THE EFFECT OF COLONIC PROPIONATE AND THE ACETATE:PROPIONATE RATIO ON RISK MARKERS FOR CARDIOVASCULAR DISEASE IN WESTERNISED AFRICAN MEN

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DECLARATION OF INDEPENDENT WORK

I, MARTIE MARGARETHA DE WET, identity number [redacted] and student number, 20044941, do hereby declare that this research project submitted to the Central University of Technology, Free State for the degree DOCTOR TECHNOLOGIAE: BIOMEDICAL TECHNOLOGY is my own independent work and complies with the Code of Academic Integrity, as well as other relevant policies, procedures and regulations of the Central University of Technology, Free State; and has not been submitted before to any institution by me or any other person in fulfilment of the requirements for the attainment of any like or other qualification.

_____ _____

SIGNATURE OF STUDENT **DATE**

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LIST OF ABBREVIATIONS

α	Alpha
β	Beta
γ	Gamma
μ	Micro
μT	Mass length ratio from turbidity
Acetyl-CoA	Acetyl coenzyme A
ADA	American Diabetes Association
ADP	Adenosine diphosphate
AIDS	Acquired Immunudificiency Syndrome
ATIII	Antithrombin III
ATP	Adult treatment panel
BMI	Body Mass Index
CAD	Coronary artery disease
CDC	Centers for disease control and prevention
CHD	Coronary heart disease
CH_4	Methane
cm	centimetre
CO_2	Carbon dioxide
CoA	Co-enzyme A
CRP	C-reactive protein
CVD	Cerebrovascular disease
DBP	Diastolic blood pressure
EDTA	Ethylenediaminetetraacetic acid
FBC	Full blood count
FFQ	Food frequency questionnaire
FM	Fibrin monomer

FVII	Factor VII
FVIII	Factor VIII
g	gram
g/L	gram per litre
H^+	ionic hydrogen
H_2O_2	hydrogen peroxide
HCT	Haematocrit
HDL-C	High-density lipoprotein cholesterol
HIV	Human immunodeficiency virus
Hb	Haemoglobin
IFG	Impaired fasting glucose
IHD	Ischaemic heart disease
JNC	Joint National Committee
kg	Kilogramme
KKV	Kort ketting vetsure
LDL-C	Low-density lipoprotein cholesterol
Max	Maximum
m	meter
Min	Minimum
MI	Myocardial infarction
ml	millilitre
mmHg	millimetre mercury
mmol/L	Millimole per litre
MS	Metabolic syndrome
NaOH	Sodium hydroxide
NCEP	National Cholesterol Education Programme
NEFA	Non-esterified fatty acids
NFC	Network fibrin content

NSP	Non-starch polysaccharides
PAI-1	Plasminogen activator inhibitor-1
pH	Percentage hydrogen
PLT	Platelets
RBC	Red blood cells
SANDF	South African National Defence Force
SBP	Systolic blood pressure
SCFA	Short-chain fatty acid
SCFAs	Short-chain fatty acids
SD	Standard deviation
THUSA	Transition and Health during Urbanisation in South Africa
TC	Total cholesterol
TG	Triglycerides
TP	Total Protein
tPA	Tissue plasminogen activator
UFS	University of the Free State
USDA	United States Department of Agriculture
VLDL	Very low-density lipoprotein
WBC	White blood cells
WHO	World Health Organisation
X	Mean

APPENDICES

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Appendix D Questionnaire for subjects who completed the study (Tolerance Questionnaire)

SUMMARY

Historically, coronary artery disease (CAD) has been remarkably rare in the black South African population. However, in recent years, major social and demographic changes have taken place in Africa. Large sections of rural black populations migrate to the cities, adopting a more western style of life, with the consequent emergence of a variety of degenerative western diseases. A diet low in saturated fat and high in fibre is regarded as one of the few controllable factors in the prevention of degenerative diseases. Long-term epidemiological and clinical intervention studies indicate that the addition of dietary fibre to the daily diet can be used as a promising therapeutic agent for the control of known coronary risk factors. The exact mechanism through which dietary fibre influences human metabolism, is not yet known. However, it is suspected that the metabolic effects of dietary fibre in humans are significantly influenced by the degree to which the fibre is fermented in the colon. Fermentation of dietary fibre results in the production of the short-chain fatty acids (SCFAs)- acetate, propionate and butyrate. These SCFAs are absorbed in the large gut where they enter the hepatic blood circulation and contribute to a number of metabolic processes, especially in the liver. It has been hypothesised that these SCFAs contribute, at least in part, to the beneficial effects of dietary fibre on human metabolism.

The main objective of the study was to measure the possible effects of the long-term intake of different combinations of colonic release SCFA supplements on lipid, carbohydrate and haemostatic risk markers in westernised African men. The concentration of SCFAs was equivalent to that normally generated by the natural fermentation of 15g of mixed fibres. It is

important to acknowledge that the ratio in which the different acids are produced, differ from fibre to fibre. This aspect was the focus of this study. Two supplements, with diferent acetate:propionate:butyrate ratios, in addition to a placebo supplement were employed for standardisation and control purposes.

The study design composed of a randomised, placebo-controlled, double blinded, clinical research trial. Seventy-five black male volunteers were recruited from the Military Base at Tempe Defence Force, Bloemfontein, and randomly assigned to three different supplementation groups. All subjects received a placebo supplement, sustained for a period of one week after the collection of baseline information and blood samples. A second baseline blood sample was collected from each individual at the end of this period to secure a stable baseline. Subjects were then randomly assigned to three different intervention groups. The three groups included a placebo supplement, an acetate(70%), propionate (15%) and butyrate (15%) supplement, as well as an acetate (50%) and propionate (50%) supplement, sustained for a period of four weeks, following the second baseline. Measurements included that of the general health status (anthropometric measurements, some biochemical parameters and blood pressure), lipid profiles (total cholesterol or TC, low-density lipoprotein cholesterol or LDL-C, high-density lipoprotein cholesterol, or HDL-C, and triglycerides), haemostatic profiles (the plasma fibrinogen concentration, factor VII & VIII activity, fibrin monomer concentrations, and some fibrin network architecture variables), as well as glycometabolic indicators (fasting serum-glucose and -insulin). The experimental supplementation phase was followed by a wash-out period of

one week, during which all subjects consumed placebo supplements.

At baseline, the study group represented a group of black African men without any apparent metabolic or physical abnormalities. All measured variables fell within the normal range. Results from the experimental phase showed that the placebo supplement caused no significant changes in any of the measured variables. The acetate-propionate (50/50) supplement caused a statistically significant decrease in the factor VII activity (from102 ± 7 to 97 ± 7%), ATIII activity (from 114 ± 13 to 108 ± 10 %), the fasting serum-glucose (from 6.65 ± 0.98 to 5.65 ± 0.42 mmol/L), a significant increase in the HDL-C (from 1.21 ± 0.24 to 1.35 ± 0.34 mmol/L) concentration, as well as a clinically insignificant, but statistically significant increase in plasma fibrinogen concentrations (from 2.98 ± 0.96 to 3.04 ± 0.89 g/L) from baseline to the end of supplementation. The high-acetate supplement induced a beneficial decrease in factor VII (from 103 ±14 to 101 ± 6 %) and factor VIII (from 93 ± 13 to 88 ± 6%) activity, ATIII activity (from 109.2 ± 16.0 to 103.0 ± 10%), the network compaction (from 14.2 ± 5 to 13.7 ± 4.0%), as well as the fibrin monomer concentrations (from 13.9 ± 2.2 to 12.1 ± 4 mg/L), during the experimental phase. This group also experienced a beneficial and statistically significant decrease of the LDL-C levels of 16% (from 3.10 ± 0.78 to 2.61 ± 0.94 mmol/L) the fasting serum-glucose concentrations (from 5.41 ± 0.52 to 4.96 ± 0.85 mmol/L), and a concomitant significant decrease in the circulating serum-insulin levels (5.80 ± 0.77 to 1.70 ± 0.36 mmol/L).

Results from this study show that supplementation with different combinations of SCFAs affect human metabolism, and more specific, the metabolism of those markers that are generally associated with the development of

cardiovascular disease. In addition, it is also evident that the specific combination (and respective ratios) in which these SCFAs are used as a supplement, play a significant role in the way it affects human metabolism. It therefore becomes clear that it is not only the presence of a single one of the SCFAs that mediate the overall metabolic effects of dietary fibre on human metabolism, but also the ratio in which they occur relative to each other, that collectively affects human metabolism.

The specific combinations of SFCAs, as applied in this study, did not affect human metabolism to the extent that it could be advised that they be used as therapeutic agents. Further research to establish the most appropriate combination in which these acids can be used to the benefit of cardiovascular disease prevention in human subjects is recommended.

OPSOMMING

Hartvatsiekte was aansienlik ongewoon onder die swart Suid-Afrikaanse populasie. Geweldige sosiale en demografiese verandering in Afrika het onlangs plaasgevind. Groot groepe landelike swart gemeenskappe het na stede migreer en sodoende ook ═nmeer verwesterse lewenstyl aangeleer, wat aanleiding daartoe gee dat verskeie degeneratiewe westerse siektes ook nou in hierdie populasiegroep voorkom. Langtermyn epidemiologiese en kliniese intervensiestudies dui daarop dat die inname van dieetvesel tot ═n mate gebruik kan word as ═n belowende terapeutiese middel vir die voordelige beheer van die algemene koronêre risikofaktore. Die presiese meganisme waardeur dieetvesel menslike metabolisme voordelig beïnvloed is egter onbekend. Daar word wel vermoed dat die metaboliese effek van dieetvesel betekenisvol beïnvloed word deur die mate waartoe die vesel in die kolon mikrobies fermenteer. Fermentasie van dieetvesel lei tot die produksie van die kortkettingvetsure (KKVs) – asetaat, propionaat en butiraat. Hierdie KKVs word in die kolon absorbeer waar dit vervolgens die hepatiese bloedsirkulasie binnegaan en deelneem aan verskeie metaboliese prosesse, veral in die lewer. Die hipotese bestaan dat hierdie KKVs, ten minste gedeeltelik, ═n bydrae lewer tot die voordelige effek van dieetvesel by die menslike metabolisme.

Die doel van hierdie studie was om die moontlike effek van die langtermyn inname van verskillende kombinasies KKV-aanvullings op die lipied-, hemostatiese- en koolhidraat risikomerkers in verwesterse swart mans te toets. Die konsentrasie KKVs in die supplemente was soortgelyk aan dit wat produseer sou word deur die natuurlike fermentasie van 15g gemengde vesel.

Dit is belangrik om waar te neem dat die verhouding vaarin die vetsure produseer word, verskil van vesel tot vesel. Dit is hierdie eienskap wat in die studie aangespreek is. Twee supplemente, waarvan die verhouding van asetaat:propionaat en butiraat verskil, asook ‗n plasebo supplement, vir kontrole- en standardisasiedoeleindes, is aan die respondente toegedien.

Hierdie studie was ‗n willekeurige, plasebo-gekontroleerde, dubbelblinde, kliniese proef. Vyf en sewentig vrywillige Afrikaan mans was by die Tempe Militêre Basis lukraak geselekteer en in drie groepe verdeel. Alle respondente het aanvanklik vir ‗ntydperk van een week die plasebo aanvulling geneem. Bloedmonsters en ander inligting is ingesamel aan die begin en einde van hierdie tydperk om sodoende ‗n meer betroubare basislyn te verseker. Respondente is vervolgens lukraak in drie groepe verdeel. Die drie groepe is onderskeidelik vir ‗n tydperk van vier weke blootgestel aan ‗n plasebo-supplement, ‗n asetaat (70%), propionaat (15%) en butiraat (15%)-supplement, asook ‗n asetaat (50%) en propionaat (50%)- supplement. Veranderlikes wat gemeet is, sluit in die algehele gesondheidstatus (antropometrie, biochemiese veranderlikes en bloeddruk), lipiedprofiele (totale cholesterol of TC, laedigtheidslipoproteïen-cholesterol of LDL-C, hoëdigtheidslipoproteïen-cholesterol, of HDL-C, asook die trigliseriedes), hemostatiese profiele (die plasmafibrinogeen konsentrasie, faktor VII & VIII aktiwiteit, fibrienmonomeer konsentrasie, asook sommige fibriennetwerk struktuur veranderlikes), asook glikometaboliese veranderlikes (vastende serum-glukose en -insulien). Die eksperimentele fase is gevolg deur ‗neen-week uitwasfase. Gedurende hierdie fase is al die respondente aan plasebo supplementasie ondewerp.

Met aanvangs van die studie was die studiegroep verteenwoordigend van ‗n groep swart mans sonder enige metaboliese of fisieke abnormaliteite. Al die veranderlikes wat gemeet is, was binne die normale reikwydte vir die gegewe veranderlike. Volgens die resultate van die eksperimentele fase het volgehoue supplementasie met die plasebo geen betekenisvolle veranderinge in enige van die gemete veranderlikes aangebring nie. Die asetaat-propionaat (50/50)- aanvulling het gepaard gegaan met ‗n statisties betekenisvolle verlaging in faktor VII aktiwiteit (van 102 ± 7 tot 97 ± 7%), ATIII aktiwiteit (van 114 ± 13 tot 108 ± 10%), vastende serum-glukose konsentrasie (van 6.65 ± 0.98 tot 5.65 ± 0.42 mmol/L), asook ‗nbetekeninsvolle verhoging in die HDL-C konsentrasie (van 1.21 ± 0.24 tot 1.35 ± 0.34 mmol/L), asook ‗n statisties betekenisvolle, maar klinies nie-betekenisvolle verhoging in die plasmafibrinogeen konsentrasies (van 2.98 ± 0.96 tot 3.04 ± 0.89 g/L) vanaf die aanvang tot aan die einde van die vier weke intervensie. Die hoë-asetaat aanvulling het ‗nvoordelige verlaging in faktor VII (van 103 ±14 tot 101 ± 6%) en faktor VIII (van 93 ± 13 tot 88 ± 6%) aktiwiteit, ATIII aktiwiteit (van 109.2 ± 16.0 tot 103.0 ± 10%), die netwerk kompaksie (van 14.2 ± 5 tot 13.7 ± 4.0%), asook die fibrienmonomeer konsentrasies (van 13.9 ± 2.2 tot 12.1 ± 3.6 mg/L), gedurende die eksperimentele fase tot gevolg gehad. Hierdie groep het ook ‗n voordelige en statisties betekenisvolle verlaging van 16% in die LDL-C konsentrasie (van 3.10 mmol/L ± 0.78 tot 2.61 ± 0.94 mmol/L), die vastende serum-glukose konsentrasies (van 5.41 mmol/L ± 0.52 tot 4.96 ± 0.85 mmol/L), en ‗n gepaardgaande betekenisvolle verlaging in die sirkulerende serum-insulienvlakke (5.80 mmol/L ± 0.77 tot 1.70 ± 0.36 mmol/L) getoon.

Volgens die resultate van hierdie studie is dit beduidend dat aanvulling met verskillende kombinasies van KKVs wel die menslike metabolisme beïnvloed. Dit kan veral die metabolisme van daardie merkers wat in die algemeen met kardiovaskulêre siektes geassosieer word, voordelig beïnvloed. Verder is dit ook duidelik dat beide die aanwesigheid van een or meer van die KKVs, asook die spesifieke kombinasie waarin hierdie KKVs as ‗n supplement aangewend word, ‗n beduidende rol speel in die wyse waarop dit menslike metabolisme beïnvloed. Dit spruit dus duidelik hieruit dat dit nie net die teenwoordigheid van ‗n enkele KKV is wat die algehele metaboliese effek van KKVs bemiddel nie, maar ook die verhouding waarin dit relatief tot mekaar voorkom wat gesamentlik die menslike metabolisme beïnvloed.

Die spesifieke kombinasies van KKVs, soos in die studie aangewend, het nie menslike metabolisme in so ‗n mate beïnvloed dat dit aanbeveel kan word om as ‗n terapeutiese middel gebruik te word nie. Verdere navorsing om die mees geskikte kombinasie te vind waarin hierdie vetsure tot voordeel vir die voorkoming van kardiovaskulêre siektes in mense aangewend kan word is nodig.

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Cerebrovascular disease (CVD) and coronary heart disease (CHD) are among the most important causes of morbidity and mortality amongst South Africans and the people of the western world (Bradshaw *et al*., 2005). Cardiovascular disease is the leading cause of disability and death in developed nations and is increasing in prevalence throughout the developing world (WHO, 2002). Murray & Lopez (1996) reported that, world-wide, Ischemic Heart Disease (IHD) was the highest cause of death in 1990 and estimated that it will remain the highest cause to 2020, while CVD was and will be the second highest cause (WHO, 2002). The South African National Burden of Disease study for the year 2000 estimated that 17% of all deaths were due to cardiovascular disease (Bradshaw *et al*., 2003). Cardiovascular disease is a major health problem in all population groups of South Africa, contributing 23%, 41%, 31% and 52% of the total age-standardised death rates in the African, white, coloured an Indian population groups, respectively (Bradshaw *et al*., 2006).

In recent years, major social and demographic changes have taken place in Africa, with large sections of rural African populations migrating

to the cities, adopting a more western style of life, and being exposed to vascular risk factors with respect to diet and level of physical activity, with the consequent emergence of a variety of degenerative ~~"~~first-world" diseases (Steyn *et al.*, 1991; Seedat *et al*., 1992; Bourne *et al.*, 1993; Mollentze *et al*., 1995; Solomons & Gross, 1995; Murray & Lopez, 1997; Kruger, Venter & Vorster, 2003; Ntyintyane *et al*., 2008). The health status of black South Africans, compared to other middle-income countries, is poor (Bradshaw, Masiteng & Nannen, 2000). The incidence of the western diseases, atherosclerosis, CHD and CVD is progressively rising in black populations in South Africa (Mollentze *et al.*, 1995; Kahn & Tollman, 1999; Bourne, Lambert & Steyn, 2002; Schutte *et al*., 2004; Vorster *et.*, 2007).

Prevention and cost-effective management of risk factors for CVD and CHD patients require an appropriate modification of life-style: avoidance of tobacco smoking, participating in regular exercise, and a health-promoting diet (Third Report of the National Education Program (NCEP) on detection, evaluation, and treatment of high blood cholesterol in adults, 2002; Erhardt, 2007).

A diet low in saturated fat and high in fibre is regarded as protective against the onset of degenerative diseases. It is suspected that the mechanism throught which diet acts, is partially mediated by its effect on the identified coronary risk factors (hyperinsulinaemia, hyperlipidaemia, hypertension, obesity, etc.) as well as raised clotting factors (Vorster, Cummings & Veldman, 1997; Ginsberg *et al*., 1998; Anderson & Hanna, 1999; Brown *et al*., 1999; Chobanian *et al.*, 2003).

The hypocholesterolaemic effect of dietary fermentable plant fibres, such as oat bran, has been attributed to, in part, to short chain fatty acids (SCFAs) generated during gut fermentation of these fibres (Bridges *et al.,* 1992; Roberfroid, Gibson & Delzenne, 1993; Anderson, 1995). In rats, fermentation derived SCFAs are well absorbed and have been suggested to suppress cholesterol synthesis in the liver (Hara *et al.*, 1999). In addition to their hypocholesterolaemic effects, absorbed SCFAs have been reported to have a beneficial effect on important factors that play a role in the coagulation cascade, including plasma fibrinogen and resulting fibrin network structure (Veldman *et al.*, 1997; Veldman *et al.*, 1999).

The *in vivo* mechanisms through which SCFAs affect lipid metabolism and haemostasis are poorly understood and in some instances, controversial. In its physical form, it is reported that dietary fibre and bile acids chelate and wash out cholesterol from the gut (Anderson *et al.*, 1991). Yet, additional studies suggest that the production and absorption of SCFAs, such as propionate, resulting from dietary fibre fermentation, would have a direct impact on reducing cholesterol synthesis in the liver (Anderson, 1995; Hara *et al.*, 1999). Some authors suggest that it is not the presence of these SCFAs alone, but rather the ratio in which they are produced and absorbed that determines the impact they have on lipid metabolism (Wolever, Fernandes & Rao, 1996). Serum acetate and -propionate concentrations are related to serum total cholesterol levels in men (Wolever, Fernandes & Rao, 1996) and the authors also suggest that

the ratio of these two products may predict the risk for CVD. Whether this is true for haemostatic risk profiles is yet to be established.

The effects of both dietary derived SCFAs and food grade SCFA supplements on lipids metabolism and haemostatic risk factors have previously been examined (Veldman *et al.*, 1997; Veldman *et al.*, 1999). Veldman *et al.* (1999) applied colonic release capsules to simulate SCFA release in the large intestine and directly monitored their effects on lipid and haemostatic risk markers in human volunteers over a period of four weeks. The main shortcoming of this study was the absence of propionate and butyrate from the supplement. In the study by Veldman *et al* (1999), supplementation caused a statistically significant reduction in serum cholesterol levels in hypercholesterolaemic subjects (Veldman *et al.*, 1999). Yet, the clinical significance of the decrease in serum total cholesterol was not sufficient to promote the use of acetate supplementation as a cholesterol-lowering agent. Yet, these results strongly suggest that the beneficial effect of soluble dietary fibre intake could only be partially mediated by the production of acetate and that the addition of both butyrate and propionate should be investigated further. In the present study, the effect of the addition of butyrate and propionate, in different proportions, to colonic release acetate capsules, was used to measure their long-term effects on haemostatic and lipid profiles in westernised African men. It is envisaged that the results from this study could provide information to better understand the protective properties of dietary fibre against the development of both CVD and CHD in

humans. Such information is also likely to be used in future for the development of short-chain fatty acid supplements that provide similar protection.

