) No. 1829/2003 and regulation (EC) No. 1830/2003.

Allergens
Neither the raw material nor the process additives used in the production of this product contain the following allergens and products thereof as listed in EU directives 2000/13/EC, 2006/142/EC and 2007/68/EC: gluten, crustaceans, eggs, fish, peanuts, soybeans, milk, nuts, celery, mustard, sesame, sulphur dioxide and sulphite, lupine and molluscs.

Kosher
Certified by Circle K.

Halal
Certified by Total Quality Halal Correct.

Analyses
Fructans (inulin/oligofructose) in food products can be analyzed by the following methods: AOAC 997.08 (AACC 32-31) and AOAC 999.03 (AACC 32-32).

Produced by
Sensus, Borchwerf 3, 4704 RG Roosendaal, The Netherlands.

APPENDIX B: Ethics permission from the eThekwini Municipality Durban

HEALTH, SOCIAL SERVICES
Health Unit
9 Archie Gumede Place
P O Box 2443, Durban, 4000
Durban, 4001
Tel: 031 311 3523, Fax: 031 311 3530
www.durban.gov.za

26 November 2012

Dear Prof Anna Coutsoudis and Ms. Chara Biggs.

Re: Permission to undertake research at Lancers Road Clinic.

The study site has been approved for:

Pilot study investigating the effect of dietary fibre on disease progression and anthropometric status of HIV infected ARV naïve adults attending a wellness program in KwaZulu-Natal, South Africa.

The following conditions are to be noted:

- Submission of the indemnity form obtainable from the EThekwini Health Unit before commencement of the study.
- Prior arrangements to be made with the facility and an assurance that all services will not be disrupted.
- Staff will not be used for data collection.
- Progress reports to be provided and the final report of the study with an oral presentation to the eThekwini Municipality Unit.
- Obtain permission from the eThekwini municipality health department for press releases and release of results to communities/stakeholders.
- The department has to receive recognition for the assistance given.
- Any amended to the study to be communicated with the Health Unit, and the relevant amendment form obtainable from the unit to be submitted.
- Withdrawal of permission to conduct research will be left to the discretion of the eThekwini Health Unit.

Yours faithfully,

Dr _____ Date: 28/11/2012.

~~Deputy~~ Head of Health: Clinical Support

APPENDIX C: Ethics approval from the Biomedical Research Ethics Committee

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YAKWAZULU-NATALI

RESEARCH OFFICE
BIOMEDICAL RESEARCH ETHICS ADMINISTRATION
Westville Campus
Govan Mbeki Building
Private Bag X 54001
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4000
KwaZulu-Natal, SOUTH AFRICA
Tel: 27 31 2604769 - Fax: 27 31 2604609
Email: BREC@ukzn.ac.za
Website: http://research.ukzn.ac.za/ResearchEthics/BiomedicalResearchEthics.aspx

22 March 2011

Ms. Chara Biggs
Dept. of Dietetics & Human Nutrition
Pietermaritzburg Campus
University of KwaZulu-Natal

Dear Ms Biggs

PROTOCOL: The effect of probiotic supplementation on disease progression and anthropometric status of HIV positive ARV naïve adults attending a wellness program in KwaZulu- Natal, South Africa. REF: BFC145/010

The Biomedical Research Ethics Committee (BREC) has considered the abovementioned application.

The study was provisionally approved by a quorate meeting of BREC on 10 August 2010 pending appropriate responses to queries raised. Your responses received on 15 December 2010 to queries raised on 31 August 2010 have been approved by the Biomedical Research Ethics Committee at a meeting held on 08 March 2011. The conditions have now been met and the study is given full ethics approval and may begin as from 22 March 2011.

This approval is valid for one year from 22 March 2011. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2004), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/ResearchEthics11415.aspx.
BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

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Email: BREC@ukzn.ac.za
Website: http://research.ukzn.ac.za/ResearchEthics/BiomedicalResearchEthics.aspx

26 July 2011

Ms. Chara Biggs
Dept. of Dietetics & Human Nutrition
Pietermaritzburg Campus
University of KwaZulu-Natal

Dear Ms Biggs

PROTOCOL: The effect of probiotic supplementation on disease progression and anthropometric status of HIV positive ARV naïve adults attending a wellness program in KwaZulu- Natal, South Africa. REF: BFC145/010

We wish to advise you that your application for amendments received by BREC on 19 June 2012 has been noted and approved by the sub-committee of the Biomedical Research Ethics Committee.

The following amendment has been noted and approved:

Change of study Title from the above to "Effect of **soluble dietary fibre** on disease progression and anthropometric status of HIV positive ARV-naïve adults attending a wellness program in KwaZulu-Natal, South Africa.

This approval **will** be ratified at the next full Committee meeting to be held on 14 August 2012.

Yours sincerely

Ms A Marimuthu
Senior Administrator: Biomedical Research Ethics

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Amended letter

18 September 2015

Ms. Chara Biggs
Dept. of Dietetics & Human Nutrition
Pietermaritzburg Campus
University of KwaZulu-Natal

Dear Ms Biggs

PROTOCOL: The effect of probiotic supplementation on disease progression and anthropometric status of HIV positive ARV naïve adults attending a wellness program in KwaZulu-Natal, South Africa. REF: BFC145/010.

I wish to advise you that your correspondence dated 24 July 2015 requesting approval of Amendments (Change of Title of Study and Change of Objective 1) for the above study has been noted and approved by the Biomedical Research Ethics Committee at a meeting held on 08 September 2015.