1.2 MOTIVATION

Cerebrovascular disease (CVD) and coronary heart disease (CHD) are among the most important causes of morbidity and mortality amongst South Africans and other people of the western world. A new political and socio-economic environment in South Africa allows for the mass movement of individuals from rural to urban and semi-urban areas, where they are exposed to factors that we collectively use to define as a westernised lifestyle. The westernised lifestyle introduces new metabolic abnormalities, such as CHD and CVD, that these population groups, in the past, enjoyed protection against through a variety of known and unknown mechanisms. Medical intervention for the treatment of CHD and CVD is expensive. The development of cheap alternative and natural methods for both the treatment and prevention of CVD and CHD is therefore, a research priority.

Previous research shows how the addition of dietary fibre to any diet can significantly improve the metabolic risk profiles that are associated with a westernised lifestyle. Yet, not all fibres exert exactly the same metabolic response. In general, the improvements include changes in the lipid, carbohydrate and protein metabolism, as well as weight loss and other physico-chemical effects that are suspected to influence

health and disease. It is believed that some of these beneficial effects may be mediated, at least in part, by the ratio of SCFAs produced during the microbial fermentation of dietary fibre in the large intestine. Not all fibres produce the same ratio of short-chain fatty acids. All short-chain fatty acids are absorbed and metabolised in the liver, where it partakes in a number of important metabolic cycles and processes, including those that affect CHD and CVD risk.

A typical westernised diet is low in dietary fibre. It is suspected that a supplement of short-chain fatty acids could, at least in part, provide protection against the development of CHD and CVD in individuals that follow a typically western diet, with a low fibre intake. These acids are easy to produce, inexpensive and available as a stable powdered crystal salt. The development of SCFA supplements would therefore, be appropriate if the intake of SCFAs holds any potential clinical benefit to human health. Yet, it is important to investigate the most appropriate ratio in which these acids should be employed.

1.3 HYPOTHESIS

The hypothesis tested in this study states that:

H_0 - The addition of propionate and butyrate in different quantities to colonic release acetate supplements will not significantly affect the effects of the supplement on haemostatic, lipid and glycomatebolic profiles of westernised African men.

H_1 - The addition of propionate and butyrate in different quantities to

colonic release acetate supplements will significantly affect the effects of the supplement on haemostatic, lipid and glycometabolic profiles of westernised African men.

1.4 AIM AND OBJECTIVES

1.4.1 Aim

The main aim of the study was to measure the possible effects of the long-term intake of different combinations of colonic release SCFA supplements on lipid, carbohydrate and haemostatic risk markers in westernised Black men. The concentration of SCFAs was standardised as equivalent to that normally generated by the natural fermentation of 15g of mixed fibres.

1.4.2 Objectives

The objectives of the study are:

- To investigate whether the exposure to different combinations of SCFAs orally administered, will have beneficial effects on the hamostatic, lipid and carbohydrate risk markers in westernised Black men. One of the supplements contained a mixture of acetate, propionate and butyrate in a ratio of 70% acetate, 15% propionate and 15% butyrate. The other supplement contained only a mixture of acetate and propionate, in a ratio of 50% acetate and 50% propionate.

- To investigate whether the ratio of acetate to propionate, as well as the absence or presence of butyrate may evoke a collaborative physiological response.

1.5 STRUCTURE OF THE THESIS

In Chapter 1 of this thesis, a short discussion is used to elucidate the rationale on which the hypothesis of this study is based. Chapter 2 provides an extensive literature survey, in which the most critical information needed to understand and interpret the hypothesis and the results of this study, is examined. In Chapter 3, a comprehensive description of the experimental methods used in the study is provided. The results of the study are reported in Chapter 4 and Chapter 5 discuss the results and compare it to previous relevant studies. Finally, in Chapter 6, conclusions and recommendations are made based on the findings reported inChapter 5.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

Coronary heart disease (CHD) and stroke are considered as degenerative western diseases which in most cases, can be prevented by a change in lifestyle, for example diet, physical activity and psychological well-being. Today, diet plays an integral position in the control of atherosclerosis, which relates to general vascular disease, CHD and stroke, especially in westernised cultures with an unhealthy lifestyle (Yussaf *et al*., 2001).

It was estimated that approximately 75% of the South Africa population would be urbanised by the turn of 2000 (Mollentze *et al.*, 1995). Richter, Norris & Swart (2006) confirmed that South Africa has reached comparatively high levels of urbanisation and the African urban and rural metropolitan population is projected to more than double between 1990 and 2010. Of all ethnic groups, the Black South African (African) group has the lowest prevalence of diseases related to lifestyle, when compared to any other groups, such as the Indian and White population groups. This phenomenon is changing, taking into consideration the adaptation of the African population to a westernised lifestyle, concomitant to urbanisation. In general, rural African groups followed a highly prudent, low-fat, high-fibre diet. Urbanisation and consequent

adoption of a western lifestyle also includes a change in diet. The western diet with its high-fat, low-fibre content already seems to increase the incidence of diseases of lifestyle among the African population group (Steyn *et al.*, 1987; Steyn, Fourie & Bradshaw, 1992; Mollentze *et al*., 1995; Jenkins *et al*., 2001; Vorster *et al*., 2007). In the early 2000s an additional factor brought the reality of raised CHD risk into the lives of the South African community. The prevalence of HIV and AIDS in the African population is considered to be amongst the highest in the world. The treatment of Human immunudifficiency virus (HIV) and Acquired immunodeficiency syndrome (AIDS) by means of antiretroviral therapy introduces metabolic changes that simulate the metabolic profile of those with increased CHD risk, such as hypercholesterolaemia, hypertriglyceridaemia and insulin resistance (Carr *et al*., 1999). Yet, it is unknown whether the cardiovascular side-effects associated with the long-term use of these drugs would cause similar effects in the presence of the HIV, something which only time would tell (Hadigan *et al.*, 2001; Samaras *et al.*, 2007).

CHD is preventable. It is important to recognise that CHD is a multi-factorial disease and manifest as a single effect with many causes (Krummel, 2008). Some of these factors (such as those we associate with lifestyle) can be controlled, by either lifestyle changes or medical intervention, others not (such as genetic predisposition). In addition, those lifestyle factors that directly affect the development of CHD have been well investigated. Prevention of CHD therefore, depends mostly upon lifestyle intervention, especially in the early phase of its development (Erhardt, 2007; Giugliano, Ceriello & Esposito, 2008). This strong link between CHD and lifestyle serves as the major force

behind the large amount of research performed to elucidate the interplay between lifestyle factors and metabolic profiles in human subjects. The National Cholesterol Education Program's Adult Treatment Panel III (NCEP ATP III) report viewed cardiovascular disease as the primary outcome of metabolic syndrome (NCEP, 2002). Most individuals who develop CHD have multiple risk factors. Mancia (2006) noted that several risk factors (eg. dyslipidaemia, hypertension, hyperglycemia, obesity) commonly cluster together. The metabolic syndrome is a particularly well-characterised example of co-excisting cardiovascular risk factors that is increasing at an alarming rate (Elabassi & Haddad, 2005). Grundy *et al.* (2004) reviewed that the metabolic syndrome can be identified by six components that relate to CHD: abdominal obesity, atherogenic dyslipidaemia, raised blood pressure, insulin resistance or glucose intolerance, proinflammatory and prothrombotic state (NCEP, 2002). The ATP III report considered the metabolic syndrome an indication for intensive lifestyle change (NCEP, 2002).

As a facet of lifestyle, dietary intervention has been a major topic of investigation. A series of recent scientific statements support this and recommend the modification of atherogenic diets as a major lifestyle intervention to reduce metabolic risk (Grundy *et al*., 2005). The prudent or low-fat, high-fibre diet is regarded by some authors as an effective tool in exercising control over CHD risk (Vorster, Cummings & Veldman, 1997; Jenkins *et al.,* 2002a; NCEP, 2002). It is important to note that the degree of control should have clinical significance, rather than to hold just statistical significance.

Protection against CHD provided by the prudent diet mainly seems to centre

on the decrease in saturated fats and on the role of dietary fibre. Dietary fibre is fermented in the large intestine to the short-chain fatty acids, acetate, propionate and butyrate (Veldman *et al.*, 1999). It is not only the individual acids, but also the combination in which they are produced and absorbed, that is believed to play an integral role in the way they act on human metabolism. A number of research trials have reported that a combination of the three major SCFAs (acetate, propionate and butyrate) in different ratios may directly affect metabolic CHD risk factors (Demignè *et al*., 1995; Veldman *et al.*, 1997; De Wet, 2000). However, in some instances results were controversial and could not be compared amongs others differences in study design, route of administration, concentrations of the acids used and duration of supplementation. These are all factors that have to be taken into consideration.

The following section will be used as an overview of those metabolic CHD risk markers that respond to lifestyle intervention, with special emphasis on diet. Other factors that do not respond to lifestyle intervention, such as genetic predisposition, will not be discussed here. In addition, the author provides an overview of those mechanisms suggested to link the production of short-chain fatty acids and CHD risk. The literature should supply the reader with a sufficient background to interpret the results of this study and the discussion at the end of the thesis.

2.2 TERMS AND DEFINITIONS

Terminology and operational definitions used in this thesis are explained here as a term of reference, for understanding the contents of this thesis.

2.2.1 Atherosclerosis

Atherosclerosis, a form of arteriosclerosis, is a complex process of thickening of the walls of arteries, particularly those of small and medium size, reducing their inside diameter and the flow of blood by the accumulation of lipids, primarily of oxidised low-density lipoprotein cholesterol (LDL-C), in the intimal or inner layer in combination with connective tissue and calcification (Meydani, 2001; Krummel, 2008).

2.2.2 Thrombosis

Thrombosis is the formation of a blood clot, or thrombus, inside a blood vessel. Forming a clump of various blood cells, the clot remains attached at its point of formation, partially or completely blocking the flow of blood through the vessel (Krummel, 2008).

2.2.3 Coronary Heart Disease

Coronary Heart Disease (CHD) or Coronary Artery Disease (CAD) is a disease involving the network of blood vessels surrounding and serving the heart. CHD is in most instances caused by the obstruction of these vessels by means of the process of atherosclerosis or thrombosis, singly or in combination, manifested in clinical end points of myocardial infarction, sudden death and angina pectoris (Krummel, 2008).

2.2.4 Stroke

Stroke is a localised neurological blood shortage due to a vascular lesion, categorised by a sudden loss of cerebral function with coma due to bleeding, thrombosis or embolism of a cerebral artery (Edlin, Golantly & Brown, 1998).

2.2.5 Myocardial Infarction (MI)

When thrombosis occurs in an artery, the tissues that the artery normally supplies with blood suffer infarction — that is, they die from a lack of blood-borne nutrients. Coronary thrombosis, the blockage of an artery that supplies blood to heart muscle, results in a myocardial infarction, or heart attack (Krummel, 2008).

2.2.6 Cerebrovascular Disease (CVD)

A cerebrovascular disease is any disease that affects an artery within the brain, or that supplies blood to the brain. The most common cerebrovascular disease is atherosclerosis, where plaques (fatty deposits) form, leading to narrowing of the arteries. There may also be a defect or weakness in a blood vessel in the brain, which can cause an aneurysm (ballooning of an artery) (Kumar, Abbas & Fausto, 2005).

2.2.7 Dietary fibre

A variety of systems exists that are used to classify dietary fibre. Dietary fibre is the substance that remains after treating plant material with human digestive-tract enzymes and reduction with acid and alkali (Ettinger, 2004). Carbohydrate rich foods can be classified into available carbohydrates which are digesed and absorbed in the small intestine and resistant carbohydrates which resist digestion in the small intestine or are poorly absorbed or metabolised. The most prominent are the non-starch polysaccharides (NSP) from plant cell walls, which are characterised of the largely unrefined plant foods (Englyst, Lui & Englyst, 2007). Insoluble fibre is not digested and is found in

vegetables, whole-grain products, and bran. It provides roughage that speeds the elimination of faeces. Soluble fibre, in contrast, is digested in the colon, by means of bacterial fermentation, that result in the formation of methane, hydrogen, carbon dioxide, and the respective short-chain fatty acids (SCFAs). These short-chain fatty acids, which include acetic, propionic, and butyric acid, are absorbed into the hepatic blood circulation. SCFAs are also known as volatile fatty acids (Cummings, Macfarlane & Englyst, 2001).

2.2.8 CHD Risk Factor versus CHD Risk Marker

Risk Factors represent both lifestyle and metabolic aspects that are believed to contribute to the development of CHD, for example diet and diabetes mellitus. Risk Markers represent those specific measurable metabolic variables in the patient‘s blood, generally associated with an increased risk of developing CHD and stroke, for example plasma fibrinogen and serum total cholesterol (Last, 1988).

2.2.9 Urbanisation

Urbanisation represents a shift of people from rural areas to cities and the resulting growth of urban areas (Le Roux & Le Roux, 1991).

2.2.10 Westernisation

Westernisation is an assimilation of western culture; the social process of becoming familiar with or converting to the customs and practices of Western civilisation (Oxford Dictionary, 2002).

2.2.11 Metabolic Syndrome

The National Cholesterol Education Program's Adult Treatment Panel III (ATPIII) identified the metabolic syndrome as a multiplex risk factor for cardiovascular disease that is deserving of more clinical attention (Grundy *et al.*, 2004). The core components of the metabolic syndrome include abdominal obesity, insulin resistance or glucose intollerance, atherogenic dyslipidaemia, prothrombotic state and hypertension (Grundy *et al.*, 2004; Alberti, Zimmet & Shaw, 2006).

2.3 METABOLIC RISK MARKERS FOR ATHEROSCLEROSIS AND THROMBOSIS

2.3.1 Introduction

Cerebrovascular disease (CVD) and coronary heart disease (CHD) are important causes of morbidity and mortality in the western world (WHO, 2002), including South Africa (Bradshaw *et al*., 2003). CHD is the prime cause of death among the South African white, coloured and Indian populations. The South African National Burden of Disease study for the year 2000 estimated that 17% of all deaths were due to cardiovascular disease (Bradshaw *et al*., 2003). The recent cause-of-death statistics show different patterns in the cardiovascular disease among population groups, with ischaemic heart disease more common among the white and Indian groups and stroke more common among black Africans (Statistics South Africa, 2007). Historically CHD has

been remarkably rare in black South Africans (Seftel, 1978). Although the black population still have the lowest CHD mortality rate in South Africa, some studies report a change in this phenomenon (Steyn *et al*., 1991, Mollentze *et al*., 1995; Jenkins *et al*., 2001; Vorster *et al*., 2007; Ntyntyane *et al*., 2008), as ongoing demographic transition and aggressive marketing of unhealthy consumer products diminishes the apparent protection against CHD they once experienced. It is this change in lifestyle and associated increase in CHD risk that makes one realise the association between them and provides an opportunity to elucidate mechanisms based on investigating population groups undergoing these changes. CHD is a multifactorial disease influenced by a mixture of genetic factors, physiological factors (such as metabolism of the arterial wall), humoral factors (lipids, lipoproteins and the complex mechanisms of blood clotting) and those associated with lifestyle (stress and similar psychological factors, cigarette smoking, diet and exercise) (Kritchevsky, 1994; NCEP, 2002; Greenland *et al*., 2003; Krummel, 2008), which will be discussed in the following section.

2.3.2 Haemostatic Risk Markers

The haemostatic system is designed to maintain blood in a fluid state under physiological conditions, but primed to react to vascular injury in an explosive manner to stem blood loss by sealing the defect in the vessel wall (Ratnoff & Forbes, 1996). Rapid, localised haemostasis is achieved by complicated systems of activation and inhibition whereby excessive bleeding and unwanted thrombosis is minimised (Hutton, Laffan & Tuddenham, 1999).

2.3.2.1 Plasma Fibrinogen

The first suggestion that raised plasma fibrinogen levels may contribute towards the development of CHD was first voiced in the 1950s (reviewed by Ernst & Resch, 1993). A prothrombotic state, characterised by raised fibrinogen was identified by the ATP III as one of the six components of the metabolic syndrome (NCEP, 2002; Devaraj, Rosenson & Jialal, 2004). Fibrinogen is a soluble glycoprotein, and is present in high concentrations in both plasma and platelet granules (Hutton, Laffan & Tuddenham, 1999; Weisel, 2005). It is the main clotting protein. The soluble protein circulating in the blood provides the source material from which an insoluble fibrin clot is formed during the polymerisation process of blood coagulation (Weisel, 2005). A high plasma fibrinogen concentration in adulthood is associated with an elevated risk of developing CHD and/or stroke (Kannel, 2005; Weisel, 2005; Aleksic *et al*., 2008). Fibrinogen may act through two independent pathophysiologic mechanisms such as thrombosis and inflammation (Best *et al*., 2008). The prospective fibrinogen-CVD association may even be a consequence, rather than a cause, of the disease process, perhaps due to an inflammatory response to progressive endothelial damage. This is why most authors suggest fibrinogen to be a marker of long-term pathophysiological changes (Fibrinogen Studies Collaboration, 2005; Aleksic *et al*., 2008).

Urbanisation and Diet as risk factor for raised plasma fibrinogen levels

The effect of urbanisation and a concomitant change in diet on circulating plasma fibrinogen concentrations in human subjects remains uncertain (James *et al.*, 2000). In addition, only a limited number of studies have investigated the haemostatic risk profiles of black South Africans. However, some authors do report that circulating plasma fibrinogen levels increase as a result of urbanisation (Pieters & Vorster, 2008). Yet, circulating fibrinogen concentration is controlled by many demographic, environmental, dietary, as well as genetic factors (Vorster, Cummings & Jerling, 1997; De Maat, 2001) and it is difficult to single out the effect of one single factor that encompasses so many other variables. It is suggested that 30-50% of the plasma fibrinogen level is genetically determined (De Maat, 2001) while Pieters & Vorster (2008) confirmed that high circulating plasma fibrinogen concentrations were observed in almost all of the studies that reported on black South Africans, despite differences in demographic location. Yet, does this imply that the process of urbanisation and concomitant change in lifestyle itself has no impact on the expression of fibrinogen production? Although dietary components have been shown to affect fibrinogen concentration, the effect seems modest and in some cases, even controversial. Results from the Transition and Health during Urbanisation in South Africa (THUSA) Study indicated that individuals following a prudent diet (low intakes of

animal protein; trans-fatty acids and higher intakes of plant protein and dietary fibre) have lower circulating plasma fibrinogen levels (James *et al.,* 2000).

2.3.2.2 Fibrin Network Formation and Architecture

It is suspected that not only fibrinogen concentration, but also the quality of fibrin networks may directly be related to CHD risk (Scott, 2004; Weisel, 2004; Collet *et al*., 2006). Patients with an elevated plasma fibrinogen concentration have a considerably lower fibrin gel permeability compared with normofibrinogenaemic patients. Fibrinogen is the main clotting protein. It forms an insoluble gel on conversion to fibrin after activation by the proteolytic enzyme thrombin, which itself is activated by a cascade of other enzymatic reactions (Sugo *et al.,* 2006). Some other proteins interact with fibrinogen to form an elastic filamentous fibrin, such as factor XIII, fibronectin, α-$_2$ antiplasmin inhibitor, plasminogen, and plasminogen activator (Weisel, 2005; Weisel, 2007).

When thrombin and fibrinogen interact, fibrin monomers are generated according to the relative amounts of the enzyme and the substrate (Wolberg, 2007; Wolberg & Campbell, 2008; Weisel & Litninov, 2008). Continuing thrombin-catalysed cleavage of the resulting fibrinopeptides from the central domain of fibrinogen leads to the formation of two-stranded polymers of fibrin, termed protofibrils, in a rapid linear bimolecular polymerisation process. This process repeats itself causing an increase in the length of the protofibrils. Additional stability of protofibrils is provided by non-

covalent interactions between the distal D-domains of subsequent fibrin molecules that are aligned in an end-to-end fashion within the same strand of each individual protofibril (Weisel, 2005a; Weisel, 2007; Crawley *et al.,* 2007). Progressive lengthening of the polymer chain occurs by a half-overlap, side-to-side approximation of fibrin monomer molecules. Separate protofibrils then interact laterally, increasing the thickness of the fibrin fibres. The first part of the polymerisation reaction therefore causes lengthening of the fibrin strands (protofibrils), whereas the second part of the reaction causes thickening of the strands. Either this could result in long, thin fibrin strands or short, broad sheets of fibrin. In mature forms, one fibrin fibre contains more or less 100 protofibrils, with a somewhat random pattern of branching that additionally links different fibres together by means of cross-linking (Ratnoff & Forbes, 1996; Weisel, 2007; Chernysh & Weisel, 2008).

Fibrin clot formation, on a molecular level, involves a series of highly ordered biochemical reactions (Sugo *et al.,* 2006):

(i) transition of fibrinogen to fibrin monomers by cleavage of the polypeptide fragments fibrinogen peptide A and B by thrombin,

(ii) construction of half-molecular overlapping linear double-stranded fibrin protofibrils, and

(iii) lateral association of the protofibrils to form thick fibrin fibres, bundles and networks (Sugo *et al.,* 2006).

It is suspected that the degree of lateral strand association probably contributes to the tensile strength of the clot. Clot resistance to plasmin degradation is believed to depend mainly on the degree of cross-linking, a process mediated by Factor XIII (Weisel, 2007). Clot rigidity is mainly determined by individual fibre size within the network, as well as the amount of cross-linking and branch points that form between different fibres within the network (Ryan *et al*., 1999).

The physical and biochemical structure of the fibrin network depends upon the polymerisation conditions (Weisel, 2007). It is known that any given network comprises of a major network of thicker fibres and a minor network of thinner fibres (Nair, Shats & Dhall, 1986; Wolberg & Cambell, 2008). According to Blombäck *et al*. (1992) these gel structures are determined by kinetic factors and modulating factors.

Kinetic factors: The kinetic factors are thrombin and fibrinogen concentrations. Increasing the kinetic factors will result in tighter, less porous networks with thinner fibres and a higher density of nodes. These structures are dense, rigid and flow of a liquid through it is impaired. Conversely, low concentrations of kinetic factors result in porous networks with thick fibres and fewer nodes. These structures are less tightly packed, deformable and plastic and consequently fluid easily escapes through the structure. It is evident that the initial fibrin polymers create nuclei for the growth of linear polymers in different spatial directions. A network structure is thus formed. The faster the activation the larger the density of

polymeric nuclei and the tighter the network structure . The rate of activation of fibrinogen by thrombin will increase significantly with increasing fibrinogen concentration and this leads to a drastic change in the fibrin gel structure (Blombäck *at al.*, 1992).

Modulating factors: The modulating factors include proteins and ions in direct contact with the fibrinogen molecule (Diamond & Anand, 1993). Modulating factors change the architecture of the network by either interacting with the surrounding fluid during fibrin formation or by binding to either fibrinogen or to the fibrin strands in the established network. Modulating factors include:

- fibrinogen composition, non-enzymatic glycosylation, carbohydrate content, and any factors causing a change in primary and/or secondary structure of the molecule (Fatah *et al.*, 1992);
- albumin (Carr, 1987);
- cations, pH and temperature (Blombäck & Okada, 1982);
- blood platelets (Van Gelder, Nair & Dhall, 1993) ;
- plasma antithrombin-III (Elgue *et al.*, 1994) ;
- homo poly (l-amino acids) (Carr, Cromartie & Gabriel, 1989);
- dextran (Dhall, Bryce and Dhall, 1976) ;
- glucose and antidiabetic drugs (Azhar *et al.*, 1990);
- gamma-globulin (Nair *et al.*, 1991a) ;
- fibronectin (Nair *et al.*, 1991b);
- insulin, growth hormone and estrogen (Joubert & Veldman, 2002);
- acetylsalicylic acid (Fatah, 1995) ; and

- a variety of long- and short-chain fatty acids (Mogongoa & Veldman, 2002).

Fibrin network structures can vary, and specific structural types are considered to be associated with an increased risk of CHD. Altered fibrin network architecture has been observed in patients with premature CAD (Collet *et al*., 2006), myocardial infarction (MI) (Fatah *et al*., 1996), and diabetes mellitus (De Maat, 2001; Dunn, Ariens & Grant, 2005), as well as in obese patients (Malan, 1999), when compared to those found in apparently healthy patients. In general, fibrin clots with thinner fibres, more branch points and smaller intrinsic pores are more dense and resistant to lysis and are associated with a raised athero-thrombotic risk (Collet *et al.*, 2000). Clots consisting of thicker fibres generally have larger pore sizes making the clots more permeable to those enzymes that control the breakdown of the clots, in addition to having more plasminogen binding sites, compared to networks having thin and dense fibres (Kemball-Cook, Tuddenham & McVey, 2004; Pieters & Vorster, 2008).

In addition, circulating LDL-C correlates inversely with permeability and the mass-to-length ratio of the fibers (Blombäck *at al.*, 1992). Significant inverse relations, which are independent of plasma fibrinogen or lipoprotein concentrations, are detected between the permeability of plasma generated fibrin networks and mass-to-length ratio within the fibrin fibres and the severity of coronary artery stenosis as determined by angiography (Fatah, 1995). Also,

Blombäck *et al.* (1992) indicated that a grossly abnormal gel structure rather than simply the tightness and rigidity of the architecture is associated with progression of the atherosclerotic process. Since these patients often have elevated plasma fibrinogen levels, an association can be found between tight and rigid fibrin networks *in vitro* and the process *in vivo*. Further research is required to elaborate on these associations.

Urbanisation and Diet as risk factor for altering fibrin network structure

The effect of diet on the fibrin network structure is not well investigated. Pectin, a soluble dietary fibre, was shown to increase the permeability and lysability of the fibrin network and to decrease tensile strength of the fibrin fibres (Veldman *et al*., 1997; Veldman *et al.,* 1999). A number of authors investigated the effect of specific molecules on the fibrin network structure, such as albumin (Nair & Dhall, 1991), anti-thrombin III (Nair & Dhall, 1991), glucose (Nair *et al.*, 1991a; Dunn, Ariens & Grant, 2005; Pieters *et al*., 2008), etc. by means of short-term clinical intervention studies. Yet, no epidemiological data is available to support the hypothesis that those factors that influence CHD risk, also affect fibrin network structure. Studies like these are complex and costly. In addition, methods used to establish fibrin network structure are not well standardised and results could differ due to the lack of external quality control samples. No published information on the role of urbanisation on fibrin network structure is available. Urbanisation is a complex

process that affects lifestyle. It is therefore suspected that dietary changes related to urbanisation may have indirect or direct effects on fibrin network structure, mediated through its effect on circulating fibrinogen concentration and the main kinetic or modulating factors affecting fibrin network structure (Pieters & Vorster, 2008). It is important to investigate the nature through which these mechanisms act. Further studies are also required to investigate the relationship between diet and network structure.

2.3.2.3 Factor VII-activity

Smith *et al.* (2005) reported that factor VII has the potential to increase the prediction of CHD and stroke in middle-aged men. A high blood factor VII-activity predisposes patients to the onset of CHD (Kannel, 2005; Miller *et al*., 2008). Karatela & Sainani (2009) found that raised factor VII levels in patients with CHD were independently associated with the metabolib syndrome. Factor VII forms part of the cascade that results in the activation of prothrombin to thrombin and thus, the eventual conversion of fibrinogen to fibrin (Swales & de Bono, 1993). It seems that hypercoagulability, especially raised plasma fibrinogen levels and factor VII-activity, may play an important role, not only in thrombosis, but also in the development of atherosclerosis and is therefore considered to be an important independent risk factor for the development of CHD (Buzzard *et al*., 1996; Wang *et al*., 2007; Miller *et al*., 2008). Higher levels of Factor VII-activity tend to cluster with additional independent CHD risk factors and is therefore, more pronounced

with increased age, obesity, high serum total cholesterol (TC), high low-density lipoprotein cholesterol (LDL-C), high serum triglycerides (TG), low high-density lipoprotein cholesterol (HDL-C), smoking, a family history of MI, high fasting insulin levels and high fibrinogen levels (Gliksman & Wilson, 1992; Cushman *et al.,* 1996*;* Junker *et al*., 1997; Ishikawa *et al*., 1997; Wang *et al*., 2007). Surgue *et al*. (1985) demonstrated that in a series of patients with heterozygous familial hypercholesterolaemia, the age adjusted mean plasma fibrinogen level and factor VII-activity were significantly higher in patients with symptomatic evidence of CHD, when compared to patients without CHD. Serum-lipid concentration did not differ significantly between the two groups.

Urbanisation and Diet as Risk Factor for raised Factor VII-activity

Very limited data is available on the effect of urbanisation on factor VII-activity. Vermaak *et al.* (1991) compared black and Caucasian subjects who had been exposed to the same environment and western diet for at least 2 years. The authors reported factor VII-activity to be significantly lower (26.6%) in adult Black Africans, compared to the Caucasian study group. Bowman *et al.* (2009) postulated that a strong association between dietary fat intake and dietary cholesterol intake and factor VII-activity (Miller *et al*., 1986). Accordingly, dietary intake of fats could influence the risk of ischaemic heart disease *via* both long-term chronic (atherogenic) and short-term (thrombogenic) pathways. Factor VII activation leads to an increase in thrombin concentration, which intensifies platelet

activation and promote a procoagulatory state. Conversely, it can be assumed that cholesterol-lowering measures also reduce the thrombotic risk associated with a high activity of factor VII-activity (Miller *et al.*, 2008). Furthermore, Tracey *et al*. (1995) and Cushman *et al*. (1996) suggested that factor VII-activity could be modifiable through lifestyle changes, which includes dietary modification, weight reduction and lipid lowering, in both men and women.

2.3.2.4 Factor VIII

Factor VIII and fibrinogen are both required for the cascade of biochemical reactions leading to fibrin formation (Aleksic *et al*., 2008). Since factor VIII is a coagulant favouring clot formation and platelet adhesion, high level might be accepted to be a risk factor for thrombosis and atherosclerosis. Factor VIII is therefore a key procoagulant and like fibrinogen, a plasma protein that participates in the inflammation response (Tracey *et al.,* 1995). However, at present there are limited data from prospective studies of factor VIII as a risk factor for the development of CHD or stroke. Conlan *et al.* (1993) reported a strong association of factor VIII with a number of known cardiovascular disease risk factors. The authors suggested that the association might not be indendent. In the Northwich Park Heart Study, the individuals in the highest tertile group of factor VIII levels were associated with a 44% increase risk for CHD (Meade *et al*., 1986a). Furthermore, a number of studies also report a strong association between factor VIII and a number of established CHD risk markers, such as glucose, insulin, LDL-cholesterol, serum total

cholesterol and serum triglycerides (Gliksman & Wilson, 1992; Conlan *et al*., 1993; Cushman *et al.,* 1996). Pan *et al*. (1997) reported a positive association between factor VIII concentration and atherosclerosis of the carotoid in the Chinese population.

Meade *et al*. (1986b) found that factor VIII was higher in Africans than Caucasians as observed in other studies (Conlan *et al*., 1993). It is suggested that factor VIII levels may be determined predominantly by genetic (ethnic group and ABO bloodgroup status) rather than environmental characteristics.

Urbanisation and Diet as Risk Factor for raised Factor VIII-activity

The effect of urbanisation and the accompanied westernised diet on factor VIII-activity is not well investigated. Van Wyk *et al*. (1998) determined that almost all the haemostatic variables, including factor VIII of San (Bushmen) who were relocated from Namibia to South Africa were statistically significantly lower than those of a Western reference group. In another study, Mezzano *et al*. (2001) found in a randomized clinical trial that levels of factor VII and factor VIII were reduced in men consuming a Mediterranean-type diet rich in olive oil, fish, fruit and vegetables, compared to those on a high saturated fat diet. Nienaber (2006) subdivided a group of 117 black girls and 78 boys, in transition, into subdivision for gender, physical activity, fat percentage and height-for-age. The author found significant changes in some haemostatic variables, but factor VIII showed no differences for any of the subdivisions.

2.3.2.5 Plasminogen activator inhibitor (PAI-1)

A variety of fibrinolytic defects has been implicated in the pathogenesis of thrombosis. These include decreased release of tissue plasminogen activator (Nilsson, Ljugner & Tengborn, 1985) and increased plasminogen activator inhibitor -1 (PAI-1) activity (Hamstern *et al*., 1987). The net effect of each of these abnormalities is impaired functional plasmin generation and a blunted fibrinolytic response to fibrin formation (De Pergola & Pannacciulli, 2002).

PAI-1 is one of the main inhibitors of the fibrinolytic process and is therefore considered to be a potential risk factor for CHD. High PAI-1 activity may especially be used to identify individuals with an increased risk of recurrent myocardial infarction (Hamstern *et al*., 1987). Components of the metabolic syndrome are associated with both coagulation and fibrinolytic proteins, with a link to an elevated PAI-1 being the most consistent (Devaraj, Rosenson & Jialal, 2004).

PAI-1 activity in black South Africans are low in general but with a tendency to increase with urbanisation (Pieters & Vorster, 2008). PAI-1 activity is furthermore consistently associated with blood lipids (Juhan-Vague *et al.*, 1996; Hoekstra *et al.*, 2004) and black South Africans have in general, a favourable lipid profile with low total cholesterol and high HDL-cholesterol (Vorster *et al.*, 2005). However, it is speculated that PAI-1 activity in black South Africans are strongly regulated by genetic factors, although this remains to be proven (Pieters & Vorster, 2008).

Urbanisation and Diet as Risk Factor for raised PAI-1 activity

Apart from metabolic risk markers associated with PAI-1, dietary factors have also been shown to influence PAI-1 levels. The intake of omega-3 fatty acids and alcohol received much attention in this regard. Most studies show that omega-3 fatty acids increase PAI-1 levels but the effect is modest and depends on the type of fat (Hoekstra *et al*., 2004). Lee & Lip (2003) demonstrated that heavy or binge drinking is associated with lower fibrinolytic capacity with increases in PAI-1 levels.

2.3.2.6 Blood Platelets

The development of thrombosis, especially arterial, is greatly influenced by the number of circulating blood platelets. This is important where vessel disease, especially atherosclerosis, is the underlying cause of any manifest pathological state. Platelet hyperaggregability is associated with increased risk of cardiovascular incidence (Mammen, 1999). This is particularly true for sticky platelet syndrome, where platelets show marked hyperaggregability with Adenosine diphosphate (ADP) and/or epinephrine (Mammen, 1995; Frenkel & Mammen, 2003). These patients have an increased risk of developing thrombosis, even while on anticoagulant therapy or with no evidence of other hyperaggregability conditions.

Urbanisation and Diet as Risk Factor for raised Blood Platelet levels

Certain aspects of blood platelet function, blood coagulability, and fibrinolytic activity are associated with cardiovascular risk, but causality has been insufficiently proven. Challen, Branch & Cummings (1983) hypothesed that dietary fibre might protect against the development of ischaemic heart disease through an effect on platelet aggregation and blood clotting. Eleven healthy volunteers took an additional 36g of pectin per day. The study by the above mentioned authors found that platelet aggregation, platelet fatty acid composition, dilute blood clot lysis time and bleeding times were unaltered during the study. They concluded that if dietary fibre does protect against heart disease it is probably not through an effect on platelet aggregation or haemostasis.

Aoki *et al*. (2006) investigated the cellular mechanism of a prothrombotic state in spontaneously atherogenic rodents kept on a Western-style high fat diet. These findings show that a high fat diet-induced prothrombotic state, endothelial dysfunction precedes both the morphologically detectable lesions and the enhancement of platelet reactivity. Furthermore, Mezzano *et al*. (2003) also found that a Mediterranean-type diet, high in fruit and vegetables and low in saturated fats had longer bleeding times as a result. The longer bleeding times in individuals on the Mediterranean diet, denotes less interaction of platelets with the vascular wall, which could be beneficial from the point of view of cardiovascular risk.

2.3.3 Blood Lipids

The value of abnormal blood lipids and apo-lipoprotein levels as predictors of CHD and mortality has been studied for decades. In general, research has focussed mainly on the role of circulating total cholesterol (TC) in relation to cardiovascular disease, but it has shifted to also include the individual components and fractions that collectively represent circulating total cholesterol (TC). Over the last 30 years, these fractions of blood cholesterol, as well as their respective carrier proteins, or lipoproteins, have also come to the forefront as important predictors of CHD risk (Krummel, 2008).

TC is a composite measure of the cholesterol content of lipoprotein particles, mainly represented by low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) (Norman *et al.,* 2007). The Third Report of the Expert Panel on Detection and Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III, or ATPIII) reaffirms that lowering blood-TC and -LDL-C reduces the risk of developing CHD (NCEP, 2002). It is estimated that a 10% reduction in serum-TC decreases CHD incidence by at least 30% (CDC, 2001). Yet, whereas TC was previously recommended as a screening tool, ATPIII now also recommends a complete lipoprotein profile for screening purposes (NCEP, 2002). The dyslipidaedemic patterns of high levels of LDL-C, associated with or without low levels of HDL-C, have been found to impart high risk for developing IHD, and justify treatment to reduce the overall risk for the development of CHD (NCEP, 2002).

LDL-C is the primary cholesterol carrier in the blood, a powerful atherogenic lipoprotein and consequently, total cholesterol levels and LDL-C levels are highly correlated. The positive relationship between serum cholesterol levels and the development of first or subsequent attacks of CHD is observed over a broad range of LDL-C levels: the higher the level, the greater the risk (Fletcher *et al.,* 2005; Marcovina and Packard, 2006; AHA, 2006). However, it is also believed that the risk associated with LDL-C is diminished when associated with high serum-HDL-C concentrations (Kaplan *et al*., 1995). Thus, high HDL-C levels conversely reduced risk. Epidemiological data taken as a whole signify that a 1% decrease in HDL-C is associated with a 2-3% increase in CHD risk. Epidemiological studies consistently show low HDL-C to be an independent risk factor for CHD (Assmann *et al.,*1996, NCEP, 2002) Cooney *et al*. (2009) confirmed an inverse, independent, strong and graded relationship between HDL-C concentration and CHD. A low HDL-C level correlats with elevations of serum triglycerides and LDL-C (Lichtenstein *et al.,* 2006*)* The LDL-C:HDL-C ratio therefore, also serves as a strong predictor of cardiac events (Hermansen *et al*., 2003).

Assmann *et al.* (1998) and Austin, Hokanson & Edwards (1998) reported a positive relationship between serum triglyceride levels and incidence of CHD. Lipoprotein metabolism is integrally linked, and elevation of serum triglycerides can be confounded by significant correlations with TC, LDL-C and HDL-C levels (Grundy, 1998; NCEP, 2002). Mazza *et al.* (2005) indicated that the combination of high

triglyceride and low HDL-C levels quadrupled the risk of CHD mortality in elderly women. According to Grundy *et al*. (2004), raised triglyceride levels and low concentrations HDL-C that manifest from routine lipoprotein analysis, can be classified as atherogenic dyslipidaemia. ATP IIII defined atherogenic dyslipidaemia as one of the components of the metabolic syndrome (NCEP, 2002). Since the metabolic syndrome comprises accepted CHD risk factors, it would be expected that the syndrome is a strong predictor for CHD (Alberti, Zimmet & Shaw, 2006).

Urbanisation and Diet as Risk Factor for abnormal Blood Lipid Profiles

In a local study (THUSA) it was demonstrated that, although the serum-TC levels of urban Africans are lower in comparison to other population groups, professional urban Africans have significantly higher serum-TC levels, when compared to their own rural counterparts (Vorster, 2002). According to Norman *et al.* (2007) the serum-TC levels of young black South Africans were more similar to that of other population groups, when compared to that of older black South Africans. This phenomenon suggests that the younger African groups have already adopted a fully westernised lifestyle. In the aforementioned study it was reported that almost 28% of all Africans, 30 years and older of age, also presented with a serum-TC level that classifies them as hypercholesterolaemic. Vorster (2002) mentioned that it could be that with more urban Africans exposed to affluence and Western lifestyles over longer periods, dyslipidaemia and obesity may increase to such

an extent that the protective mechanisms against IHD are no longer active.

Historically, CHD has been remarkably rare in black South Africans (Seftel, 1978). This low prevalence of CHD in the rural African population was initially ascribed to their favourable lipid profiles (Seftel, Raal & Joffe, 1995), marked by low serum-TC, –LDL-C and high HDL-C levels (Steyn *et al*., 1987; Vermaak *et al.,* 1991; Oosthuizen *et al.*, 2002). The ratio of serum-HDL-C to -TC has generally been found to be higher in the black African population group, when compared to any other ethnic groups (Mollentze *et al.*, 1995; Oelofse *et al.*, 1996). This suggests that the black African population group with its lower serum-TC levels experiences additional protection against atherosclerosis and -related disease, by virtue of relatively higher proportions of the protective HDL-C fractions (Norman *et al*., 2007). Seftel, Raal & Joffe, (1995) reported that the serum-TC of almost all black Africans comprise of more than 20% HDL-C, which protects them against IHD. However, Ntyintyane *et al.* (2008) reported that with slightly elevated TC and LDL-C levels, the urbanised black African population group also had lower HDL-C concentrations.

An unfavourable lipid profile is accepted as an independent risk factor for the development of CHD and as reported by Oostuizen *et al.* (2002), serum lipid levels of black South Africans increase with urbanisation. Urbanisation is mainly associated with the shift from a traditional lifestyle and prudent eating habits, to a westernised lifestyle and diet (McMurry *et al.,* 1991; Bourne *et al.*, 1993). The traditional

prudent diet is low in fat, high in unrefined carbohydrates and dietary fibre. A western diet can be defined as high in animal fat and refined carbohydrates and low in dietary fibre (Walker, 1981; Bourne *et al*., 1993; Solomons & Gross, 1995). For more than 40 years, epidemiologic and clinical trials have managed to show that numerous dietary risk factors affect the circulating components of serum lipids.