It is noted that the title of study has changed as follows:

"A randomized double blind placebo controlled trial to determine the effect of soluble dietary fibre (inulin-type fructans) on disease progression and body composition of HIV positive ARV naïve adults attending a wellness clinic in KZN South Africa"

Yours sincerely

Ms A Marimuthu
Senior Administrator: Biomedical Research Ethics

APPENDIX D: Participant information sheet - isiZulu

UQULU WOLWAZI

Isihloko socwaningo: Imiphumela ye – soluble fibre supplementation kulabo abanezifo futhi bephila negciwane lengculaza bengena lwazi ngemishanguzo

Sanibona

Siphuma eNyuvesi yakwaZulu – Natal senza ugcwaningo ngempushana ifibre sifuna ukuthola ukuthi ngabe ingakwazi yini ukwehlisa ukukhishwa isisu iphinde yenze amasosha omzimba (CD4) angehli ngokushesha. Ucwaningo yindlela yokufunda kuphendulwe imibuzo, lolugcwaningo luyingxenye ye – PhD eyiziqu zika Chara Biggs okunguye umcwaningi omkhulu noma onguyena mphathi wocwaningo kulolucwaningo oluyisifundo sifuna ukufunda ukuthi ngabe impushana ifibre ingakwazi yini ukukugcina isikhashana uphile kahle ngakho-ke sifuna ukuhlola ukuthi ingabasiza yini abantu abaphila negciwane lengculaza. Uma uvuma ukuba yingxenye yalesisifundo uyocelwa ukuthi usebenzise lempushana njalo kanye ngosuku isikhathi esingangezinyanga ezintathu Uhafu wabantu abazongenela lesisifunda banikezwa yona lempushana esiyihlolayo omunye uhafu unikezwa efana nayo kodwa engesiyo. Ngekesazi ukuthi ubani othola yiphi futhi nawe ngekewazi Lokhu sikwenza ngoba sifuna ukubona ukuthi umkhiqizo ifibre iyawenza yini umehluko. Lokhu kuyadingeka ukuzesiqiniseke ukuthi ucwaningo luhamba ngendlela eyiyo nokuthi sithole imiphumela eyiqiniso.

Sicela noma ubani oza eLancers Road kliniki ahlanganyele kulolucwaningo uma nje amasosha omzimba wakhe ewu-350 nangaphezulu.

Uma uvuma ukuhlanganyela kulesisifundo siyobesesikunikeza usuku oyoza ngalo ekliniki oyobe uzongenela ngalo ucwaningo. Siyokunikeza ibhodlela oyosiphathela ngalo indle yakho. Sicela usiphathele eseyitsha oyiqoqe ekuseni noma-ke ebusuku. Siyocela ukuthi ungadli ungaphuzi lutho isikhathi esingangehora. Ngalesosikhathi uyothathwa amagazi okuhlola izakhamzimba uchofozwe sibheke i-iron uphinde uhlolwe udokotela siyokukala isisindo kanye nobude bakho siyobe sesikuphuzisa amanzi anekhemikheli eyokwazi ukusikhombisa imisipha yakho lamanzi aphephile aseke asetshenziswa kwezinye izifundo eziningi. Sicela ungadli amahora amathathu ngoba siyocela isampula lamathe ngaphambu kokuba uphuze amanzi nasekupheleni kwamahora amthathu. Lokhu kuhlolwa kuyo phindwa emveni kwezinyanga ezntathu.

Uyobe usunikezwa leyompushana oyoyisebenzisa isikhathi esingangenyanga kanye nephepha oyosibhalela kulo uma kwenzekile kwabakhona ushitsho okungaba ukugula noma ukukhishwa isisu uyokhonjiswa indlela yokulicwalisa.

Siyodinga ubuye njalo ngenyanga kuze kuphele izinyanga ezintathu. Ngasosonke isikhathi uma ufika ekliniki siyokukala siphinde sixoxisane sithole ukuthi impilo injani nokuthi asikaze sikukhiphe yini isisu. Siyokunikeza enye impushana kanye nephepha lokucwalisa ushitsho lwempilo. Kubalulekile ukubika noma yini ewushitsho ethimbeni

locwaningo noma kukhona imithi oyisebenzisayo ozithengele yona mona uyinikezwe udokotela. Uma sekuphele izinyanga ezintathu uyophinda uhlolwe njengasekuqaleni.

Abukho ubungozi ngokuhlangnyela ocwanigweni/esifundweni.

Uyohlomula ngokuba sesifundweni ngoba uyobonwa udokotela izikhathi eziningi kanye nabeluleki.

Njalo uma uze ekliniki uyonikezwa uR80.00 wokugibela. Uma uzohlala isikhathi eside ngoba kuthathwa isampula lamathe uyonikezwa uR100.00 kanye nesidlo sasemini nesiphuzo.

Awuphoqiwe ukuba umhlanganyeli kulolucwaningo uyolungenela ngokuzithandela akusho ukuthi uma ungavumi uyophucwa amalungelo akho. Uyoqhubeka nokunakekelwa ekliniki noma ungahlangnyelanga ocwaningweni.

Uma ucwaningo seluphelile uyobe usunikezwa imiphumela yokho uma isikhona. Imiphumela iyobhalwa phansi kuxoxwe ngayo eimihlanganweni kodwa igama lakho liyogcinwa liyimfihlo.

IMFIHLO: Siyokwenza isiqiniseko sokuthi lonke ulwazi luyimfihlo kodwa uma ulwazi ludingwa umthetho singeke sakuqinisekisa lokho.

Inhlangano iyohlola ikopishe imiqulu yocwaningo ukuqinisekisa nokubhekisisa. Ihlangano iResearch Ethic Committee kanye nophiko lwezempilo

Thinta uChara Biggs uma udinga ulwazi olwengeziwe ku 0845248062.

Xhumana ne BREC Administrator or chair – ukubika noma izikhalazo /izinkinga:

Biomedical Research Ethics, Research Office, UKZN, Private Bag X54001, Durban 4000

Telephone: +27 (0) 31 260 4769 / 260 1074

Fax: +27 (0) 31 260 2384

Administrator: Ms Ngwenya Email: ngwenyap@ukzn.ac.za

Chair: Prof D R Wassenaar Email: c/o ngwenyap@ukzn.ac.za

Participant information sheet – English

INFORMATION DOCUMENT

Study title: The effect of soluble dietary fibre supplementation on morbidity and immune status in HIV positive ARV naive individuals.

Good day.

We are from the University of KwaZulu-Natal, and are doing research on whether this fibre powder can reduce the number of times you are sick and/or have diarrhoea and keep your CD4 count from dropping too quickly. Research is just the process to learn the answer to a question and this research is part of a PhD degree of the principal investigator Chara Biggs. In this research study we want to learn whether this fibre powder can keep you well for longer so we are testing to see if this fibre powder could be helpful to people with HIV infection. If you agree to be part of the study you will be asked to take this powder once a day every day for 3 months. Half of the people in the study will receive the powder that we are testing and the other half will receive a powder that looks the same but does not contain the fibre product we are testing. We will not know who is taking which powder and you will not know. This will help us see whether the fibre product is making a difference. We need to do this to make sure that our research is done properly and we find out the true results.