The main elements of a prudent or anti-coronary diet include a reduction of saturated fat, cholesterol and energy intake, relative to the typical westernised intake (Berger & Marais, 2000; Giugliano, Ceriello & Esposito, 2008). A Westernised diet is generally associated with detrimental metabolic consequences (Meisenberg & Simmons, 1998; Grundy *et al*., 2005). Saturated fats, derived mainly from animal fats, tend to elevate the presence of cholesterol in all lipoprotein fractions (i.e. both LDL-C and HDL-C) when substituted for carbohydrates or other fatty acids (Van Horn & Ernst, 2001). In general, low levels of serum-TC, -LDL-C and -HDL-C and raised triglyceride levels are all associated with a carbohydrate intake of 50 to 60 % of the total energy intake (Hallfrisch *et al*., 1988; Turley *et al*., 1998). Although high-carbohydrate low-fat diets are associated with lower HDL-C levels, it seems as if the ratio of HDL-C:LDL-C slightly increases (Pietinen & Huttunen, 1987). When carbohydrates are consumed along with high-fibre diets, however, the rise in triglycerides or fall in HDL-C has been reported to reduce (Jenkins *et* al., 1993; Turley *et* al., 1998, Vuksan *et* al., 2000). Soluble fibres, like oat bran and beans, significantly reduce the serum-TC and LDL-C levels (Brown *et al.,* 1999; Anderson *et al*.,

2000; Aller *et al*., 2004) , but have no consistent effect on HDL-C and serum-TG levels (Aller *et al*., 2004). However, it has been reported that soluble dietary fibre could cause a reduction in serum-TG and an increase in HDL-C levels (Sing *et al.,* 1992; Lui *et al*., 2000; Jenkins *et al*., 2002b).

2.3.4 Possible Relationship between Haemostatic and Other Risk Markers

CHD is a multicausal disease manifested as atherosclerosis and/or thrombosis (Krummel, 2008). Besides their independent association with CHD, it seems as if the risk markers often co-exist (Steyn, Rossouw & Joubert, 1990). In addition, the collective risk of CHD seems more than would be expected if the risk calculation were based on the sum of the contribution of individual risk markers (WHO, 1990). A High CHD risk is therefore, usually defined only in the presence of two or more CHD risk factors, and requires more vigorous intervention (NCEP, 2002).

The value of plasma fibrinogen as risk marker for CHD is controversial and neglected. In general, a plasma fibrinogen assay is not requested as part of a clinical CHD risk profile assessment. Yet, both clinical and epidemiological data provide sufficient evidence to support the use of plasma fibrinogen as risk marker for the development of CHD. When the plasma fibrinogen concentration is added to the risk profile for CHD, the individual prediction of coronary risk may be markedly improved (Kannel *et al*., 1987; Heinrich *et al.,* 1994). High levels of fibrinogen clusters with most of the major coronary risk factors, such as

abdominal obesity (Vorster *et al*., 1998), dyslipidaemia (Møller & Kristensen, 1991; Kannel, 1997), diabetes (Haffner, 2003) and hypertension (Best *et al*., 2008). It is suggested that fibrinogen represents one mechanism by which various other risk factors lead to CHD, based on the premise that other risk factors are able to mediate a fibrinogen effect (Ernst & Resch, 1993). This hypothesis seems highly valid. A raised plasma fibrinogen concentration is linked to an unfavourable lipoprotein profile, as well as associated with the serum-TC levels (Ernst & Resch, 1993). Several other studies support this positive relationship between fibrinogen and raised plasma cholesterol (Cushman *et al.,* 1996; Alikmets, Parik & Teesalu, 1996; Ridker, 2000; Fibrinogen Studies Collaboration, 2005). Stone & Thorp (1985) implicates the risk of CHD to increase six times if both elevated TC and plasma fibrinogen levels are present. This relationship extends to other clotting proteins. Factor VII activity (Wilhelmsen *et al.*, 1984; Prisco *et al.*, 1996; Cushman *et al*., 1996) and factor VIIIc activity (Pan *et al.*, 1997) were also positively associated with TC and LDL-C. Halle *et al.* (1996) and other studies reported a significant association between elevated fibrinogen levels, factor VII activity and reduced HDL-C levels. Although Møller & Kristensen (1991) indicates that HDL-C levels are independently related to the plasma fibrinogen levels, both serum triglyceride levels and obesity act as confounders within the formula (Halle *et al*., 1996).

Furthermore, Veldman (2008) reviewed that not only fibrinogen itself but also the quality of resultant fibrin networks may be a predisposing

risk factor for the development of cardiovascular disease. Fibrin network architecture as a CHD risk is discussed earlier. However, Veldman (2008) reported that changes in plasma fibrinogen concentrations need not be present to induce alteration in fibrin network architecture. Studies confirmed that a cardiovascular risk reducing diet promoted the formation of ~~h~~ealthier" fibrin networks, believed to be less atherogenic, without inducing a fibrinogen lowering effect (Veldman *et al*., 1997; Veldman *et al*., 1999). This aspect requires further research.

2.3.5 Other cardiovascular risk factors

Risk factors mediate their effect by causing change in the quality and/ or concentration of an established metabolic risk marker. These effects can then be measured experimentally. In the previous section, diet and urbanisation as risk factor for CHD have been reviewed. Yet, as mentioned earlier, CHD is a multifactorial disease. Other risk factors generally associated with an increased CHD risk, not discussed here in detail, include:

- Smoking (Haapenen *et al*., 1989; Homer *et al*., 1991; Ingall *et al*., 1991; Lichtenstein *et al*., 2006; Karaolis *et al*., 2010; Bowen, 2010);
- Obesity (Lowe *et al*., 1988; Lee *et al*., 1990; Lowe *et al*., 1992; De Pergola & Pannacciulli, 2002; Bowen, 2010) ;
- Diabetes mellitus (Colwell, 1988; Ostermann & Van de Loo, 1996; Hu *et al*., 2000; Bowen, 2010);

- Hypertension (Kannel, 1991; Rodgers & MacMahon, 1999; Van den Hoogen *et al*., 2000; Karaolis *et al*., 2010);
- Stress, or type A personality (Siltanen, 1987; Dimsdale, 1988);
- Gender (Beard *et al*., 1989; Stampfer *et al*., 1991; Wilson *et al;* 1998) ;
- Physical inactivity (Powell, Thompson & Casperson, 1987; Møller & Kristensen, 1991; Kannel, 1997; Lichtenstein *et al*., 2006) and
- Age (Bush, Fried & Barrett-Connor, 1988; Witterman *et al*., 1989; Barrett-Connor & Bush, 1991; Wilson *et al*., 1998).

2.4 DIETARY FIBRE

2.4.1 Introduction

A large quantity of material describes how a diet rich in fibre is beneficial to human health, including a decreased risk of CHD and improved laxation (Dietary Guidelines for Americans, 2005). Rimm *et al.* (1996) suggests that fibre independent of fat intake is an important dietary component for the prevention of CHD. The "Fibre Hypothesis," first described by Dennis Burkitt & Hugh Trowell in the early 1979, suggests that a diet rich in fibre helps protect against certain diseases preventable in the affluent Western communities (Burkitt & Trowell, 1986).

Dietary fibre can affect satiety, blood glucose, lipid metabolism and through fermentation exert a major control on colonic function, including bowel habit, transit, the metabolism and balance of the commensal flora and large bowel health (Cummings & Stephen, 2007). During their transit through the alimentary tract, dietary fibres have many opportunities to interact with the substrates, effectors and products of digestion as well as a variety of other substances progressing towards absorption or evacuation (reviewed by Deskens, 1996).

Addition of fibre for treatment of disorders such as atherosclerosis and colon cancer could hold therapeutic value (Wrong, 1995). Adding 3g per day of soluble fibre from oat bran can reduce total cholesterol by 0.13mmol/L (Blake & Triplett, 1995).

Current dietary guidelines recommend a total daily intake of at least 20 to 30 grams for adults, with 25 percent of the fibre being soluble fibre (NCEP, 2002). These levels may be attained with a proposed six or more daily servings of grain products and five or more daily servings of fruit and vegetables.

2.4.2 Classification, chemistry and sources of dietary fibre

Dietary fibre is defined as plant material, mainly derived from plant cell walls, that is resistant to digestion by human gastrointestinal enzymes (Englyst, Liu & Englyst, 2007). Food chemists prefer to define fibre as ~~–li~~gnin and non-starch-polysaccharides (NSP)", where NSP includes cellulose, hemicelluloses, pectin, gums and mucilage, found in food

(Cummings & Stephen, 2007). NSP is not hydrolysed by small-intestinal enzymes and is a suitable substrate for the bacterial production of short chain fatty acids (SCFAs), acetate, propionate and butyrate in the large bowel, together with incompletely digested starch, lactose and proteins (Champ *et al*., 2003; Fuller & Perdigon, 2003). Soluble fibres such as pectin, gums and certain hemicelluloses, are almost completely fermented in the colon to SCFAs (Walker, 1993).

2.4.3 Production of Short-Chain Fatty Acids

Short-chain fatty acids are the final product of microbial fermentation of dietary fibre within the digestive tract. SCFAs are biochemically more closely related to carbohydrates than fats. Some of them are not constituents of natural fats, and they are not ~~"f~~atty", as the nonprofessional envisages the term, as they are completely miscible in water (Wrong, 1995). SCFAs (acetate, propionate and butyrate) and gases (H_2, CH_4 & CO_2) are the main products of the anaerobic breakdown of complex polysaccharides by colonic bacteria (Topping & Clifton, 2001). SCFAs make up the predominant anions in the large intestine of mammals and create a slightly acidic pH level (6.0-7.0). SCFAs vary widely in their relative proportions, depending upon the fibre source in the diet. Various population data show that SCFA production is in order of acetate > propionate > butyrate in a molar ratio of approximately 60:20:20 or 3:1:1, respectively in the proximal and distal colon (Cummings, 1981; Cummings *et al*., 1987; Topping & Clifton, 2001).

Fermentation of dietary fibre involves a variety of reactions and metabolic processes in the anaerobic microbial breakdown of organic matter, yielding metabolisable energy for microbial growth and maintenance and other metabolic products for host use (Hijova & Chmelarova, 2007).

Carbohydrates are fermented (Fig.2.1) by saccharolytic bacteria primarily in the proximal colon producing linear SCFAs, CO_2 and H_2 (Macfarlane & Macfarlane, 2003), and both the presence of carbohydrates in the colon and their fermentation can alter the colonic physiology. Fermentation of proteins and amino acids by proteolytic bacteria yield branched SCFAs, SCFAs, CO_2, CH_4 and H_2, phenols, and amines (Roberfroid, 2005). The primary effect of SCFAs on colonic function is the result of their uptake and metabolism by colonocytes, although SCFAs are also metabolic substrates for other host tissues. The production of SCFAs is determined by many factors, including the numbers and types of micro flora present in the colon (Roberfroid, 2005), substrate source (Cook & Sellin, 1998), and gut transit time.

Other predominant factors that affect SCFA production from polysaccharides and protein by bacteria in the large intestine are as follows:

- chemical composition of the fermentable substrate;
- amount of substrate available;
- type of bacteria present in the large gut;
- rate of depolymerisation;

- colonic transit time;
- availability of inorganic electron acceptor;
- pH of the gut content, particularly in the proximal colon;
- fermentation strategies of substrate-utilising bacteria;
- substrate specificities and catabolite regulation mechanism of individual competitive and co-operative particle size;
- solubility and association with indigestible complexes such as lignin, tannins and silica (Macfarlane & Gibson, 1995).

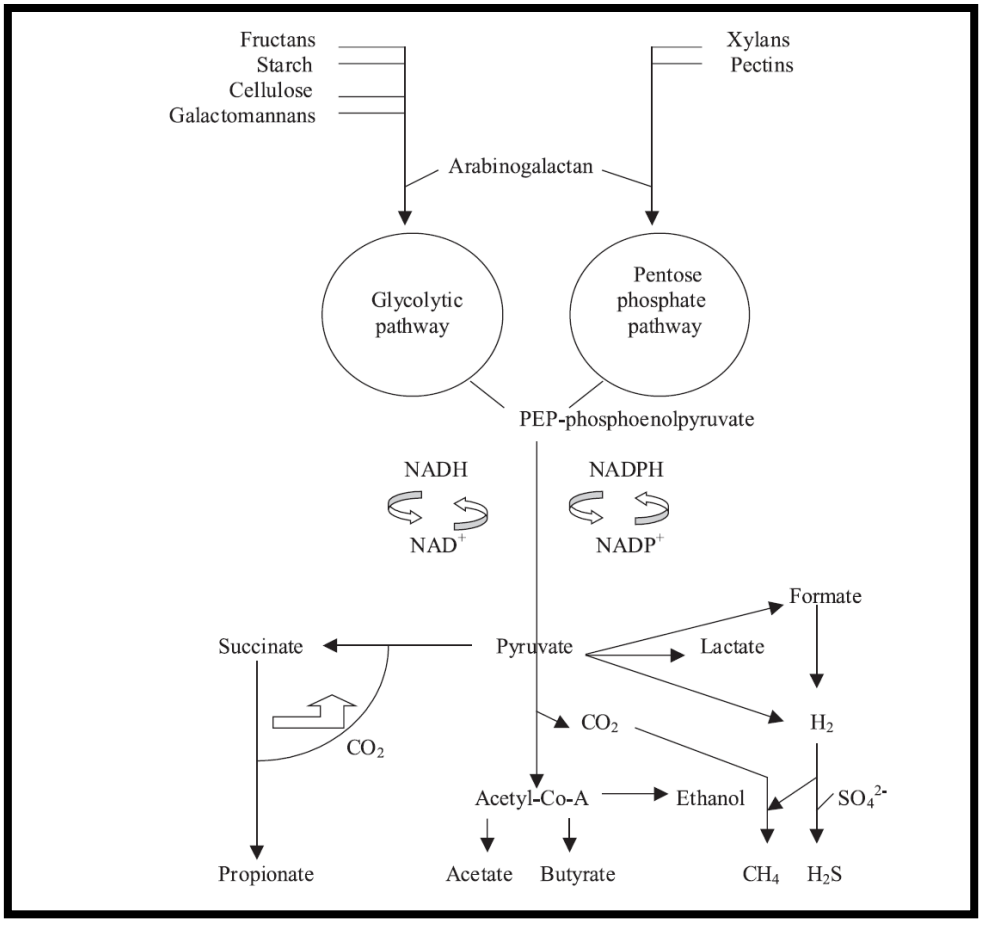

Figure 2.1 SCFA production in the colon. The basic pathway for production of a variety of SCFA metabolites by bacterial fermentation in the colon lumen (Macfarlane & Gibson, 1996).

2.4.4 Absorption and functions of SCFAs

Short-chain fatty acids are rapidly absorbed in the caecum and colon with only 5% to 10% excreted in the faeces (Cook & Sellin, 1998). SCFA absorption in the large intestine involves several processes: non-ionic diffusion, carrier-mediated mechanisms, diffusion after protonation, carrier mediated SCFA anion exchange with bicarbonate, and diffusion coupled with sodium absorption (Scheppach, Fabian & Kasper, 1987; Fuller & Perdigon, 2003). It is widely believed that the transmural movement of SCFAs is a concentration-dependent, passive diffusion process, whereby SCFAs, at least in part, are transported in the protonated form. Hydrogen ions are needed for SCFA protonation, because 99% of SCFA (pKa = 4.8) are in the ionised form for hydration of luminal CO_2 to HCO_3^- and H^+. In addition, the mechanism of uptake into enterocytes is undoubtedly a complex process involving a number of factors and mechanisms (Engelhardt, 1995). The precise mechanism for the absorptive process of SCFAs remains undefined, but all these above outlined factors seem to play a role (Brøbech-Mortensen & Clausen, 1996). SCFAs uptake is associated with the transport of water that seems to be higher in the distal than in proximal colon (Hijova & Chmelarova, 2007).

The role of SCFAs has expanded to include their role as nutrients for the colonic epithelium, as modulators of colonic and intracellular pH, cell, volume, and other functions associated with ion transport, and as regulators of proliferation, differentiation, and gene expression (Cook & Sellin, 1998). Increases in SCFAs result in decreases of pH, which

indirectly influence the composition of the colonic micro flora, decrease solubility of bile acids, increase absorption of minerals (indirectly), and reduce the ammonia absorption by the protonic dissociation of ammonia and other amines (i.e., the formation of less diffusible NH_4^+ compared with the diffusible NH3) (Vince, Kingley & Wong, 1978; Jenkins *et al*., 1987).

2.4.5 SCFAs Metabolism

The SCFAs that escape colonic metabolisation enter the hepatic portal blood, where their concentration varies over a wide range, depending on their intestinal production rates, and therefore on the diet. The major site of SCFA metabolism is the liver; where butyrate and propionate are almost entirely absorbed. However, the percentage of acetate uptake is lower (frequently less than 50%). SCFA concentrations in the portal vein are closely dependent on digestive fermentation (Brøbech-Mortensen & Clausen, 1996). The relative proportions of the three major acids in the portal blood reflect the relative proportions of those found in the intestinal contents (Bugaut & Bentéjac, 1993). Portal vein concentrations of butyrate are approximately tenfold higher when compared to the peripheral vein concentration, while propionate concentrations are 20 - 40 fold higher and acetate concentrations only approximately fourfold higher.

The major SCFAs, acetate, propionate and butyrate are absorbed at comparable rates in different regions of the colon. Once absorbed, SCFAs are metabolised at three major sites in the body (Hijova & Chmelarova, 2007). :

1. cells of the ceco-colonic epithelium that use butyrate as a major substrate for the maintenance of energy producing pathways;
2. liver cells that metabolise residual butyrate with propionate used for gluconeogenesis; 50% to 70% of acetate is also taken up by the liver;
3. muscle cells that generate energy from the oxidation of residual acetate (Hijova & Chmelarova, 2007).

2.4.5.1 Acetate metabolism

Acetate, the principal SCFA in the colon, is readily absorbed and transported into the liver, and therefore less metabolised in the colon (Hijova & Chmelarova, 2007). The metabolic fate of acetate and its role in the liver is connected to the general orientation of metabolism towards carbohydrate or lipid utilisation (Rèmésy, Demigné & Morand, 1995). Acetate is the primary substrate for cholesterol synthesis. In the host, it may be absorbed and utilised by peripheral tissues (Pomare, Branch & Cummings, 1985), further, bacteria isolated from the human intestine are capable of utilising acetate for the production of butyrate in the colon (Duncan *et al.,* 2002).

Furthermore, acetate can stimulate gluconeogenesis from lactate. A substantial fraction of acetate from digestive fermentation is not taken up by the liver. Splanchic balance of acetate is always positive, even during fasting periods. Most of the extrasplanchnic tissues can metabolise acetate, for example, adipose tissue, mammary gland (cytosolic), muscle, kidney, and the heart (mitochondrial). The presence of acetyl-CoA synthesis in the cytosol of adipose and

mammary glands allows the use of acetate for lipogenesis once it enters the systemic circulation, whereas the mitochondrial localisation favours its utilisation for energy supply in the Krebs cycle (Rèmésy, Demigné & Morand, 1995; Hijova & Chmelarova, 2007).

2.4.5.2 Butyrate metabolism

Butyrate is an important source of energy for colonocytes and thus extensively metabolised by the colon (Kruh, Defer & Tichonicky, 1995). Butyrate is found in a lower proportion in the blood draining the large intestine when compared to blood draining the lumen (Brøbech-Mortensen & Clausen, 1996). This reflects the fact that a large part of absorbed butyrate is metabolised by the mucosa (to carbon dioxide and ketone bodies). Butyrate is an important fuel for the colonic mucosa whereas acetate and propionate are released into the portal blood. Butyrate is exclusively metabolised in mitochondria (carnitine-independent source of acetyl-CoA) and acts as a potentially ketogenic substrate during the post-absorptive period. In fed subjects, butyrate may represent a precursor for lipogenesis. High concentrations of butyrate inhibit propionate utilisation. Because of the provision of acetyl-CoA in mitochondria, butyrate is an effective activator of gluconeogenis from lactate, and of ureogenesis; thus butyrate probably thwarts some of the inhibitory effects of propionate on gluconeogenesis (Rèmésy, Demigné & Morand, 1995).

2.4.5.3 Propionate metabolism

Propionate can be found in portal blood, although some may be metabolised in the colonic epithelium and may be a differentiating factor, but with less power than butyrate (Champ *et al.*, 2003). Propionate is produced via two main pathways (Cummings, 1981) :

1. fixation of CO_2 to form succinate, which is subsequently decarboxylated;
2. from lactate and acrylate (Cummings, 1981).

Much of the knowledge about the nutritional fate of propionate comes from studies of ruminants. Intestinal glucose uptake is minimal in ruminants because of the presence of microbiota in their rumen for the digestion and fermentation of carbohydrates (Hijova & Chmelarova, 2007). Production of SCFA constitutes the major source of ruminant energy (Hooper, Midtvedt & Gordon, 2002) where propionate is a primary precursor for gluconeogenesis. Propionate metabolism in humans is not understood very well.

Under normal conditions, propionate is completely metabolised by the liver (Rèmésy, Demigné & Morand, 1995). This process is certainly favoured by facilitated diffusion, which is efficient even in the presence of relatively low propionate concentrations (Fafournoux, Rèmésy & Demigné, 1985).

2.4.6 Biological Effects of SCFAs

Various epidemiological and case control studies have shown that the intake of dietary fibre has some beneficial effects on the health of

human subjects (Brøbech-Mortensen & Clausen, 1996). Colonic fermentation of dietary fibre results in SCFAs (Bourquin, Titgemeyer & Fahey, 1992). After absorption, each of the primary SCFAs produced is metabolised by the body and many biological effects of SCFAs have been reported (Bourquin, Titgemeyer & Fahey, 1992). SCFAs influence carbohydrate and lipid metabolism, and may therefore contribute to the protective effect of NSP against degenerative western diseases associated with fibre intake (Burkitt & Trowell, 1986; Wolever, Spadafora & Eshuis, 1991).

Especially the cholesterol-lowering effects of dietary fibre intake have been the topic of many studies. Veldman *et al.* (1997) found that pectin supplementation caused significant decreases in total cholesterol, LDL-C, apo A, apo B and lipoprotein (a). Vorster *et al.* (1988) also suggested that SCFA production, which is quickly absorbed and transported to the liver, is one of the possible mechanisms by which NSP may influence the synthesis of coagulation factors.

2.4.6.1 Acetate

When acetate is administered through different routes, for example oral, rectal and intravenous, to healthy human volunteers a decrease in glycerol levels and a significant decrease in serum concentrations of free fatty acids is seen. Acetate appears to compete with long-chain fatty acids for oxidation in certain tissues, promote hepatic cholesterol synthesis and decrease lipolysis (Topping & Pant, 1995). Acetate is the primary substrate for cholesterol synthesis. Veldman *et al*. (1999) postulated that acetate affected fibrin network structure

in a positive way. The networks were more permeable and had lower tensile strength and are believed to be less atherogenic. These changes are partly accounted for by the direct effect of acetate on fibrin network structure.

2.4.6.2 Propionate

Propionate supplemented diets have been shown to lower blood cholesterol in rats (Chen, Anderson & Jennings, 1984; Illman *et al.*, 1988) and pigs (Boila *et al.,* 1981), but in humans the effects are less clear. Venter, Vorster & Cummings (1990) administered 7.5g of propionate daily in capsule form to healthy subjects for seven weeks. Propionate use did not affect serum cholesterol levels but significantly increased HDL-C.

Venter *et al*. (1997) undertook a study where baboons were fed a western diet with either a 2% propionate or 5% soluble dietary fibre concentrate supplement. Total serum cholesterol values were increased in the baboons fed a non-supplemented western diet. Soluble fibre supplementation prevented this increase, while propionate did not. However, both propionate and fibre intake increased the serum HDL-C concentration. Furthermore, the liver cholesterol concentration was lowered with propionate and fibre supplementation. Experimental studies in animals indicate that feeding oat bran has hypocholesterolaemic effects (Brøbech-Mortensen & Clausen, 1996). Concomitant to this, oat bran significantly increases portal vein propionate concentration. In comparison, cellulose that does not lower cholesterol levels does not

increase the propionate concentration. *In vitro* studies indicate that propionate at physiological concentrations, significantly decreases hepatic cholesterol synthesis (Anderson, 1995). Other conflicting results show that propionate administered rectally, has no effect on either the circulating total cholesterol or the triglyceride concentration (Wolever, Spadafora & Eshuis, 1991). When administered orally, propionate is reported to have no effect on serum cholesterol levels, but causes an increase in the triglyceride concentration (Brøbech-Mortensen & Clausen, 1996). Conflicting results show that the total serum cholesterol concentration is slightly decreased and the triglyceride concentration slightly increased when propionate is administered (Anderson, 1995).

A number of mechanisms have been suggested to be responsible for the observed lipid lowering effect, with an increased propionate production being one of the possible mechanisms (Hijova & Chmelarova, 2007). Increased production of propionate, through fermentation, may inhibit hepatic cholesterol synthesis. It seems possible that one of the determinants of action of propionate in serum lipids is the ratio of propionate to acetate (Cheng & Lai, 2000; Wolever, Fernandes & Rao, 1996).

2.4.6.3 Butyrate

The effect of butyrate on haemostasis is not known, while the effect on lipid metabolism has not been extensively studied. In cultured cell lines, butyrate is a well-recognised antitumor agent, whereas the other SCFAs are much less active in this respect (Young & Gibson,

1995; Brøbech-Mortensen & Clausen, 1996). Sodium butyrate exerts an antiproliferative activity on many cell types that have demonstrated preventative effects of butyrate on colon cancer and adenoma development (Bornet *et al*., 2002). At a molecular level, butyrate affects gene expressions via the phosphorylation and acylation of histone proteins (Archer & Hodin, 1999). Butyrate also stimulates immunogenicity of cancer cells (Hijova & Chmelarova, 2007).

2.5 SUMMARY

In this chapter, the author provides a summary of the most important theoretical concepts that relates to this study. Risk factors that provide some evidence to increase the risk of cerebrovascular and CHD and how these are linked to diet and urbanisation have been reviewed. Cerebrovascular disease and CHD are of the most important causes of morbidity and mortality amongst South Africans. It is evident that black populations of South Africa are now migrating and adopting a more westernised lifestyle, which includes a diet low in fibre and high in fat. This urbanisation and adoption of a westernised diet, is one of the reasons responsible for the rise in cardiovascular disease in the black population of South Africa.

In general, it is suggested that lifestyle management should be the first choice when having to treat patients at risk of CHD. Diet is one of the controllable factors in the treatment and prevention of westernised diseases such as CHD. Based on current understanding, it is apparent

that western diets do not contain enough dietary fibre. Furthermore, research has shown that the addition of dietary fibre may have a beneficial effect on some known coronary risk factors. The physiological effects of dietary fibre in humans are significantly influenced by the degree to which fibre is fermented to SCFAs, acetate, propionate and butyrate, in the colon. It will be discussed how these SCFAs are believed to be one of the possible mechanisms through which dietary fibre protects against heart disease.

CHAPTER 3

METHODOLOGY

3.1 INTRODUCTION

The main objective of this study was to measure the possible metabolic effects of the intake of different combinations of colonic release SCFA supplements on lipid and haemostatic risk profiles in westernised black men. The diet of a group of westernised African male volunteers was supplemented with different combinations of short-chain fatty acids, for a period of six weeks, in addition to supplementation with placebo for one week at the beginning and end of the study. Blood was drawn at various intervals and analysed to assess changes in the metabolic profiles of the volunteers. In this chapter, the author provides a comprehensive summary of all methods and materials used in this study.

3.2 STUDY DESIGN

The study design was that of a randomised, placebo-controlled, double blinded, clinical research trial (represented in Figure 3.1). Seventy-five volunteers that adhered to a predetermined set of inclusion criteria (see section 3.6.1) were recruited from the Military Base at Tempe Defence Force,

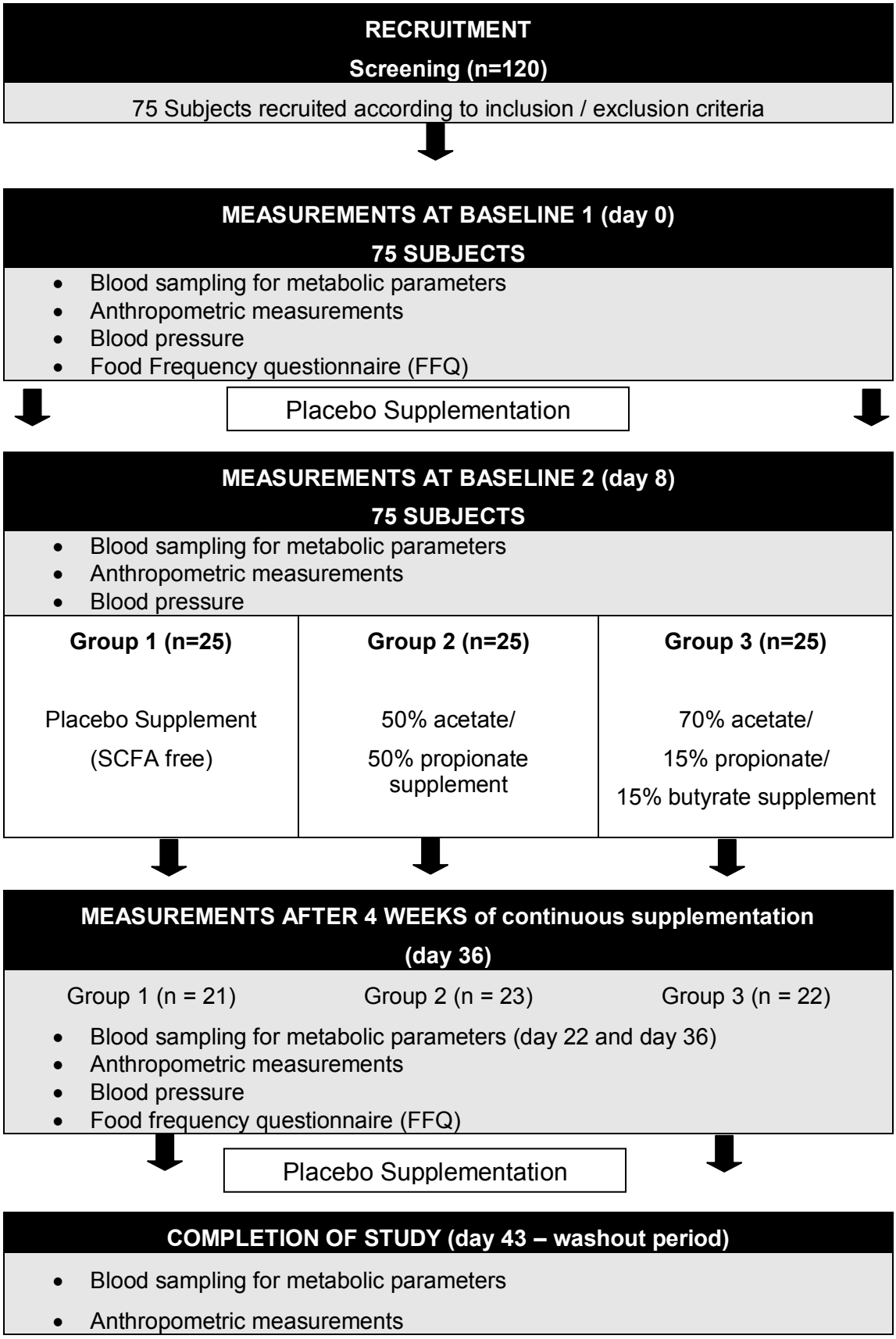

Figure 3.1 Schematic representation of the study design

Bloemfontein, and randomly assigned to three different supplementation groups. Group 1 was assigned a placebo supplement filled with non-fermentable cellulose. Group 2 was assigned a supplement containing short-chain fatty acids in a ratio of 50% acetate and 50% propionate. Group 3 was assigned a SCFA supplement in a ratio of 70% acetate, 15% propionate and 15% butyrate. Researchers, field-workers and the volunteers were unaware of the assignment. It was also not possible to differentiate between supplements based on the capsules, or the physical characteristics of the content. The study was therefore of a double-blind nature.

For standardisation purposes, experimental supplementation followed after collection of two subsequent baseline samples on day 0 and 8, during which all subjects took placebo supplements. in order to obtain a stable reference baseline. On day 8, the subjects were randomised into three different experimental groups of 25 subjects each. The groups were assigned to either one of the two experimental SCFA combinations, or the placebo. Supplementation was then sustained for four weeks (days 8-36). During this experimental phase, blood samples were drawn after two weeks of supplementation (on day 22) and again at the end of the four-week period (day 36). An additional washout period followed, during which all subjects received the placebo supplement for an additional period of seven days (day 36 to day 43), after which blood was drawn to assess the recovery of measured variables after supplementation.

The study was undertaken at a time specifically designed to minimise possible seasonal effects on human metabolism. The dietary intake of the group was under strict control during the study. What was of importance, is that no major

changes in their intake occurred during the intervention period. All meals were therefore served at the same mess of the Tempe Defence Force. In addition, the principle investigator of this study, as a registerd dietitian, were responsible for working out their menue at this mess. Food Frequence Questionnaires were completed at the beginning and end of the study, but used only for control purposes and will not be discussed in this thesis. At the end of the study, the subjects completed a questionnaire to report their experiences during the study, in addition to any side effects (Appendix D).

3.3 SUBJECT SELECTION

A recruitment day was scheduled at the premises of the Tempe South African National Defence Force base in Bloemfontein, during which blood samples were drawn, dietary intake, social and demographic background questionnaires were completed, as well as anthropometric measurements taken as part of the screening process. The inclusion criteria used for the selection of subjects in this study are discussed under section 3.6.1. The scientific value of the study and expectations of the researchers were also explained to the volunteers, in both English and their preferred mother tongue. Inclusion criteria were strictly adhered to for selection of participants.

3.4 ETHICAL CONSIDERATION

This study proposal was submitted to and approved by the Ethics Committee of the University of the Free State, reference number: ETOVS 227/98. The

study was conducted according to International Good Clinical Practice Guidelines and adhered to the contents of the Declaration of Helsinki. All subjects participating in the study received information leaflets that explained the relevance of the study. Subjects were requested to give written consent (Appendix A). A record of patients and their appropriate assignments were kept at the NESTEC Research Laboratory, Lauzanne, Switzerland, for safety purposes, in case of an emergency.

3.5 OPERATIONAL DEFINITIONS

Variables defined to meet the objectives of this study, included the general health profiles, lipid profiles, haemostatic profiles, and glycometabolic indicators.

3.5.1 General Health Profiles

For the purpose of this study, health status includes the anthropometric measurements, a selection of biochemical parameters, and blood pressure as general markers of health and nutritional status.

3.5.1.1 Anthropometric measurements

Body mass inex (BMI), a definition of the degree of adiposity is a validated measure of nutritional status (see Table 3.1) (Hammond, 2008), even though it is not very accurate in that sense. BMI accounts for differences in body composition by defining the level of adiposity according to the relationship of weight to height, thus elimination dependence on frame size (Lee & Nieman, 2003; Gibson, 2005).

Table 3.1 Classification of BMI (Laquatra, 2004)

Classification	BMI
Underweight	< 18.5 kg/m^2
Normal weight	18.5 – 24.9 kg/m^2
Overweight	25.0 – 29.9 kg/m^2
Obesity, class I	30.0 – 34.9 kg/m^2
Obesity, class II	35.0 – 39.9 kg/m^2
Obesity, class III	>40 kg/m^2

Waist circumference measurements assess abdominal fat content. A measurement of greater than 102 cm for men is an independent risk factor for disease when out of proportion to total body fat (Centre for Disease Control and Prevention, 2002).

3.5.1.2 Blood Pressure

Blood pressure is dynamic and measured as an indicator of the physical and emotional state of the subject at the time of the measurement (De Bono & Boon, 1991). A general definition of hypertension is a systolic blood pressure (SBP) of 140 mmHg or higher or a diastolic blood pressure (DBP) of 90 mmHg or higher, or both. In the seventh Report of the Joint National Committee on Detection, Evaluation, and Treatment of High Blood Pressure (JNC VII) (Joint National Committee, 2004), hypertension is classified in stages based on the risk of developing CVD (see Table 3.2).

Table 3.2 Classification of Blood Pressure (Joint National Committee, 2004)

Blood Pressure Classification	SBP mmHg	DBP mmHg
Normal	<120	and <80
Prehypertension	120-139	or 80-89
Stage 1 Hypertension	140-159	or 90-99
Stage 2 Hypertension	≥160	or≥100

3.5.1.3 Biochemical parameters

Serum-total protein (TP) and -albumin, have been used for decades now as biochemical risk markers for general health and nutritional status (Lindsay, 1996).

Serum total protein is a biochemical test for measuring the total amount of protein in blood plasma or serum. Protein in the plasma constitutes of albumin and globulin (Lindsay, 1996).

Serum total albumin is the most abundant and most often measured plasma protein and a strong predictor of health (Lindsay, 1996).

Full blood count is one of the most commonly performed blood tests, which can be used as an indication of general health status. A full blood count examines the different components of blood, including red blood cells, white blood cells and platelets (Lindsay, 1996) and can be used to indicate the presence of infection, or even chronic diseases. It is important to note that in this study, the Full Blood Count data was analysed as part of the general health profiles and not as part of the haemostatic profiles. A Full Blood Count is used to establish the general health of patients under normal clinical

conditions. It was therefore used in that respect, as it was not expected that the intervention would affect any of these parameters.

3.5.2 Lipid profiles

Serum-total cholesterol (TC), serum low-density lipoprotein cholesterol (LDL-C), serum high-density lipoprotein cholesterol (HDL-C), serum triglycerides (TG) as part of the lipid profile (NCEP, 2002).

3.5.2.1 Serum total cholesterol (TC)

A total cholesterol measurement captures cholesterol contained in all lipoprotein fractions: 60% to 70% is carried on LDL, 20% to 30% on HDL, and 10% to 15% on very low-density lipoprotein cholesterol (VLDL). There is a direct, positive relationship between total cholesterol and CHD (Krummel, 2008). The clinical classification of serum total cholesterol is provided in Table 3.3.

Table 3.3 Classification of serum Total Cholesterol (NCEP, 2002)

Total Cholesterol mmol/L	
Desirable	**<5.17**
Borderline High	**5.17-6.18**
High	**≥6.20**

3.5.2.2 Serum Low-Density Lipoprotein Cholesterol (LDL-C)

Lipoproteins are the major cholesterol carrier in the blood. High levels are associated with increased risk of CHD (Krummel, 2008). The clinical classification of serum LDL-Cholesterol is provided in Table 3.4.

Table 3.4 Classification of serum LDL-Cholesterol (NCEP, 2002)

LDL-Cholesterol mmol/L	
Optimal	**<2.59**
Near Optimal/ above optimal	**2.59-3.34**
Borderline High	**3.35-4.1**
High	**4.14-4.89**
Very High	**≥4.9**

3.5.2.3 Serum High Density Lipoprotein Cholesterol (HDL-C)

A plasma lipoprotein containing mostly protein and less cholesterol and triglyceride. High levels of HDL-cholesterol are associated with a decreased risk of CHD (Krummel, 2008). The clinical classification of serum HDL-Cholesterol levels are provided in Table 3.5.

Table 3.5 Classification of serum HDL-Cholesterol (NCEP, 2002)

HDL-Cholesterol mmol/L	
Low HDL-Cholesterol	**<1**
High HDL-Cholesterol	**≥1.55**

3.5.2.4 Serum Triglycerides

Triglycerides are the most common type of glyceride in which the three alcohol groups in the glycerol molecule are attached to fatty acids. The triglyceride-rich lipoproteins, include chylomicrons, VLDL and any remnants or intermediary products formed in catabolism (Krummel, 2008). Many prospective epidemiological studies have reported a positive relationship between serum triglyceride levels and incidence of CHD (reviewed by NCEP, 2002). The classification of serum triglycerides is provided in Table 3.6.

Table 3.6 Classification of serum Triglycerides (NCEP, 2002)

Triglycerides mmol/L	
Normal	**<1.7**
Borderline High	**1.7-2.25**
High	**2.26-5.6**
Very High	**≥5.6**

3.5.3 Haemostatic profiles

Plasma fibrinogen, a collection of fibrin network architecture variables, factor VII activity and factor VIII activity, D-Dimer, c-reactive protein and fibrin monomers were measured as part of the haemostatic risk profile (see Table 3.7):

3.5.3.1 Plasma fibrinogen

Raised plasma fibrinogen levels are associated with increased risk of cardiovascular disease (Viles-Gonzalez, Fuster & Badimon, 2004; Fibrinogen Studies Collaboration, 2005).

3.5.3.2 Factor VII and VIII activity

Elevated FVII (Heinrich *et al*. 1994) and FVIII (Pan *et al*. 1997) are associated with increased risk of cardiovascular disease.

3.5.3.3 Antithrombin III (AT III)

AT III is considered a natural anticoagulant; its deficiency is associated with thrombosis.

3.5.3.4 Fibrin monomer and D-Dimer

Both fibrin monomer and d-dimer levels are considered as potential risk markers for development of cardiovascular events. D-dimer is a product of clotted fibrin breakdown, and is usually found in increased levels in patients with hypercoagulable disease states occurring before and after cardiovascular events.

3.5.3.5 Fibrin network architecture

Three independent methods have been used in this study to characterise the fibrin network architecture. The mass-length ratio serves as a measure of fibrin thickness (Nair *et al*., 1991a). Compaction characterises the degree of cross-linking and tensile properties of the fibrin network (Nair & Shats, 1997). The network lysis rate, which could be used to assess the overall structure of the fibrin network in terms of lytic rate under controlled conditions. Gabriel, Muga & Boothroyd (1992) have shown that thin fibrin fibres are lysed more slowly when compared to thick fibrin fibres.

3.5.3.6 C-reactive protein

C-reactive protein (an acute phase protein) was measured to ensure that possible changes in other haematological parameters may not be due to inflammation or any other unknown acute phase response, and also because it is associated with CHD.

Table 3.7 Normal ranges for haemostatic profiles (Department Haematology and Cell Biology, University of the Free State & NHLS, 2003)

	Normal Range
Plasma fibrinogen	2.0 -4.0 g/L
Favtor VII	50 – 150%
Factor VIII	50 – 150%
AT III	76 – 122%
D-Dimer	< 500 µg/dL
C-reactie protein	0 – 03mg/dL

3.5.4 Glycometabolic indicators

Fasting serum glucose and -insulin levels were measured as part of the carbohydrate risk profile.

3.5.4.1 Fasting Serum Glucose

According to the Framingham Heart Study, the association between elevated plasma glucose and CHD risk is a continuous variable. Some investigators view impaired fasting glucose (IFG) to be an independent risk factor for cardiovascular disease (NCEP, 2002). According to the American Diabetes Association (ADA) (2008) IFG can be defined between 5.6mmol/L to 6.9 mmol/L.