We are asking everyone who comes to the Lancers Street Clinic to take part if their CD4 count is >350 cells/mm^3.

If you consent to be in this study you will be given an appointment to return to the clinic for a study visit and you will be given a specimen bottle and be asked to bring a stool sample that was collected any time after lunch the day before or on the morning of your visit. At the clinic when you begin the study you will be asked not to eat or drink anything for an hour. In the meanwhile, the doctor will do an examination and take blood to do a CD4 count, albumin, C-reactive protein and for lipoprotein polysaccharides. Your finger will also be pricked to see what your iron levels are. You will be asked questions about yourself and what you eat. When this is done your height and weight will be measured. You will then be asked to drink a small amount of water which contains a chemical marker to help us work out how much of your body is muscle. This water with the special maker is safe and has been used in many other studies. You may not eat and drink for another 3 hours. A saliva sample will be taken just before you drink the solution and at the end of 3 hours. This test will be repeated at the 3 month visit.

You will then be given the powder for one month and shown how to fill in a form to record how many times you were sick and had diarrhoea over the last month.

You will need to come back every month for 3 months. You will be given money for transport so that it will not cost you anything extra. At each visit you will be weighed and we will discuss the diarrhoea/infection diary with you and give you a new one. We will then give you the next month's supply of powder. It is important that you tell the research team if there has been any changes in the medication that you have been taking and whether you are taking any other medications that have not been prescribed by a doctor.

After 3 months we will redo the tests that were done when you started the study.

There are no risks to you taking part in the study.

You will benefit from the study by being able to attend the clinic more frequently and being able to see the doctors and counselors more often.

For every clinic visit during the study you will be given R100 to help pay for travel costs and for the days that you have stayed on a bit longer because we are taking saliva samples you will be given R150 as well as lunch and a drink.

You do not have to be part of the study. Participation is voluntary and if you don't want to take part you may still attend the clinic as usual and receive the same care. You may also choose to stop being in the study at any time and you will still be able to attend the clinic and receive the same care.

When the study is over you will be given the results when they become available. The results of the study will also be published and presented at meetings but your name will not be used so no one will know who you are.

Confidentiality: Every effort will be made to keep personal information confidential. However absolute confidentiality cannot be guaranteed. Personal information may be disclosed if required by law.

Organizations that may inspect and/or copy your research records for quality assurance and data analysis include groups such as the Research Ethics Committee, Data Safety Monitoring Committee and the Medicines Control Council (where appropriate).

Contact details if you need further information: Chara Biggs 0845248062
Contact details of BREC Administrator or Chair – for reporting of complaints/ problems:
Biomedical Research Ethics, Research Office, UKZN, Private Bag X54001, Durban 4000
Telephone: +27 (0) 31 260 4769 / 260 1074
Fax: +27 (0) 31 260 2384
Administrator: Ms P Ngwenya Email:
mailto:ngwenyap@ukzn.ac.zaChair: Email: Prof D R Wassenaar c/o
ngwenyap@ukzn.ac.za

APPENDIX E: Consent Form - isiZulu

UMQULU OYIMVUME

Imvume yokuhlanganyela ocwanigweni.

Sanibonani.

Ulwazi ngomcwaningi uChara Biggs kanye nohlelo lwesifundo semiphumela ye soluble dietary supplementation ezifweni kanye nezivikela mzimba kwabaphila negciwane lengculaza bengakabi nalwazi ngemishanguzo futhi bengakayinikezwa.

Uceliwe ukuthi uhlanganyela ocwaningweni oluyisifundo lapho sibheka khona ukuthi ngakube ukwelashwa esikunikeza khona kungakusiza yini ukuthi uphile isikhathi esengeziwe. Besekuchaziwe kuwe ukuthi uma uvuma ukuhlanganyela kuyodinga uchithe amahora ayisithupha ekliniki osukwini oyobe uzoqala ngalo kanye nolokugcina esifundweni.

UChara Biggs ubese ekuchazele ngesifundo.

Ungaxhumana noChara Biggs ku 1845248062 noma ingasiphi isikhathi uma unemibuzo ngocwaningo.

Akukho ayokukhokha okungaphezulu ngokungenela lesisifundo.

Ungathintana namahovisi eBiomedical Research Ethics kule namba 031 260 4769 noma 260 1074 uma unemibuzo ngamalungelo akho njengomhlanganyeli ocwaningweni.

Ukuhlanganyela kulolucwaningo kuwukuzithandela, ngeke ujeziswe noma uphucwe amalungulo akho uma wenqaba ukuhlanganyela noma unquma ukuyeka noma nini.

Siyacela futhi ukuthi sigcine amagazi ayosala uma sesikwenzile ukuhlolwa okudingekayo kulesisifundo. Siyowagcina elabharetri yaseNyuvesi ku freezer iminyaka emihlanu emveni kokuba sesiphelile isifundo ukuze kuthi uma kukhona imibuzo noma izimo lapho kudingeka kwenziwe okunye ukuhlola okungase kusisize siqonde kangcono ezinye izimo. Isampula lendle liyogcinwa elabhoretri yaseNyuvesi kufreezer kuze kucubungulwe ukuze sithole ushitsho kwimigudu (gut). Uma kungenzeka singakwazi ukucubungula kulabhoretri yethu sicela imvume yokuthi siyithumele kwenye ilabhoretri. Uma sesiqedile siyowalahla amasampula. Sicela imvume yokusebenzisa amakhadi akho akadokotela khona sizokwazi ukubona ushitsho uma lwenzeka noma imithi akunikeza yona.

Uma uvuma uyonikezwa umqulu ofingqiwe wolwazi wocwaningo.

Ucwaningo lwesifundo, kanye nolwazi olungenhla, ngichazelwe ngomlomo. Ngiyaqonda ukuthi ukuhlanganyela kwami kulesisifundo kusho ukuthini futhi ngizivumele ngokuzithandela. Nginikiwe ithuba lokubuza imibuzo ebenginayo ngokuhlanganyela esifundweni.