3.5.4.2 Fasting Serum Insulin

The metabolic syndrome is closely associated with a generalised metabolic disorder called *insulin resistance,* in which tissue

responsiveness to the normal action of insulin is impaired (Haffner, 1999). According to Alberti, Ziimmet and Shaw (2006) insulin resistance correlates with the risk of CHD. The mechanisms underlying the link between insulin resistance and CHD require further investigation.

3.6 MATERIALS

3.6.1 Study population

Approximately one hundred and twenty members of the SANDF were screened for participation in the study. The subjects had to be permanent African male staff residing at the Tempe Military base, Bloemfontein. Subjects were selected based on the following inclusion criteria:

- No smokers
- No previous history of cardiovascular incidences, diagnosed diseases of lifestyle (diabetes, hypercholesteraemia, etc.) or apparent genetic disorders
- No usage of chronic medications
- No usage of medication or other supplementation during the study, without the knowledge of the principal investigator
- No apparent lifestyle changes during study and at least six months prior the study
- Subjects should have been permanent staff for at least one year prior the study

- Subjects with a plasma fibrinogen level lower than 2.5 g/L were excluded from the study
- Subjects with a fasting blood glucose levels above 5.8 mmol/L were excluded from the study
- No alcohol abusers (<3 drinks/day or 28g alcohol/day)

The background diet of all subjects had to be controlled. All subjects in this study received their meals from the same mess.

3.6.2 Sample size

Seventy-five volunteers were recruited to participate in the study. The total sample size (3 x n) was prescribed by the statistical services of the Research Centre, NESTEC LTD PTY, Lauzanne. A calculation based on the results of previous experiments was only of partial value for obtaining sample size, since effects of propionate supplementation on outcomes measured in this study are not available. For power calculations a reference of 15% change in circulating blood cholesterol was used (Veldman, 2000). It was estimated that a group of 25 individuals per supplement would allow for expected drop-outs without a significant loss of statistical power.

3.6.3 Supplements

Volunteers obtained SCFA supplements in capsules equivalent to the fermentation of 15 grams dietary fibre. The capsules were designed in such a way that their contents were released only in the large intestine. This simulates the generation of SCFA from soluble dietary fibre, in the large intestine. The capsules were designed in such a way that they

Table 3.8 Ratio of SCFAs in the different supplements

Placebo Supplement
Sodium chloride, calcium chloride and CMC sodium carmelel DV (S), a non fermentable form of cellulose. The capsules weighed approximately 1.0288g each. Number of capsules per day: 8
Supplement 1: 50% acetate; 50% propionate; 0% butyrate
50% acetate, consisting of 0.3299g sodium and 0.2135g calcium acetate; 50% propionate, made up of 0.2328g sodium and 0.2257g calcium propionate. The capsules weighed 1.0019g each. Number of capsules per day: 8
Supplement 2: 70% acetate; 15% propionate; 15% butyrate
70% acetate, consisting of 0.4618g sodium and 0.2989g calcium acetate; 15% propionate, made up of 0.0698g sodium and 0.0677g calcium propionate; 15% butyrate, made up of 0.0801g sodium and 0.0779g calcium butyrate. The capsules each weighed 1.0562g. Number of capsules per day: 8

contained equal amounts of the sodium and calcium salts, in order to protect subjects against excess intake of these ions. The ratio of SCFAs in the different supplements is presented in Table 3.8.

Compliance was monitored directly by the dieticians (Captains M. de Wet (priniciple researcher) and A. van Onselen) – who were involved in the study, as the entire subject group ate their meals at the same mess hall of Tempe Military Base, Bloemfontein.

3.6.4 Specimen collection

Fasting blood samples were required from all subjects on the appropriate sampling days. Venepuncture was performed between 8:00 and 10:00 am. Subjects were requested to fast from 22:00 the previous night. For the full blood count and C-reactive protein analysis, blood was drawn into one 5 ml EDTA tube. Two 10 ml red top serum tubes were drawn for analysis of biochemical markers, and an additional six 5 ml tri-sodium citrate (3.8%) tubes for coagulation and network assays.

3.6.5 Apparatus

All laboratory analyses were performed using commercially available apparatus. Relevant information with a description of the apparatus used for the respective laboratory analyses is provided in table form (Table 3.9).

Table 3.9 The apparatus used for laboratory analyses of blood specimens

Apparatus	Description	Brand Name	Supplier
Coagulation analyser	Coagulation Timer	Behring	Dade Behring
Chemical analyser	Hitachi 902	Hitachi	Roche Diagnostics
Cell counter	Coulter MD 18	Coulter	Beckman Coulter
Microplate reader	EL 312e	Biotek	Biotek Instruments
CRP analyser	MICROS CRP	ABX CRP-100	ABX Diagnostics
Spectrophotometer	Shimadzu UV-1201	Shimadzu	Scientific Group

3.6.6 Standards and controls

The quality of all laboratory analyses was under strict control. Apparatus were regularly cleaned and control samples analysed according to the prescribed protocol of each variable. Relevant information with a description of the appropriate standards and controls used for specific laboratory analyses is provided in table form (Table 3.10).

Table 3.10 Standards and controls used for different laboratory investigations

Standard or control	Catalogue no.	Supplier
Fibrinogen Standards 1-4#	OWCS 11	Dade Behring, Marburg, Germany
Standard Human Plasma#	ORKL 17	Dade Behring, Marburg, Germany
Control Plasma N#	ORKE 35	Dade Behring, Marburg, Germany
Control Plasma P#	OUPZ 13	Dade Behring, Marburg, Germany
D-Dimer Standard Plasma#	OQXA 11	Dade Behring, Marburg, Germany
D-Dimer Control Plasma I#	OQKA 15	Dade Behring, Marburg, Germany
D-Dimer Control Plasma II#	OQKB 17	Dade Behring, Marburg, Germany
S.F.A.C.*	759350	Roche Diagnostics, Mannheim, Germany
Percinorm® U*	171735	Roche Diagnostics, Mannheim, Germany
Percipath® U*	171760	Roche Diagnostics, Mannheim, Germany

the reagents used for calibration and control of coagulation assays, while * where used for chemical assays.

3.6.7 Reagents and consumables

Relevant information with a description of the reagents used for each laboratory analysis is provided in table form (Table 3.11).

Table 3.11 Reagents and consumables used during the study

Reagents & consumables	Catalogue no.	Supplier
Trasylol®/Aprotinin	H2912	Bayer-miles, Germany
Glass beads	267 02 50	Saarchem, South Africa
Sodium hydroxide	582 31 80	Saarchem, South Africa
Sodium carbonate	582 20 40	Saarchem, South Africa
Folin & Ciocalteu's reagent	243 300	Saarchem, South Africa
Albumin	T-3379	Sigma, USA St. Louis
Thrombin	101141	ICN, USA
Streptokinase	10114	ICN, USA
CRP latex reagent	7003077	ABX Diagnostics, France

3.7 METHODS AND TECHNIQUES

3.7.1 Metabolic risk markers

3.7.1.1 Specimen preparation

Full blood count analysis was performed within four hours post venepuncture, after which the specimen was centrifuged, plasma separated and stored in 1.5 ml Eppendorf® vials at –70°C for CRP analysis. Clotted specimens were centrifuged; serum separated and stored in 1.5 ml Eppendorf® vials at –70°C. Platelet free plasma was obtained by centrifuging citrated blood twice, which was aliquoted and stored in 1.5 ml Eppendorf® vials at –70°C.

3.7.1.2 Fibrin network architecture variables

Fibrin network structure variables were performed using citrate plasma. Thirty-five micro-litres of 10.000 KIU/ml Trasylol®/aprotinin per nine volumes of citrate plasma was added, and thus used as an

inhibitor of fibrin(ogen)olysis. Plasma with trasylol was used for analysis of fibrin network content, compaction and mass-length ratio from turbidity. Permeability was not performed because of the great degree of variation and the complexity of the method. Network lysis rate was performed on plasma without Trasylol®.

3.7.1.2.1 Network fibrin content

The method of Ratnoff & Menzies (1951) was used for duplicate determination of the network fibrin content. For network fibrin content 0.9 ml plasma was pipetted into test tubes containing 1g glass beads and clotted by addition of 100 µL Thrombin Reagent (1 IU/ml Thrombin final concentration, 25 mM Ca^{++} final concentration). Samples were left overnight for maximum polymerisation. All samples were centrifuged at 1300 x g and the supernatants discarded. The isolated networks were washed three times with saline solution. One ml 2.5 M NaOH was dispensed into each test tube and the networks dissolved by heating the tubes for 15 minutes at 95°C. The samples were left to cool at room temperature. Seven millilitres water and 3 ml 1.9 M sodium carbonate were dispensed into each tube, containing 200 µL of the NaOH-fibrin suspension. The tubes were vortexed and mixed with one ml Folin-Ciocalteu's phenol reagent. Samples were incubated at room temperature for 20 minutes and the absorbance measured at 650 nm. Different concentrations of albumin were dissolved in 2.5 M NaOH and used to prepare a

standard curve. This standard curve was used to calculate the concentration of fibrin present in unknown samples.

3.7.1.2.2 Mass-length ratio from turbidity (μT)

The mass-length ratio from turbidity (μT) was determined in triplicate for each sample, using the method as described by Nair *et al*. (1991a). Nine hundred μL of platelet-poor plasma was pipetted into micro-cuvettes of 1 cm path length. The plasma was mixed and clotted by addition of 100 μL Thrombin Reagent (1 IU/ml Thrombin final concentration, 25 mM Ca^{++} final concentration). Samples were left overnight for maximum polymerisation.

The intercepts, A, in plots of $c/T(\lambda)^3$ as a function of $1/(\lambda)^2$ were used to calculate μT according to the equation:

$$\mu T = [10/1.48xA] \times 10^{12} \text{ daltons/cm}$$

with c the network content, T the absorbance $\times$ 2.304 and λ the wavelength. Turbidity (optical density) was measured at a range of wavelengths between 600 and 800 nm.

3.7.1.2.3 Fibrin network compaction

Compaction was measured in duplicate using the method as described by Dhall, Bryce and Dhall (1976). Nie hundred μL of plasma was pipetted into 1.5 ml Eppendorf® vials, pre-sprayed with lecithin-based aerosol (Spray-a-Cook®) to render the surface non-adhering. The plasma was clotted by introduction of 100 μL Thrombin Reagent (1 IU/ml Thrombin final concentration, 25 mM Ca^{++} final concentration). Samples were left overnight for

maximum polymerisation. After centrifugation at 8000 X g for 45 seconds the volume of expelled sample from the fibrin networks was determined using a one ml syringe and expressed as a percentage of the initial volume.

3.7.1.2.4 Fibrin network lysis rate

The network lysis rate was measured in duplicate using a method developed at the Fibrinogen Unit, Research laboratory. Ninety µL of plasma was mixed and clotted by the introduction of 10 µL thrombin reagent (1 IU/ml thrombin final concentration, 25 mM calcium final concentration) in microtiter plates. After total polymerisation took place, 50 µL of streptokinase with a final concentration of 100 U/ml was introduced to start lysis of networks. The absorbance was measured with an ELISA plate reader at 608 nm for six hours at 10 minute intervals. The lysis rate was determined by plotting the time versus change in absorbance.

3.7.1.3 Coagulation factor determinations

All the coagulation measurements were performed on the Behring Coagulation Timer auto-analyser, using the methods listed in Table 3.12.

Table 3.12 Methods used for coagulation factor determinations

Measured variable	Catalogue no	Brand name	Principle
Factor VII	OUHP 17	Thromborel® S	Clot formation
Factor VIII	OQGS 17	Pathrombin® SL	Clot formation
Antithrombin III	OWWR 17	Berichrom® AT III	Chromogenic
Fibrinogen	OWZG 15	Multifibrin® U	Clauss' method
Fibrin monomers	OWXZ 11	Berichrom® FM	Chromogenic
D-Dimers[$]	OQWW 11	D-Dimer Plus	Turbidimetric

[$]D-Dimer analyses were performed only on samples drawn on day 8 and 36. These reagents were all provided by Dade Behring, Marburg, Germany

3.7.1.4 Blood Chemistry analyses

All the chemical analyses were performed on the Hitachi 902, automatic analyser, using the methods as listed in Table 3.13.

Table 3.13 Methods used for chemical variables determination

Variables	Cat. no.	Principle	Supplier
Total cholesterol	1489323	Enzymatic colorimetric	Roche Diagnostics
LDL-Cholesterol	1985604	Enzymatic colorimetric	Roche Diagnostics
HDL-Cholesterol	1930672	Enzymatic colorimetric	Roche Diagnostics
Triglycerides	1488872	Enzymatic colorimetric	Roche Diagnostics
Free fatty acids	FA115	Enzymatic colorimetric	Randox Laboratories Ltd

3.7.1.5 Full blood counts and C–reactive protein measurements

A full blood count was performed on the Coulter® MicroDiff 18, automatic cell counter. CRP is determined using the CRP Latex Reagent, Cat. No. 7003077 (ABX Diagnostics, France).

3.7.2 Anthropometric Measurements

Anthropometric variables included body mass index (BMI) and waist circumference. All the anthropometric measurements were performed by a paid field-worker, for standardisation purposes. The researcher trained the field-worker. The field-worker was a student in Dietetics at the University of the Free State and had prior experience taking anthropometric measurements.

BMI refers to a relationship of weight in kilogram to the square of the height in meter (Lee & Nieman, 2003)

BMI = Weight (kg) ÷ Height $(m)^2$

Weight: Body weight was measured according to standard method described by Lee & Nieman (2003) using a calibrated Seca digital electronic scale which weighs to the nearest 0.1 kg. The weight of the subjects wearing light clothing and no shoes was measured before blood samples were collected prior to breakfast, and after the subjects went to the bathroom. The weight was measured at baseline and after four weeks of supplementation.

Height: Standing height of subjects wearing light clothing and no shoes was measured to the nearest 0.5 cm using a stadiometer as described by Lee & Nieman (2003). The subjects stood with their feet together,

heels against the measuring board. They stood erect, neither slumped nor stretching, looking straight ahead, without tipping the head up or down. The top of the ear and outer corner of the eye were in a line parallel to the floor (~~F~~rankfort plane"). The top of the stadiometer was lowered to rest flat on the top of the head.

Waist Circumference is obtained by measuring the distance around the smallest area below the rib cage and above the umbilicus with the use of a non-stretchable tape measure (Gibson, 2005).

3.7.3 Blood Pressure

Blood pressure was recorded by a qualified nurse using a sphygmomanometer and a stethoscope, before blood sampling, using the method as described by De Bono and Boon (1991). The subjects were seated with their back supported. The cuff was applied to the right upper arm, with the bag over the branchial artery and connected to a mercury or aneroid manometer. The stethoscope was placed over the brachial artery and the cuff was inflated to a level well above that which abolishes the Korothov sounds. The pressure in the cuff was then allowed to drop slowly and the point of return of the sounds was taken as the systolic pressure. As the pressure drops further, the sounds become louder and then usually suddenly becomes muffled and later disappears, at which stage the diastolic pressure is measured. Three intermittent readings were taken at two-minute intervals, and the lowest value was recorded.

3.7.4 Questionnaires

It is important to acknowledge that this study was performed on exactly the same study population as a previous study, during which the principal investigator of this study was also involved, as a Masters student in Dietetics. The questionnaires used in this present study, are very similar to those used as part of the previous study. Yet, none of them are exactly the same and were the current questionnaires adapted for this study.

Questionnaires used in this study included:

i) a screening /recruitment questionnaire (Appendix B),

ii) a food frequency questionnaire (Appendix C) and

iii) a tolerance questionnaire (Appendix D).

Questionnaires were designed in collaboration with the University Free State and NESTEC PTY LTD. The questionnaires were tested during a pilot study (see section 3.10), on a group of random individuals from the study population, during which the average time to take each questionnaire was measured, as well as its clarity. All the questionnaires were completed during an interview by the main researcher and four trained, qualified dieticians.

3.7.4.1 Screening / recruitment questionnaire

The screening questionnaire was developed to select subjects with specific characteristics according to the inclusion and exclusion criteria of the study. Answers to questions regarding age, activity level, smoking habits, alcohol intake and a medical history were recorded. All the volunteers were from exactly the same socio-

economic background (based on income, where they live, working environment, etc.). The socio-demographic status of the respondents was therefore not a variable or considered relevant for the purpose of this study. All questions relating to socio-demographic information, were therefore included as part of the screening questionnaire.

3.7.4.2 Food Frequency Questionnaire (FFQ)

Nelson (2000) states that the purpose of dietary assessment is to estimate food consumption of dietary intake in individuals or groups of people. The dietary intake of the subjects in this study was measured by means of a FFQ adopted from the THUSA study (North-West University) and standardised for use in the African population in the Free State Province (Hatting, 2000). This FFQ was adjusted to include cultural food preferences and habits of the study population (Macintyre, Venter & Vorster, 2000). According to Dwyer (1998), the FFQ provides an overall picture of food intake. The FFQ were completed during and interview by the trained fieldworkers. In this study, the FFQ (Appendix C) was used exclusively to control for the homogeneity of the study population. No detailed analyses of the FFQ were performed, or related to any of the blood parameters, as it was not within the scope of this study. It is important here to acknowledge that all volunteers followed exactly the same meal plan, which was developed by the principal investigator of this study. A short discussion of the dietary intake of the study group will be provided in Chapter 4.

3.7.4.3 Tolerance questionnaire

The tolerance questionnaire (Appendix D) was completed at the end of the study to determine whether any of the subjects experienced any possible side effects or discomfort during the intervention period, whether suspected to be related to the supplements or not. Subjects were asked whether they experienced any of the following symptoms during the study: nausea, constipation, diarrhoea, decrease or increase in appetite, and whether the number of capsules was acceptable.

3.8 FIELDWORKERS AND STANDARDISATION OF TECHNIQUES

3.8.1 Pilot Study

A pilot study was conducted on a group of 20 volunteers chosen randomly from the study population. However, all measurements where simulated and no blood samples were taken from any of the volunteers. The FFQ was tested on five members of the 20 volunteers in order to ensure the clarity of quetsions. This simulation was performed in order to give the research team a clear indication of possible problem areas and identify time- and other practical limitations and implications. Based on the results of this study, the research group could assess how much time is required for each volunteer to progress through each separate assessment of the study. It was then also possible to establish the total number of subjects that could be

assessed on a daily basis. The pilot study was performed one month prior the actual study.

3.8.2 Fieldworkers

The fieldworkers used in the study included:

- A qualified nurse (registered with the South African Health Professions Council). The nurse worked within a general clinic setting, so required additional training to deal with the large number of blood samples taken from each volunteer, in addition to those samples that were specifically prepared for the study (and not part of general clinical investigations). The time limitations for blood sampling were also strictly adhered to (all samples were taken before 10 a.m. in order to minimise the impact of daily variation on the measured variables.
- A group of four trained medical personnel of the SANDF who were also responsible for taking the blood samples and measuring blood pressure. The group was trained with the study nurse.
- Four qualified dieticians (fourth-year students from the University of the Free State, Department of Human Nutrition) who were trained by the principal investigator of this study, to use the validated FFQ.
- A bilingual primary health care worker of the SANDF who helped with the translation of the questionnaires, when necessary. All questionnaires were thoroughly discussed with the Principal Investigator of this study, before starting the actual study.

- A post-graduate dietetics student trained specifically to take anthropometric measurements (for standardisation purposes the anthropometric data were collected only by one single individual).
- A full-time medical team on the premises of the SANDF. The subjects had 24 hour access to the medical team. The team itself did not participate in the study itself, but were aware that it is taking place and that any of the subjects could develop side-effects that require medical attention.

3.9 VALIDITY AND RELIABILITY OF MEASUREMENTS

Validity refers to the degree to which a research procedure or tool measures what it is supposed to measure (Mosdøl & Brunner, 2005; Gibson, 2005; Koh & Owen, 2000). Reliability refers to the degree to which the same results can be reproduced after repeating the measurement (Gibson, 2005). The validity and reliability of all laboratory analyses were discussed with their appropriate methodology, which include the use of commercially available standards (validity) and controls (reliability).

3.9.1 Anthropometric measurements

As discussed under section 3.7.2., anthropometric measurements were taken by a trained fieldworker in order to perform measurement technique correctly, and to ensure validity and reliability.

3.9.1.1 Validity

The validity of a test or instrument refers to its ability to measure the phenomenon it intends to measure (Monsen, 1992). To ensure validity in this study the appropriate measuring tools and techniques were used as recommended in the literature and discussed under section 3.7.2. The student was trained beforehand by a trained research biokineticist.

3.9.1.2 Reliability

The reliability of a test or instrument is determined by its consistency of results when applying to the same specimen repeatedly, administered by either the same or different persons. The reliability of physical examinations can be assessed by comparing the data from the same subjects gathered by two or more observers (Monsen, 1992). To ensure reliability in this study the fieldworkers used the same, standardised techniques as recommended by Lee & Nieman (2003). It was required of the fieldworker to repeat each measurement three time, after which an average was used as a final value.

3.9.2 Questionnaires

Dietary methods designed to characterise usual intakes of individuals are the most difficult to validate because the ~~"tr~~uth" is never known with absolute certainty. There is no guarantee that a subject's true usual food intake can be assessed with precision (Gibson, 2005).

A dietary assessment method is considered precise (i.e. reliable/reproducible) if it gives very similar results when repeated in the same situation (Koh & Owen, 2000).