Kusayina obambiqhaza :_____ Usuku:_____

Kusayina ochazayo:_____ Usuku:_____

Kusayina ufakazi:_____ Usuku:_____

Consent Form - English

CONSENT TO PARTICIPATE IN RESEARCH

Good day.

Information about the researcher Chara Biggs and the planned study on the effect of soluble dietary fibre supplementation on morbidity and immune status in HIV positive ARV naive individuals has been provided to you.

You have been asked to participate in a research study to see whether the treatment we give you will help prolong your wellness. It has been explained to you that if you agree to participate you will need to spend 6 hours at the clinic on the first and last day of the study.

You have been informed about the study by Chara Biggs.

You may contact Chara Biggs at 0845248062 any time if you have questions about the research or if you are injured as a result of the research.

There will be no extra cost to yourself if you take part in this study.

We will tell if we discover something during the research that may mean that you may not want to continue being in the study.

You may contact the Biomedical Research Ethics Office on 031 260 4769 or 260 1074 if you have questions about your rights as a research participant.

Your participation in this research is voluntary, and you will not be penalized or lose benefits if you refuse to participate or decide to stop at any time.

We are also requesting permission from you to store any blood samples that are left over after we have conducted the tests that are needed for this study. We will store these samples in the University laboratory freezer for a further 5 years after the study ends in case a situation arises where there may be some other tests that we can do to help us understand the issues a bit better. At the end of the 5 years we will discard the samples. The stool samples will be stored in the University laboratory freezer until they are analysed to detect any changes in gut inflammation. In the event that these analyses cannot be performed in our laboratory we request permission to send them to another laboratory. After analysis these samples will be destroyed. We are also requesting permission to access your medical records so that we may see what medical conditions you have suffered from and what medical treatment you have received.

If you agree to participate, you will be given a signed copy of this document and the participant information sheet which is a written summary of the research.

The research study, including the above information, has been described to me orally. I understand what my involvement in the study means and I voluntarily agree to participate. I have been given an opportunity to ask any questions that I might have about participation in the study.

Signature of Participant: _____ Date: _____

Signature of Witness: _____ Date: _____

Signature of Translator (where applicable): _____ Date: _____

Enrollment

DD/MM/YYYY

PIMA CD4

To be enrolled in the study a **YES** must be answered to each of the following criteria

Signed informed consent form	Yes	No
African adult ≥18 years (born 1995 or later) and ≤ 44 years (born 1969)	Yes	No
HIV positive and never taken ARV's	Yes	No

To be enrolled in the study a **NO** must be answered to each of the following criteria

Are they currently taking medications/ traditional medicine/supplements/immune boosters/herbs?	Yes (fill in table below)	No

Name (Refer to PI to see if use excludes participation)	**Start/stop date**	**Reasons for use**

Pregnant or lactating?	Yes	No
Tuberculosis or on INH prophylaxis?	Yes	No
Chronic disease eg cancer, diabetes?	Yes	No
Antibiotics during the previous month?* α	Yes	No
Dietary supplements with pre and/or probiotics during the previous month? *	Yes	No

α Commonly used antibiotics include Cotrimoxazole, Dapsone, Fluconazole, Ethambutol, Streptomycin.

*ask these patients to return next month to be included in the study

APPENDIX G: Clinical Examination Form

Normal health 100% Minor symptoms 90% Normal activity with some effort Unable to carry on normal activity. Requires help with personal needs	Disabled 50% Considerable assistance, medical care Severely disabled, in hospital 30% Very sick, active support needed 20%

Karnofsky Score | | | | %

Examinations	**Normal**	**Abnormal**	**Not done**	**Comments/descriptions**
Ear, nose, throat				
Head and neck				
Cardiovascular				
Lungs				
Abdomen				
Lymph Nodes				
Skin				
Urogenital				
Musculoskeletal				
Neurological				

Comments:

APPENDIX H: Food Frequency Questionnaire

Soluble fiber/probiotic intake – in the past month have they used any of the following regularly?

Maas	Yes	No	If yes then record how much and how often	¼ box (125 ml)	½ box (250 ml)	1 box (500 ml)	Daily	2-3 X per week	1 per week	<1 per week	Brand Name
Yoghurt	Yes	No		1 tub (125 ml)	2 tubs (250 ml)	1 tub (500 ml)	Daily	2-3 X per week	1 per week	<1 per week	Brand Name
Nutren Active	Yes	No		½ glass (125 ml)	1 glass (250 ml)	2 glasses	Daily	2-3 X per week	1 per week	<1 per week	
Ensure	Yes	No		½ glass (125 ml)	1 glass (250 ml)	2 glasses	Daily	2-3 X per week	1 per week	<1 per week	
Coffee	Yes	No		1 cup (250 ml)	2 cups (500 ml)	3 cups (750 ml)	Daily	2-3 X per week	1 per week	<1 per week	Brand Name
Future life	Yes	No		½ cup (125 ml)	1 cup (250 ml)	2 cups (500 ml)	Daily	2-3 X per week	1 per week	<1 per week	
Pronutro	Yes	No		½ cup (125 ml)	1 cup (250 ml)	2 cups (500 ml)	Daily	2-3 X per week	1 per week	<1 per week	
Bread	Yes	No		4 slices	8 slices (¼ loaf)	14 slices (½ loaf)	Daily	2-3 X per week	1 per week	<1 per week	Brand Name
Garlic	Yes	No		¼ clove	½ clove	1 clove	Daily	2-3 X per week	1 per week	<1 per week	
Banana	Yes	No		1 small	1 medium	1 large	Daily	2-3 X per week	1 per week	<1 per week	
Onions	Yes	No		¼ medium	½ medium	1 medium	Daily	2-3 X per week	1 per week	<1 per week	
Leeks	Yes	No		¼ medium	½ medium	1 medium	Daily	2-3 X per week	1 per week	<1 per week	
Oats	Yes	No		½ cup (125 ml)	1 cup (250 ml)	2 cups (500 ml)	Daily	2-3 X per week	1 per week	<1 per week	
Barley	Yes	No		½ cup (125 ml)	1 cup (250 ml)	2 cups (500 ml)	Daily	2-3 X per week	1 per week	<1 per week	
Asparagus	Yes	No		1 to 3 spears	4 to 6 spears	7 to 10 spears	Daily	2-3 X per week	1 per week	<1 per week	
Beetroot	Yes	No		1 table spoon	2 table spoons	3 table spoons	Daily	2-3 X per week	1 per week	<1 per week	
Imifino	Yes	No		1 serve spoon	2 serve spoons	3 serve spoons	Daily	2-3 X per week	1 per week	<1 per week	
Turnips	Yes	No		1 small	1 medium	1 large	Daily	2-3 X per week	1 per week	<1 per week	
Nectarines	Yes	No		1 small	1 medium	1 large	Daily	2-3 X per week	1 per week	<1 per week	
Melon	Yes	No		1 small slice	1 medium	1 large slice	Daily	2-3 X per week	1 per week	<1 per week	