3.9.2.1 Validity

The performance of any dietary assessment method is markedly influenced by the motivation and compliance of the respondents (Gibson, 2005). Careful attention must be given to the choice of foods, the clarity of the questions and the format of the frequency response section, when constructing the FFQ (Koh & Owen, 2000). In this study, a FFQ was used that was adjusted specifically for African population groups, based on results from the THUSA survey. This FFQ lists commonly consumed foods of the target population. Yet, it is important to acknowledge that the food given to the study population at the mess, classify as westernised, and not traditional. The FFQ were tested during the pilot study on five individuals from the same group at the SANDF and who did not participate in the study (but came from the same population). For the purpose of this study, the FFQ was only used as a measure of control.

3.9.2.2 Reliability

A FFQ is not sensitive to day-to-day variations in intake, because it is designed to assess the usual food intake of an individual over an extended period of time. Gibson (2005) suggests that the reproducibility of the FFQ is good. Reliability is influenced by the method of administration. Interviewer administration is likely to

appear more reliable than self-administration (Koh & Owen, 2000). The FFQ in the present study was completed by trained dieticians, but the principal researcher interpreted and coded the questionnaires. The portion sizes used in the FFQ were compared to the portion sizes used in the mess and three dimentional food models, portion sizes, cups, plates and spoons usually used in the mess were used to estimate and calculate exact portion sizes. The results from the FFQ could be compared to the actual intake of the population group. It is important to acknowledge that it is only the energy distribution of the macronutrients (including the dietary fibre intake) that is of importance here.

3.10 MANAGEMENT OF THE STUDY AND ROLE OF THE RESEARCHER

This study formed part of a commercial endeavour, funded by the NESTEC PTY LTD Research group, Lausanne, Switzerland. The study was performed by a group of individuals, headed by Prof. FJ Veldman, the then Director of the Fibrinogen Unit, School of Health Technology, Central University of Technology. The researcher of this study, as a registered dietitian with the South African Health Professions Council, working on the premises of the SANDF where this study was performed, was responsible for all activities at the premises of the SANDF (letters of permission, handing out the supplements, recruitment of volunteers, activities at the mess, etc.). Yet, is is important to acknowledge that this was a large study and required the assistance of other research personnel and field workers. In addition, the

Principle Investigator of this thesis actively participated in the laboratory analyses of biological material. Yet, it should also be appreciated that most of the analyses were performed using automated equipment, and that in such case, the investigator of this study was assisted by trained Biomedical Technology students from the School of Health Technology, Central University of Technology.

Daily management is an essential component of quality assurance (Dennis & Kris-Etherton, 1992). A strong, capable investigative team is the key to avoiding problems that might prejudice the study. Good management includes: organisation, communication, clear delineation and coverage of duties and responsibilities, contingency plans, and procedures for dealing with problems (Dennis & Kris-Etherton, 1992). The following management measures were taken to ensure that the objectives of the study were met:

- Volunteers were informed of the content of the study, the importance of their roles, as well as the practical arrangements that might help the flow of the study.
- A placebo group was included in the study to exclude the effect of other factors such as seasonal changes, etc. on the measured metabolic variables;
- A late breakfast was arranged at the mess after the blood samples were drawn to make sure that volunteers attended the sample collection in a fasting state;
- The Department of Biostatistics at the UFS randomly divided the subjects into the experimental and placebo groups;

- The capsules were counted beforehand by an outside party that neither participated in the project, nor in the execution of any aspects of the project. All capsules looked similar. Packages were numbered by an outsider and kept off-site. The blind information was made available once all the results were supplied to the statistician in charge. This ensured that both participants and researchers were blinded for the duration of the study.
- The SANDF section head of the members ensured that the capsules were taken daily. Capsules were kept in separate bags, clearly marked with individual numbers.
- If was crucial that the subjects be continuously motivated throughout the study. The following measures were taken by the researcher in order to keep the subjects motivated:
    - The researcher encouraged the members with each visit to take their supplements daily. The researcher gave the subjects supplements on a daily basis when they had their meals at the mess. This was also a measure of compliance. The bags were transparent and were marked clearly with each participant number. All capsules were exactly similar in shape and colour. Capsules were counted beforehand and the exact number of capsules was given to each participant on a daily basis.
    - Informal social functions for volunteers were arranged at regular intervals.
    - The subjects were followed up weekly by the researcher. This helped with the evaluation of the progress of the study, as well as

with the participation of the subjects; and also identified any unwanted but inevitable problems.

- At the end of the study, each subject received a gift in acknowledgement for taking part in the study.

3.11 STATISTICAL ANALYSES

Results were analysed by the Research Centre, NESTEC PTY LTD., Lauzanne. Results were summarised using means and standard deviations (SD), and frequencies and percentages (categorical variables). Changes within groups, from baseline one (day 0) to baseline two (day 8); and from baseline 2 (day 8) to the end of the intervention period (day 36), were compared using paired-t-tests, using a p-level of <0.05 as significant. Level of significance: The chosen level of significance sets the likelihood of detecting a treatment effect when no effect exists (leading to a so-called "false-positive" result) and defines the threshold "*P* value". Results with a *P* value above the threshold lead to the conclusion that an observed difference may be due to chance alone, while those with a *P* value below the threshold lead to rejecting chance and concluding that the intervention has a real effect. The level of significance in this study was set at 5% (that is, $P = 0.05$). This means the investigator is prepared to accept a 5% chance of erroneously reporting a significant effect.Between-group differences were compared using the student t-test, also using a p-level of <0.05 as significant. Power calculations were performed to establish the minimum number of volunteers required to participate in each study group. Calculations indicated that 15 individuals are

required for each of the three intervention groups. The power of a study is its ability to detect a true difference in outcome between the standard or control arm and the intervention arm. This is usually chosen to be 80%. By definition, a study power set at 80% accepts a likelihood of one in five (that is, 20%) of missing such a real difference. Thus, the power for large trials is occasionally set at 90% to reduce to 10% the possibility of a so-called "false-negative" result. The effect of treatment in a trial can be expressed as an absolute difference. That is, the difference between the rate of the event in the control group and the rate in the intervention group, or as a relative reduction, that is, the proportional change in the event rate with treatment. If the rate in the control group is 6.3% and the rate in the intervention arm is 4.2%, the absolute difference is 2.1%; the relative reduction with intervention is 2.1%/6.3%, or 33%. In this study, the effect of treatment was fixed at 15%. From these components, sample size can be calculated. Based on these calculations, it was decided to recruit 25 volunteers for each group, taking into consideration that not all volunteers will complete the study.

In this chapter, the author provides a concise summary of all methods, procedures and materials used throughout the study. In the next Chapter, the results of the different analyses will be provided, followed by a discussion thereof in chapter 5.

CHAPTER 4

RESULTS

4.1 INTRODUCTION

Chapter 3 was used to describe the experimental procedures of a clinical intervention trial during which the metabolic effects of the long-term intake of two different combinations of short-chain fatty acid supplements were tested on westernised black male volunteers. The results of this randomised clinical intervention trial will be presented in this chapter. The aim is now to describe the baseline characteristics of the subject group as a whole, as well as the metabolic changes measured in physical- and metabolic risk markers, during different intervals after intake of the respective supplements.

A summary of the baseline measurements (day 0 and 8) for the entire study group will be presented, followed by the results of the individual supplements, measured at the separate intervals. The results are summarised as follows: (i) general health profiles, (ii) haemostatic profiles, (iii) lipid profiles, and (iv) glycometabolic profiles.

4.2 BASELINE RESULTS OF THE ENTIRE STUDY GROUP

Two baseline measurements were performed for each volunteer. Each measurement was separated by a time-interval of seven days, during which subjects consumed placebo supplements for standardisation purposes. The results of the two baseline measurements will be presented in order to provide information regarding the health status of the study population as a whole (Tale 4.1). The reference ranges of the different variables are included as an aid to judging the results.

4.2.1 Demographic characteristics of the study group as a whole

All the volunteers of this study were members of the South African National Defence Force (SANDF), Tempe, having been based in Bloemfontein for at least one year prior the study. The subjects all lived on the premises of the SANDF, and followed exactly the same basic diet, supplied at the same mess on the premise of the SANDF. The researcher monitored the food intake of the study group in order to limit any major changes of dietary intake during the study, as well as for general control purposes.

4.2.2 Dietary intake of the entire study group

The dietary intake of the study group was determined by means of a FFQ at the start of the study (day 0) and at the end of the intervention washout period (day 36) as a measure of control. It is important to acknowledge that what is of importance here, is not the dietary intake

of the respective supplementation groups, but rather that of the group as a whole. The random selection of individuals allocated to specific supplementation groups, would also imply that a change of dietary intake within the group as a whole, would be equally distributed between the respective supplementation groups. All subjects followed exactly the same meal plan during the entire study. It was therefore important to evaluate whether the dietary intake of the study group remained constant throughout the study.

The results of the FFQ showed that at baseline the mean energy intake of the subjects was ~16 570kJ per day, with the total mean carbohydrate intake below 50% of the total energy intake. The dietary protein intake contributed to approximately 16% of the total daily energy intake. The total mean fat intake of the group was higher than 30% of their total energy intake. The mean dietary fibre intake of the study group was reported as 32.5g per day. A tendency was therefore observed towards a high fat (>30% of total energy) and low carbohydrate intake (< 50% of total energy), which is characteristics of an atherogenic, westernised diet. Assessment of the dietary intake at the end of the study showed that there was no significant change in dietary intake during the study. The results indicated that the usual dietary intake of the subjects did not differ significantly and remained somewhat similar from baseline (day 0) to the end of supplementation (day 36), with an almost similar energy intake of approximately 16 000kJ per day.

4.2.3 General health profiles of the entire study group for both baseline 1 and 2 (Table 4.1)

All subjects were male, between the ages of 18 and 45 years. The mean age of the study group was 27 years. None of the subjects used any chronic medication, had a history of previous lifestyle illness (such as coronary heart disease, diabetes) or had known or visible physical disabilities prior to the study. A total of 66 suject were include in the results

No overweight or obese subjects were included (see section 3.6.1) in the study group. Anthropometric measurements, blood cell counts and CRP concentrations of the two baseline visits for most of the study group were within the normal reference range, except for a few individual outliers. However, none of these outliers could be classified as clinically abnormal and there was no reason to exclude them from the study. The results of baseline one were comparable to those of baseline two, with no statistically significant differences between the two visits, for any variable (Table 4.1).

Table 4.1 General health profiles of the entire study group for both baseline 1 and 2

VARIABLE	Normal Range	Baseline 1 (Day 0) n= 66				Baseline 2 (Day 8) n = 66			
		X	SD	Min	Max	X	SD	Min	Max
Length (cm)		170.0	6.5	157.0	189.0				
Weight (cm)		66.3	8.9	51.4	87.5	67.2	8.9	52.3	86.9
BMI (kg/m^2)		22.8	2.3	17.3	29.0	23.0	2.4	18.0	29.5
Waist circumference (cm)		77.2	6.9	66.0	94.0	77.7	7.1	66.0	94.0
Hip circumference (cm)		95.4	5.9	74.0	110.0	95.9	6.2	74.0	110.0
SBP (mmHg)	125±13.6[a]	118	11	90	160	118	12	91	162
DBP (mmHg)	78±9.9 [a]	81	16	60	130	83	12	60	130
TP (g/L)	60-82	80.5	6.30			79.9	5.60		
Albumin (g/L)	34-48	45.20	3.80			44.80	2.60		
WBC (x10^3/µL)	2.5-8.5[b]	5.95	2.05	3.20	12.00	5.66	1.51	2.90	9.00
RBC (x10^3/µL)	4.5-5.9 [b]	5.16	0.53	4.31	6.77	5.17	0.57	4.25	6.93
Hb (g/dL)	13.7-17.8 [b]	15.9	1.1	13.0	18.1	15.8	1.5	12.8	22.6
Haematocrit (%)	41-52 [b]	45.8	4.1	37.9	60.2	45.2	4.1	35.7	61.0
PLT (x10^3/µL)	50-400 [b]	281	56	182	396	297	67	149	498
CRP (mg/dL)	0 - 0.3[c]	0.22	0.29	0.01	1.35	0.27	0.33	0.01	1.91

(X = mean; SD = standard deviation; BMI = Body Mass Index; SBP = Systolic Blood Pressure; DBP = Diastolic Blood Pressure; TP = Total Protein; WBC = White Blood Cell Count; RBC = Red Blood Cell Count; CRP = C-reactive protein; Hb = Haemoglobin; PLT = Platelet count).

a = Solomon, Schmidt & Adragna, 1990; **b =** Department Haematology and Cell Biology, University of the Free State & NHLS, 2003; c = Department of Chemical Pathology, University of the Free State & NHLS, 2003.

4.2.4 Haemostatic profiles of the entire study group for both baseline 1 and 2 (Table 4.2)

Haemostatic profiles for both baseline visits of the group as a whole fell within the normal reference range. No clinical reference values for markers of fibrin network architecture are available. Due to cost implications, D-Dimer concentrations were only measured from baseline two. The results of baseline one was comparable to that of baseline two, with no statistically significant changes that took place within any variable between the two visits.

Table 4.2 Haemostatic profiles of the study group as a whole for both baseline 1 and 2

VARIABLE	Normal Range	Baseline 1 (Day 0) n = 66				Baseline 2 (Day 8) n = 66			
		X	SD	Max	Min	X	SD	Max	Min
FVII (%)	50-150 [b]	101	8	121	82	103	10	122	85
FVIII (%)	50-150 [b]	88	8	108	73	92	11	116	76
Fibrinogen (g/L)	2.0-4.0 [b]	2.77	0.91	4.93	1.80	3.28	1.26	6.72	1.56
ATIII (%)	76-122 [b]	113	12	132	78	113	13	133	83
DDimer (µg/dL)	<500 [b]					98	70	271	26
Fibrin Monomer (mg/L)		13.6	1.8	19.2	10.5	13.7	2.4	20.5	9.2
Compaction (%)		16	6	26	8	14	5	23	7
µT $x10^{12}$Da/cm		11.8	6.3	32.3	2.7	7.2	5.6	28.1	2.0
Network Fibrin Content (g/L)		2.0	0.5	1.0	3.8	2.9	1.3	5.8	1.0

(X = mean; SD = standard deviation; FVII = blood clotting factor VII; FVIII = blood clotting factor FVIII; ATIII= antithrombin III; µT = mass/length-ratio of fibrin strands in fibrin networks)
b = Department Haematology and Cell Biology, University of the Free State & NHLS, 2003.

A graphic presentation of the changes that occurED during fibrin network lysis, as measured by means of turbidimetry, for both baseline 1 and 2 of the group as a whole, is provided in Figure 4.1. Similar rates of network lysis were measured up until 10 minutes, after which the curves both plateau at 180 minutes. However, in comparison with baseline one, baseline two plateaus at a higher absorbance.

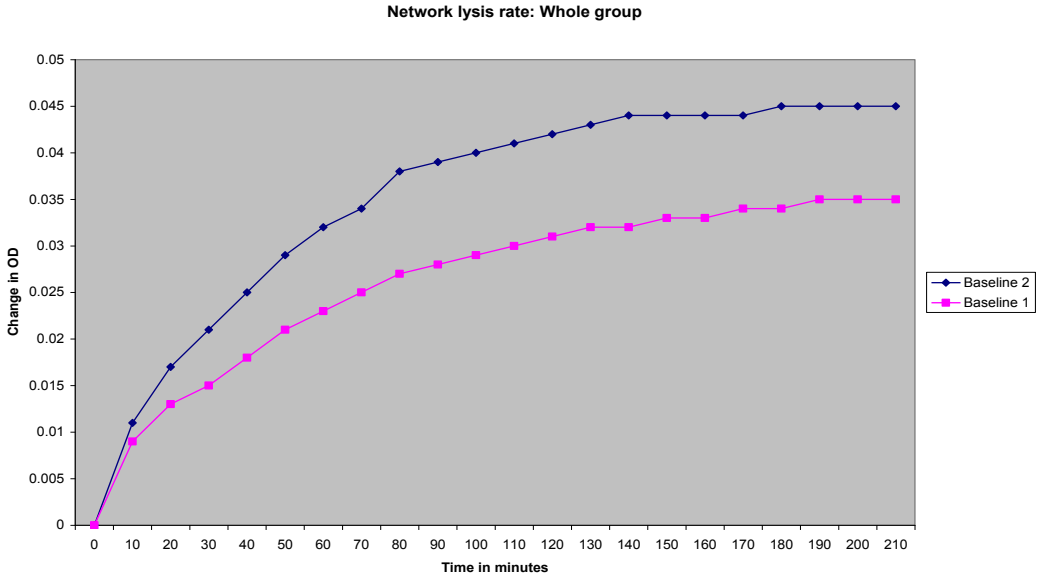

Figure 4.1 A turbidimetric presentation of the mean fibrin network lysis rate of the entire study group at baseline one and two (Day 0 and 8)

4.2.5 Lipid profiles of the entire study group for both baseline 1 and 2 (Day 0 and 8)

Mean lipid measurements of the two baseline visits for the entire group fell within the normal reference range. Subjects with outlier values were not considered clinically abnormal, and therefore not excluded from the study. The results of baseline one was comparable to those

of baseline two, with no statistically significant differences measured between the two visits (Table 4.3).

Table 4.3 Blood lipid profiles of the study group as a whole for both baseline 1 and 2

VARIABLE	Normal Range	Baseline 1 (Day 0) n = 66				Baseline 2 (Day 8) n = 66			
		X	SD	Max	Min	X	SD	Max	Min
TC (mmol/L)	2.10-5.32	4.36	0.88	6.3	2.3	4.46	0.84	7.2	2.6
TG (mmol/L)	0.30-1.70 [c]	1.17	0.50	2.21	0.50	1.17	0.61	2.72	0.53
HDL-C (mmol/L)	0.75-1.9 [c]	1.41	0.47	3.6	0.7	1.24	0.28	2.0	0.7
LDL-C (mmol/L)	2.24-4.6 [c]	2.35	0.83	4.51	0.8	2.67	0.78	3.64	1.24
% HDL-C (%)		33.7	9.5	65.5	16.4	28.5	7.3	47.1	14.8
NEFA (mmol/L)		4.78	2.74	9.10	1.40	3.68	1.70	9.10	1.20

(X = mean; SD = standard deviation; TC = serum-Total Cholesterol; TG = serum-Triglycerides; HDL-C = serum-High-Density Lipoprotein Cholesterol; LDL-C = serum-Low-Density Lipoprotein Cholesterol; %HDL-C = HDL-C/TC * 100; NEFA = Non-Esterified Fatty Acids, The measurement unit for all the lipid variables was mmol/L except for % HDL-C which was percentage) [c] = Department of Chemical Pathology, University of the Free State & NHLS, 2003.

4.2.6 Glycometabolic profiles of the entire study group for both baseline 1 and 2 (Day 0 and 8)

The fasting serum-glucose levels and fasting serum-insulin levels of the study group as a whole fell within the normal healthy reference range (Table 4.4).

Table 4.4 Glycometabolic indicators of the study group as a whole for both baseline 1 and 2

VARIABLE	Normal Range	Baseline 1 (Day 0) n =66				Baseline 2 (Day 8) n = 66			
		X	SD	Max	Min	X	SD	Max	Min
Glucose (mmol/L)	4.1-5.9 [c]	5.32	0.83	6.12	3.25	5.47	0.88	6.30	3.88
Insulin (µIU/mL)	6-27[c]	10.7	16.6	28.0	4.8	13.7	21.3	5.20	40.2

(X = mean; SD = standard deviation;)
[c] = Department of Chemical Pathology, University of the Free State & NHLS, 2003.

4.3 RESULTS OF THE INTERVENTION STUDY

This section will be used to present the results measured in metabolic risk markers after supplementation with the placebo and two different short-chain fatty acid supplements. Results for short-term changes (after 14 days of supplementation), at the end of supplementation changes (after 28 days of supplementation) and an additional washout period of one week will be reported, for each supplement. Attention will mainly be given to the differences between day 8 (baseline 2; start of supplementation) and day 36 (at the end of 4 weeks supplementation).

A full-time medical team on the premises of the SANDF monitored any apparent changes in the health of the subjects. Subjects had access to this team both during and after the study.

Any drugs, medication or supplements taken during the intervention phase were reported on the individual subject files. Subjects were examined for any possible side effects that may have been directly or

indirectly caused by the respective supplements (allergic reactions, abdominal iscomfort, nausea, etc.). In addition, subjects were asked to report side effects, observations, or anything out of the ordinary. Records were kept of these reports on the tolerance questionnaire (Appendix D).

4.3.1 The Placebo Supplement Study Group Results

The group consumed a placebo for the entire study period. The group composed of 25 volunteers, of which only 21 completed the study.

4.3.1.1 Changes in the general health profiles within the placebo supplement study group

No significant changes were measured in any of the general health indicators from day 8 (start of supplementation phase) to day 36 (end of supplementation phase) within the placebo supplement group (Table 4.5).