Enrollment

DD/MM/YYYY

Study Number

Deuterium and saliva testing

When did they last eat and drink?

<1 hour ago	<2 hours ago	>2 hours ago

Time dose taken

Time of post saliva (add 4 hours)

Bottle number:

Do saliva collection and deuterium dose before doing other measurements

Anthropometry

	1st	2nd	3rd	Average
Weight (kg) (must urinate first and preferably give stool sample)				
Height (cm)				
MUAC (cm)				
TST (mm)				

Stools collected:

Yes	No

APPENDIX J: Chart of affordable culturally acceptable food sources high in iron

Iron rich foods


Crossbow
SUGAR BEANS
GRADE 1
CLEANED 3 TIMES
500 g

APPENDIX K: Gastrointestinal Symptom Rating Score

Study Number

Symptoms	**0**	**1**	**2**	**3**	**Score**
Diarrhoea	Normal	Somewhat loose	Runny	Watery	
Increased stools	Once a day	Three times a day	Five times a day	≥3 Seven times a day	
Increased urgency to defecate (when you gotta go in a hurry)	Normal control	Occasional feelings of urgency	Frequent feelings of urgency with sudden need for a toilet interfering with social activities	Inability to control defecation	
Decreased stools	Once a day	Every third day	Every fifth day	≥Every seventh day	
Constipation	Normal	Somewhat hard	Hard	Hard and fragmented	
Feeling of incomplete evacuation.	Feeling of complete evacuation without straining	Defecation somewhat difficult; occasional feelings of incomplete evacuation	Defecation definitely difficult; often feelings of incomplete evacuation	Defecation extremely difficult; regular feelings of incomplete evacuation	
Nausea/vomiting.	No nausea	Occasional of short duration	Often and prolonged	Continuous nausea;	

			nausea; no vomiting	frequent vomiting	
Burping	No or transient	Occasional troublesome	Frequent interfering with social activities	Frequent seriously interfering with social activities	
Excessive flatulence	No or transient	Occasional troublesome	Frequent interfering with social activities	Frequent seriously interfering with social activities	
Stomach pain	No or transient	Occasional interfering with some social activities	Prolonged/troublesome; requests relief/ interfering with many social activities	Severe or crippling with impact on all social activities	
Stomach rumbling	No or transient	Occasional troublesome of short duration	Frequent and prolonged but without interfering with social activities	Continuous severely interfering with social activities	
Bloating	No or transient	Occasional discomfort of short duration	Frequent and prolonged/ can be mastered by adjusting the clothing	Continuous discomfort seriously interfering with social activities	
Heartburn (burning near stomach).	No or transient	Occasional discomfort of short duration	Frequent episodes of prolonged discomfort; requests for relief	Continuous discomfort with only transient relief by antacids	
Acid Reflux (acid in mouth)	No or transient	Occasional troublesome	Once or twice a day; requests for relief	Several times a day; only transient and insignificant relief by antacids	

APPENDIX L: Bristol Stool Chart

Bristol Stool Chart

Type 1		Separate hard lumps, like nuts (hard to pass)
Type 2		Sausage-shaped but lumpy
Type 3		Like a sausage but with cracks on the surface
Type 4		Like a sausage or snake, smooth and soft
Type 5		Soft blobs with clear-cut edges
Type 6		Fluffy pieces with ragged edges, a mushy stool
Type 7		Watery, no solid pieces. **Entirely Liquid**

Follow up

DD/MM/YYYY

Visit number

Packets returned

Have they been well over the past month?

Have they visited the clinic or a doctor or traditional healer?

Have they taken any medication? And if so what for? Please record the name and dose of the medication.

Pregnant or lactating?	Yes	No
Tuberculosis or on INH prophylaxis?	Yes	No
Antibiotics during the previous month?* α	Yes	No
Dietary supplements with pre and/or probiotics during the previous month? *	Yes	No

α Commonly used antibiotics include Cotrimoxazole, Dapsone, Fluconazole, Ethambutol, Streptomycin.

*ask these patients to return next month to be included in the study

Do they have any questions about the study?

Are they willing to continue in the study?

APPENDIX N: Preparation of calibration standards for deuterium dilution according to the standard operating procedures of the International Atomic Energy Agency

9.0 Procedures: Preparation of the calibration standard

A large volume of a calibrating or standard solution of approximately 1000 mg/kg (ppm), or 1 g/L should be prepared (gravimetrically) by weighing 99.8 atom % deuterium oxide (D_2O) and diluting in normal drinking water from the region. Do not use distilled water to make the calibration standard. Distilled water is subject to fractionation.

9.1 Using an analytical balance (accurate to 0.0001 g), weigh a clean, dry 50 mL volumetric flask with its stopper in place, or another similar container e.g. a clean, dry glass bottle with a cap.

9.2 Add a small volume (~20 mL) of drinking water to the flask, replace the cap and weigh again.

9.3 Add 1 g of D_2O to the bottle. If you are using an adjustable pipette to transfer 1 g D_2O, then the volume selected should be 0.9 mL, as the density of D_2O is higher than water (1.105 g/mL and 1.000 g/mL respectively at 25°C). Replace the stopper or cap to avoid losses by evaporation, and note the weight. Calculate the weight of D_2O in the bottle.

9.4 Weigh a clean dry 1 L volumetric flask with its stopper. At this stage a balance weighing to 0.1g can be used.

9.5 Quantitatively transfer the water from the 50 mL container into the 1 L volumetric flask using a funnel. Add local drinking water to the smaller container and pour it into the larger container. Repeat this at least three times to ensure that all the deuterium oxide is transferred. Be careful not to spill any.

9.6 Add local drinking water to the 1 L volumetric flask up to the mark. Replace the stopper and weigh again.

9.7 After noting the weight, transfer the calibration standard to a clean, dry glass bottle with a PTFE lined screw cap.

9.8 Keep a similar volume of the local drinking water to use as a zero-standard or blank to measure the background spectrum.

9.9 Calculate the enrichment of the calibration standard as follows:

9.9.1 If (A) is the weight of D_2O, (B) is the weight of drinking water plus D_2O in the 1L flask, then the weight of added drinking water is (B-A).

9.9.2 Enrichment of D_2O in the calibration standard = $A/(B-A) \times 10^6$ mg/kg

See Appendix 1 for example calculation.

9.10 Transfer the contents of the volumetric flask into four 250 mL borosilicate glass bottles with PTFE-lined screw caps. Save a similar quantity of the water used to make the dilution. Use one of each of these as "working standards". The remainder will remain unopened until required. This has the advantage of only exposing a small portion of the calibration standard to the atmosphere at any time.

9.11 Write the date, D_2O enrichment and the initials of the person who prepared the standard on the label.

9.12 The shelf-life of the calibration standards will depend on the quality of the local drinking water. The bottles should be stored in a cool dark place out of direct sunlight, but not in the same refrigerator as the D_2O. Wrapping bottles in aluminium foil helps to protect the contents from light.

10.0 Accuracy and precision: preparation of a standard curve

10.1 The accuracy and precision of deuterium analysis over the range of enrichments likely to be encountered should be checked periodically (as a training exercise and whenever the FTIR has not been used for 6 months or more) using gravimetrically prepared standards. The enrichment should range from 0 (natural abundance drinking water) to 2000 mg/kg, i.e. an enrichment above that likely to be encountered in saliva samples.

10.2 Standards should be made (in 100 mL local drinking water) according to the following table. The D_2O can be pipetted into the volumetric flask (column 2), but it must be accurately weighed (column 3). Also note the weight of the drinking water added to make up the volume (column 4). The actual enrichment (mg/kg) can be calculated from the weights as described previously.

PREPARATION OF FTIR STANDARDS

Target enrichment (mg/kg D_2O)	µL D_2O	Weight D_2O (g) to 4 decimal places	Weight drinking water added (g)
0	0		
100	10		
200	20		
400	40		
600	60		
800	80		
1000	90		
1500	140		
2000	180		

10.3 Standards should be analysed in triplicate according to the procedure described in sections 11 and 12.

10.4 Plot the calculated enrichment on the x-axis and the measured enrichment on the y-axis of the calibration curve (see Appendix 2).

10.5 Accuracy is determined from the gradient and intercept of the linear regression line through the data. The gradient should be close to 1 (>0.95) and the intercept should be close to 0 (-0.5 to + 20). If not, there is a problem with the weighing, with the calculations or with the analysis. Check the data input and, if necessary, start again and make new standards.

10.6 Precision is the standard deviation (SD) of repeated measures of the same sample. Precision is usually expressed as the coefficient of variation (CV), which is the standard deviation expressed as percent of the mean, SD/Mean x 100. The CV should be less than 1%.

10.7 Within-day and between-days precision of analysis can be determined by repeated measures of the same standard.

APPENDIX O: Preparation of the FTIR for measurement according to the standard operating procedures of the International Atomic Energy Agency

13.0 Preparing the FTIR for measurement

13.1 The FTIR should be switched on 30 minutes before using to allow the electronics to stabilise.

13.1.1 Firstly ensure that both the interface and the mirror are working properly.

13.1.2 The spectrometer settings should be checked. Ensure that the following are set:

Measurement mode:	Absorbance
Apodization:	SqrTriangle
No of scans:	32
Resolution:	2.0
Range (cm^{-1}):	
Minimum	2300
Maximum	2900

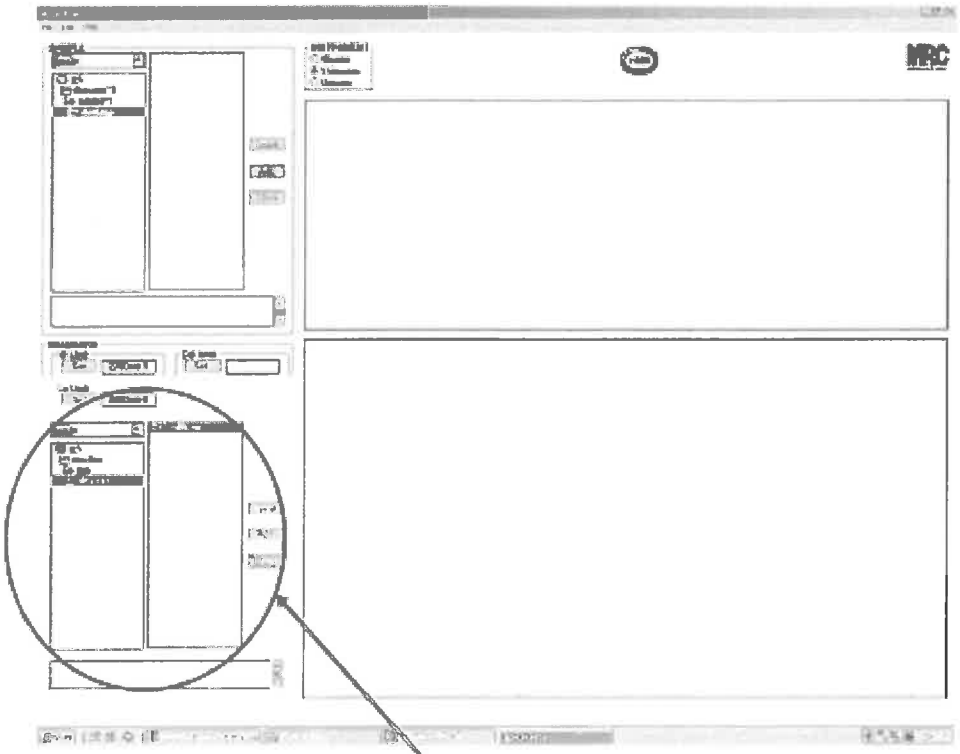
13.1.3 The 'Background' unenriched (natural abundance) water spectrum can now be taken.

13.1.4 Next prepare to calibrate the instrument. Use the calibration standard with deuterium enrichment about 1000 mg/kg (ppm) deuterium oxide. Obtain the spectrum. Remember to set a suitable filename. The peak due to deuterium should have a maximum at about 2510cm^{-1}.

There will be interference from CO_2 on the low energy (right hand) side of the peak. This might be either positive or negative (in the example shown it is positive).

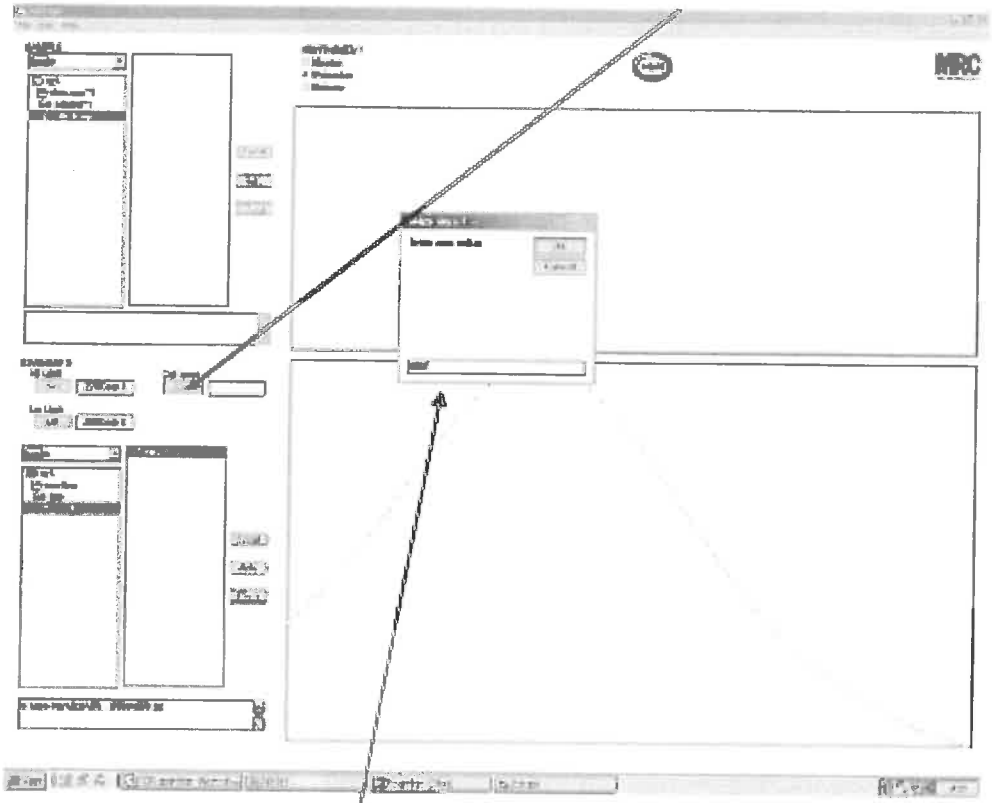
13.1.5 This spectrum should now be saved to disc in a form that can be read by the special MRC software. Choose 'File' from the main menu bar at the top, and then 'Export'. Choose a filename. Ensure that the exported filetype is 'ASCII Simple Text (*.txt)'. Then click on 'Save'.

13.1.6 Now launch the "isotope.exe" programme.



In the section for the standard select the file that has just been exported, and Load it.

13.1.7 Now set the calibration level. Click on the "Cal level set" button

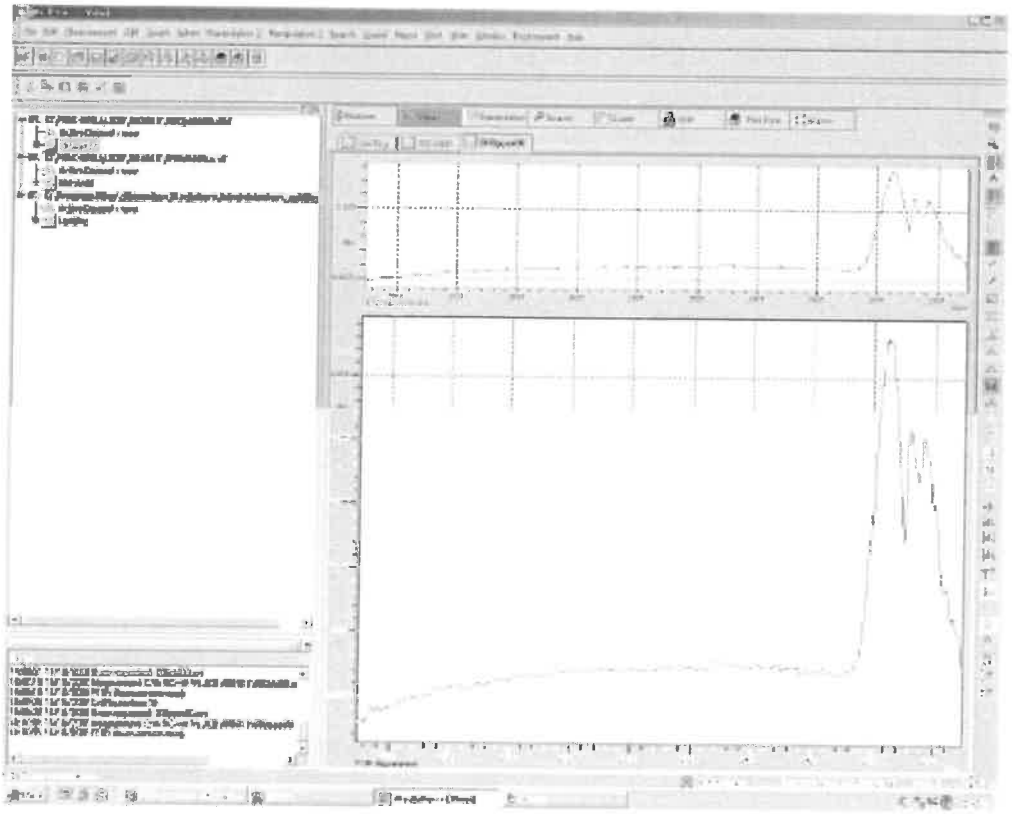


Enter the calculated enrichment of the calibration standard in the dialogue box and then click on "OK".

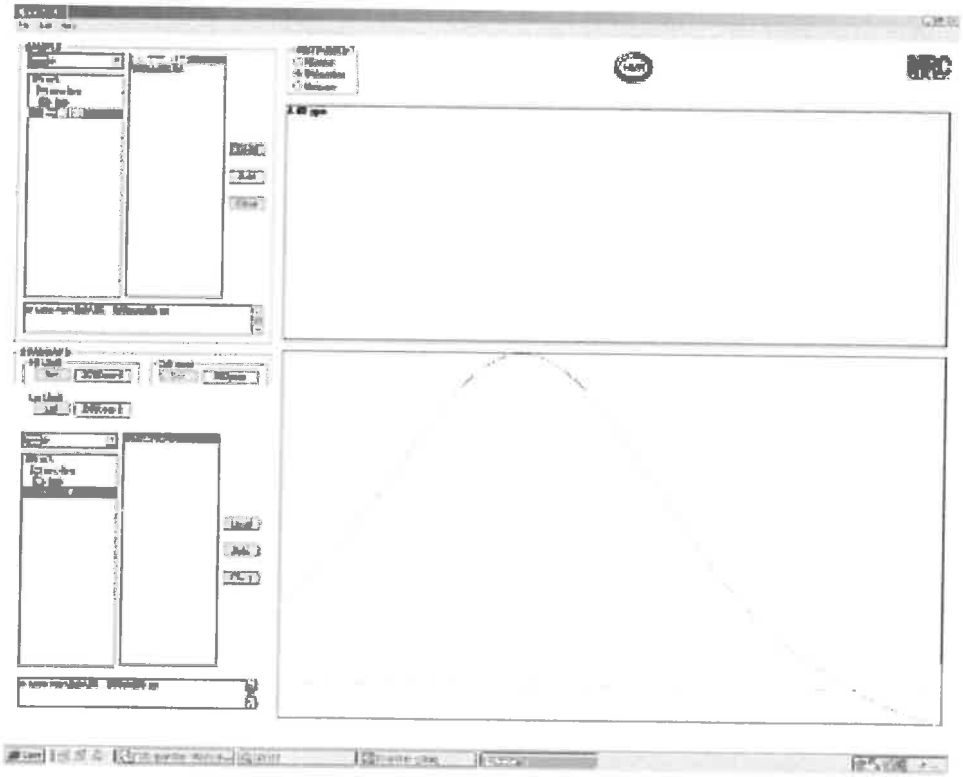
The system is now ready to make measurements of deuterium isotope composition of water or saliva samples.

13.1.8 To check that the reference and calibrant levels have been correctly set the following two steps should be performed.

Refill the sample cell with the background unenriched water sample, and measure it as though it were a sample. The spectrum should be almost featureless, apart from the possibility of CO_2 interference.



Now export this file and read it into the "isotope.exe" software.



The reported concentration of D_2O should be small, in the range −10 to +10 mg/kg (ppm).

This is the most difficult measurement to make since it is heavily influenced by any slight imperfection in sample cell filling.

Now re-measure the calibration standard water in the same way. The answer obtained should be within 1% of the set value (i.e for 1000 mg/kg (ppm) should lie between 990 and 1010 mg/kg.

14.0 Supporting information:

14.1 IAEA Human Health Series No.3. Assessment of Body Composition and Total Energy Expenditure in Humans Using Stable Isotope Techniques.

14.2 IAEA Human Health Series No.7: Stable Isotope Technique to Assess Human Milk Intake in Breastfed Babies.

14.3 IAEA Human Health Series (in press): Introduction to Body Composition Assessment using the Deuterium Dilution Technique with Analysis of Saliva Samples by FTIR

14.4 IAEA distance learning module on analysis of deuterium enrichment by FTIR.

APPENDIX P: Example calculations of body composition using deuterium dilution from the International Atomic Energy Agency

Table 6 shows example data from two adults with the same BMI, but different body composition. An example of data from a child is also shown. The dose of deuterium oxide consumed is lower in children than adults. The hydration of FFM appropriate for the age of the child is used.

TABLE 6. EXAMPLE CALCULATIONS

	Adult 1	Adult 2	Child
Study ID	A001	A002	Child1
Study date	15 Aug. 2007	15 Aug. 2007	15 Aug. 2007
Date of birth	9 Sep. 1944	29 Apr. 1979	1 Apr. 2000
Age (years)	62	28	7
Body weight (kg)	90	90	25
Height (cm)	180	180	130
BMI (kg/m^2)	27.8	27.8	14.8
Dose weight (g) = Weight D_2O (g) consumed	30.03	29.99	10.05
Dose weight (mg) = Dose (g) ×1000	30030	29990	10050
^2H enrichment in saliva (mg/kg)	674	498	610
^2H pool space (V_D, kg) = Dose (mg)/^2H conc. (mg/kg)	44.6	60.2	16.5
Non-aqueous exchange factor	1.041	1.041	1.041

	Adult 1	Adult 2	Child
Total body water (kg) = V_D (kg)/1.041	42.8	57.8	15.8
Hydration factor (adults 0.732; child age 7 female 0.776)	0.732	0.732	0.776
FFM (kg) = TBW (kg)/hydration factor	58.5	79.0	20.4
Fat mass (kg) = Body weight (kg) – FFM (kg)	31.5	11.0	4.6
Fat mass (% body weight) = FM (kg)/ body weight (kg) ×100	35.0	12.2	18.4
FFM (% body weight) = FFM (kg)/body weight (kg) × 100	65.0	87.8	81.6
TBW (% body weight) = TBW (kg)/ body weight (kg) × 100	47.6	64.3	63.3