Table 4.5 Changes in the general health profiles within the placebo supplement study group (n = 21)

VARIABLE		Day 1	Day 8	Day 22	Day 36	Day 43
Length	**X**	171.0				
(cm)	**SD**	6.6				
Weight	**X**	69.0	70.0	70.3	9.8	70.0
(kg)	**SD**	10.6	10.2	11.3	10.4	10.5
BMI	**X**	23.5	23.8	24.0	23.8	23.9
(kg/m^2)	**SD**	3.1	3.0	3.2	2.9	3.0
Waist	**X**	79.8	80.7	79.1	79.11	79.1
(cm)	**SD**	8.6	8.5	8.1	8.3	8.0
Hip	**X**	96.2	96.8	97.9	97.5	98.2
(cm)	**SD**	8.4	8.7	6.3	6.2	6.7
SBP	**X**	120	124	122	116	116
(mmHg)	**SD**	14	14	13	11	10
DBP	**X**	85	87	85	83	83
(mmHg)	**SD**	15	15	15	10	11
TP	**X**	76.8	79.8	77.5	77.0	79.7
(g/L)	**SD**	4.7	3.4	6.6	4.4	4.9
Albumin	**X**	43.9	45.1	43.9	43.9	45.8
(g/L)	**SD**	2.1	2.8	3.4	2.3	1.7
WBC	**X**	6.24	5.91	5.27	5.29	5.82
($x10^3/\mu L$)	**SD**	2.06	1.62	1.52	1.24	1.79
RBC	**X**	5.25	5.10	5.00	5.00	5.19
($x10^3/\mu L$)	**SD**	0.44	0.50	0.52	0.42	0.62
Hb	**X**	16.1	15.3	15.2	15.6	15.8
(g/dL)	**SD**	1.1	1.0	1.3	1.2	1.2
Haematocrit	**X**	46.4	44.2	43.3	43.6	44.9
(%)	**SD**	3.6	3.0	3.1	3.2	3.6
Platelets	**X**	307	304	296	300	348
($x10^3/\mu L$)	**SD**	58	66	61	56	52
CRP	**X**	0.17	0.24	0.21	0.11	0.16
(mg/dL)	**SD**	0.17	0.15	0.14	0.12	0.23

(X = mean; SD = standard deviation; BMI = Body Mass Index; SBP = Systolic Blood Pressure; DBP = Diastolic Blood Pressure; TP = Total Protein WBC = White Blood Cell Count; RBC = Red Blood Cell Count; Hb = Haemoglobin; CRP = C-reactive protein)

4.3.1.2 Changes in the haemostatic profiles within the placebo supplement study group

A statistically significant decrease ($p = 0.047$) in the circulating plasma fibrinogen concentration was measured from the start of supplementation (day 8) to the end of supplementation (day 36) within

the placebo supplement group (Table 4.6). No other significant changes in any of the other haemostatic variables were measured.

Table 4.6 Changes in the haemostatic profiles within the placebo supplement study group (n=21)

VARIABLE		Day 1	Day 8	Day 22	Day 36	Day 43
FVII activity	**X**	102.3	104	99	100	99
(%)	**SD**	9	8	12	7	10
FVIII activity	**X**	89	94	87	89	90
(%)	**SD**	10	10	8	7	10
Fibrinogen	**X**	2.80	3.39*	2.80	2.79*	2.90
(g/L)	**SD**	0.88	1.17	0.71	0.76	1.24
Albumin/	**X**	17.2	15.5	16.8	16.8	19.2
Fibrinogen	**SD**	4.7	5.2	4.9	4.1	7.2
ATIII	**X**	114	114	109	108	110
(%)	**SD**	10	10	9	11	10
D-Dimer	**X**		89.9		138.7	
(µg/dL)	**SD**		64.2		126.0	
Fibrin	**X**	13.2	13.4	12.3	12.2	12.4
Monomer	**SD**	1.4	3.0	2.5	3.1	3.7
(mg/L)						
Compaction	**X**	18	14	15	14	15
(%)	**SD**	7	6	5	4	5
µT (x10^{12}	**X**	11.8	7.4	5.5	5.0	5.8
Da/cm)	**SD**	5.6	5.1	2.5	2.0	2.9
Network Fibrin	**X**	2.6	2.9	2.0	2.7	2.6
Content (g/L)	**SD**	0.9	1.4	0.6	1.6	1.9

(X = mean; SD = standard deviation; FVII = blood clotting factor VII; FVIII = blood clotting factor FVIII; ATIII= antithrombin III; CRP = C-reactive Protein; FM = Fibrin Monomer; µT = mass/length-ratio of fibrin strands in fibrin networks; NFC = Fibrin Content of Fibrin Networks; * = differs significantly with $p<0.05$; ** = differs significantly with $p<0.01$)

A graphic presentation (Figure 4.2) of the turbidimetric changes that occured during fibrin network lysis, for all visits for the placebo supplemented group, followed more or less the same pattern. Similar rates of lysis were initially measured up until 20 minutes, after which the graphs all reached a plateau at 190 minutes. However, in comparison to all the other study periods, day 22 (after 2 weeks of experimental placebo supplementation) reached the plateau phase at a much lower absorbance.

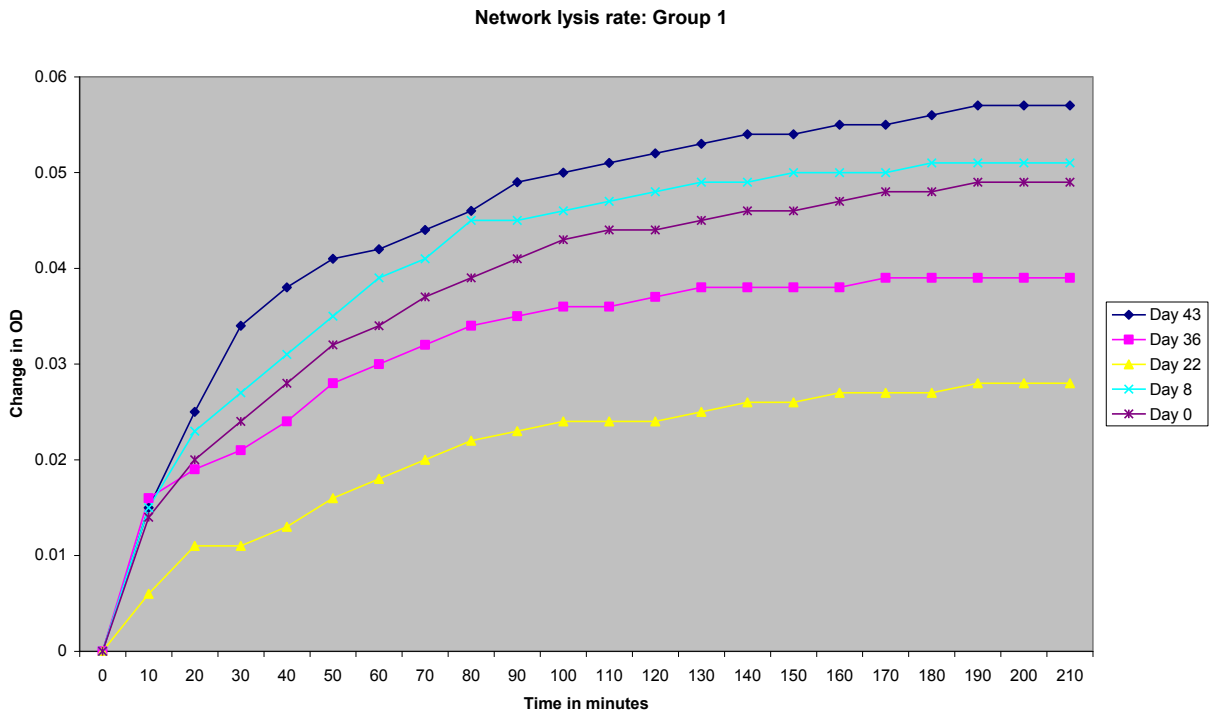

Figure 4.2 A turbidimetric presentation of the mean fibrin network lysis rates of the placebo group for the entire study period

4.3.1.3 Changes in the lipid profiles within the placebo supplement study group

Compared to baseline (day 8) no significant changes in any of the lipid variables were measured at the end of supplementation (day 36) within the placebo supplemented study group (Table 4.7).

Table 4.7 Changes in the lipid profiles within the placebo supplement study group (n = 21)

VARIABLE		Day 1	Day 8	Day 22	Day 36	Day 43
TC	**X**	4.21	4.31	4.29	4.32	4.43
(mmol/L)	**SD**	0.95	0.70	0.97	0.47	0.89
TG	**X**	1.26	1.30	1.12	1.27	1.18
(mmol/L)	**SD**	0.40	0.84	0.47	0.41	0.75
HDL-C	**X**	1.22	1.18	1.34	1.26	1.44
(mmol/L)	**SD**	0.27	0.27	0.27	0.25	0.31
LDL-C	**X**	2.27	2.47	2.42	2.47	2.55
(mmol/L)	**SD**	0.73	0.65	0.76	0.50	0.79
%HDL-C	**X**	31.3	28.1	32.3	29.6	32.9
(%)	**SD**	7.1	7.8	7.1	6.5	8.8
NEFA	**X**	4.99	3.83	3.68	3.59	3.66
(mmol/L)	**SD**	1.94	1.62	1.99	2.16	2.44

(X = mean; SD = standard deviation; TC = serum-Total Cholesterol; TG = serum-Triglycerides; HDL-C = serum-High-Density Lipoprotein Cholesterol; LDL-C = serum-Low-Density Lipoprotein Cholesterol; %HDL-C = HDL-C/TC * 100; NEFA = Non-Esterified Fatty Acids)

4.3.1.4 Changes in the glycometabolic profiles within the placebo supplement study group

No significant changes in any of the glycometabolic parameters of the placebo group were measured during the entire intervention period. The measured decrease in serum-insulin levels from day 8 to day 22 was not significant (Table 4.8).

Table 4.8 Changes in the glycometabolic profiles within the placebo supplement study group (n = 21)

VARIABLE		Day 1	Day 8	Day 22	Day 36	Day 43
Glucose	**X**	5.08	5.65	5.12	5.15	5.52
(mmol/L)	**SD**	0.57	1.16	0.93	1.21	1.07
Insulin	**X**	8.30	10.10	21.60	9.10	14.5
(µIU/mL)	**SD**	8.90	14.90	20.70	15.30	9.70

(X = mean; SD = standard deviation)

4.3.2 The Acetate-Propionate (50%/50%) Supplement Study Group Results

This study group consisted of 25 volunteers that consumed placebo supplement for a period of one week, followed by four weeks of experimental supplementation, which composed of 50% acetate and 50% propionate salts, after which an additional one week of supplementation with placebo followed. Two volunteers dropped out, bringing the number to 23 participants.

4.3.2.1 Changes in the general health profiles within the acetate-propionate supplement study group

No significant changes were observed in any of the general health indicators between start of supplementation (day 8) and end of supplementation (day 36) for the acetate-propionate supplement group (Table 4.9).

Table 4.9 Changes in the general health profiles within the acetate-propionate (50%-50%) supplement group (n = 23)

VARIABLE		Day 1	Day 8	Day 22	Day 36	Day 43
Length	**X**	170.0				
(cm)	**SD**	6.2				
Weight	**X**	65.1	66.1	65.3	65.7	66.6
(kg)	**SD**	7.4	8.2	8.1	7.8	7.5
BMI	**X**	22.6	22.9	22.8	22.8	23.0
(kg/m^2)	**SD**	1.9	2.1	2.1	2.1	1.9
Waist	**X**	75.5	76.4	75.9	76.5	75.6
(cm)	**SD**	5.2	5.1	5.5	4.9	4.8
Hip	**X**	95.5	96.5	96.2	96.4	96.0
(cm)	**SD**	4.3	5.1	5.1	4.7	4.8
SBP	**X**	120	116	117	116	118
(mmHg)	**SD**	10	12	7	8	8
DBP	**X**	81	81	79	79	83
(mmHg)	**SD**	9	9	7	8	9
TP	**X**	82.10	78.90	79.90	77.80	77.40
(g/L)	**SD**	6.30	6.10	7.40	9.50	8.10
Albumin	**X**	44.80	43.90	44.70	46.46	43.50
(g/L)	**SD**	3.20	2.50	3.90	3.30	2.40
WBC	**X**	5.96	5.78	5.89	5.88	5.65
($x10^3/\mu L$)	**SD**	2.47	1.42	1.30	1.63	1.43
RBC	**X**	5.33	5.32	5.17	5.33	5.23
($x10^3/\mu L$)	**SD**	0.65	0.63	0.49	0.54	0.56
Hb	**X**	16.2	16.1	15.5	15.7	15.6
(g/dL)	**SD**	1.2	1.9	1.3	1.2	1.5
Haematocrit	**X**	47.3	46.1	44.4	45.6	44.8
(%)	**SD**	5.0	5.4	4.3	4.4	4.5
Platelets	**X**	264	301	282	331	340
($x10^3/\mu L$)	**SD**	50	79	85	83	90
CRP	**X**	0.23	0.25	0.28	0.27	0.15
(mg/dL)	**SD**	0.29	0.28	0.24	0.49	0.18

(X = mean; SD = standard deviation; BMI = Body Mass Index; SBP = Systolic Blood Pressure; DBP = Diastolic Blood Pressure; WBC = White Blood Cell Count; RBC = Red Blood Cell Count; Hb = Haemoglobin; CRP = C-reactive protein)

4.3.2.2 Changes in the haemostatic profiles within the acetate-propionate supplement study group

A statistically significant decrease in plasma factor VII activity ($p=0.016$), antithrombin III ($p<0.001$), an increase in plasma fibrinogen concentration ($p=0.048$), and a decrease in the albumin/fibrinogen ratio ($p=0.044$), was measured from day 8 to day 36 within the

acetate-propionate supplementation group (Table 4.10). No other changes in any of the other haemostatic variables were significant.

Table 4.10 Changes in the haemostatic profiles within the acetate-propionate (50%-50%) study group (n = 23)

VARIABLE		Day 1	Day 8	Day 22	Day 36	Day 43
FVII activity	**X**	101	102*	99	97*	98
(%)	**SD**	7	7	7	7	8
FVIII activity	**X**	89	91	90	91	89
(%)	**SD**	6	11	11	8	8
Fibrinogen	**X**	2.90	2.98*	2.58	3.04*	2.69
(g/L)	**SD**	1.15	0.96	0.61	0.89	0.70
Albumin/	**X**	17.8	17.4*	17.9	15.7*	18.6
Fibrinogen	**SD**	5.7	5.9	3.9	4.7	5.5
ATIII	**X**	113	114**	107	108**	109.1
(%)	**SD**	13	13	7	10	8
D-Dimer	**X**		118.6		121.2	
(µg/dL)	**SD**		89.3		75.7	
FM	**X**	13.5	13.7	12.4	12.0	12.6
(mg/L)	**SD**	1.3	2.1	2.3	2.2	2.6
Compaction	**X**	16	14	15	14	15
(%)	**SD**	6	5	4	4	4
µT	**X**	13.0	8.5	8.0	6.7	5.7
(x10^{12} Da/cm)	**SD**	7.4	7.5	5.7	2.3	3.3
Network Fibrin	**X**	1.8	3.0	2.5	2.6	2.7
Content (g/L)	**SD**	0.6	1.1	0.8	1.3	1.5

(X = mean; SD = standard deviation; FVII = blood clotting factor VII; FVIII = blood clotting factor FVIII; ATIII= antithrombin III; CRP = C-reactive Protein; FM = Fibrin Monomer; µT = mass/length-ratio of fibrin strands in fibrin networks; NFC = Fibrin Content of Fibrin Networks; * = differs significantly with $p<0.05$; ** = differs significantly with $p<0.01$)

A turbidimetric presentation of the changes in the network lysis rate of all the study periods, for the acetate-propionate study group, showed a sharp rise in optical density for the first 40 minutes and reaching a plateau phase at approximately 190 minutes, except for day 22, where the maximum plateau phase was reached at 240 minutes (Figure 4.3). In comparison with the other study periods day 1 reached a much lower maximum absorbance, while day 43 reached the highest maximum absorbance.

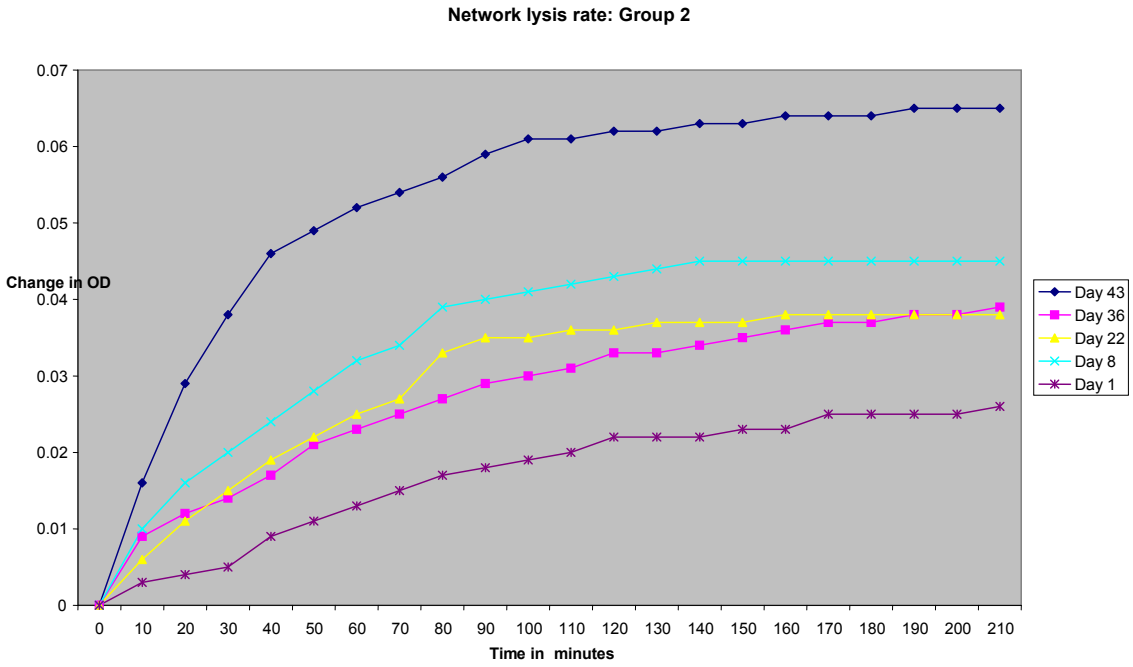

Figure 4.3 A turbidimetric presentation of the mean fibrin network lysis rate of the acetate-propionate group for the entire study period

4.3.2.3 <u>Changes in the lipid profiles within the acetate-propionate supplement group</u>

A slight decrease in the LDL-C from day 8 to day 36 within the acetate-propionate supplement group was measured. The serum-TC levels of this group remained unchanged. However, a statistically significant increase in HDL-C ($p<0.05$) accompanied by a statistically significant decrease in NEFA ($p<0.01$) were found during the same period for this group of subjects (Table 4.11).

Table 4.11 Changes in the lipid profiles within the acetate-propionate (50%-50%) supplement group (n = 23)

VARIABLE		Day 1	Day 8	Day 22	Day 36	Day 43
TC	X	4.37	4.14	4.82	4.25	3.92
(mmol/L)	SD	0.96	0.81	1.00	1.77	0.80
TG	X	1.05	1.01	1.10	1.09	0.98
(mmol/L)	SD	0.4	0.39	0.42	0.36	0.27
HDL-C	X	1.39	1.21*	1.37	1.35*	1.37
(mmol/L)	SD	0.36	0.24	0.32	0.34	0.31
LDL-C	X	2.43	2.37	2.37	2.31	2.10
(mmol/L)	SD	0.90	0.58	0.32	0.71	0.71
%HDL-C	X	33.70	30.56	30.10	33.53	35.80
(%)	SD	9.40	6.14	7.90	6.38	8.70
NEFA	X	5.01	5.02**	4.06	4.14**	2.43
(mmol/L)	SD	2.23	1.72	2.38	3.43	1.18

(X = mean; SD = standard deviation;TC = serum-Total Cholesterol; TG = serum-Triglycerides; HDL-C = serum-High-Density Lipoprotein Cholesterol; LDL-C = serum-Low-Density Lipoprotein Cholesterol; %HDL-C = HDL-C/TC * 100; NEFA = Non-Esterified Fatty Acids;* = differs significantly with $p<0.05$; ** = differs significantly with $p<0.01$)

4.3.2.4 Changes in the glycometabolic profiles within the acetate-propionate supplement group

The fasting serum-glucose levels of this group decreased significantly ($p < 0.05$) from baseline (day 8) to the end of the experimental supplementation (day 36) (Table 4.12).

Table 4.12 Changes in the glycometabolic indicators of the acetate-propionate (50%-50%) supplement group (n = 23)

VARIABLE		Day 1	Day 8	Day 22	Day 36	Day 43
Glucose	X	5.87	6.65*	5.77	5.65*	5.03
(mmol/L)	SD	0.60	0.98	0.94	0.42	0.53
Insulin	X	7.90	12.62	7.10	14.01	13.80
(μIU/mL)	SD	4.60	22.61	5.50	24.83	9.70

(X = mean; SD = standard deviation; * = differs significantly with $p<0.05$)

4.3.3 The High Acetate Supplement Group Results

The baseline results of this group were comparable to the results of the other two groups. Three members of this group dropped out of the

study. This group of volunteers also consumed placebo supplements for a period of one week, followed by four weeks of supplementation with 70% acetate, 15% propionate and 15% butyrate, concluded by one additional week of placebo supplementation.

4.3.3.1 Mean changes in the general health indicators of the high acetate supplement study group

Table 4.13 Changes in the general health profiles within the high acetate supplement group (n = 22)

VARIABLE		Day 1	Day 8	Day 22	Day 36	Day 43
Length	**X**	170.6				
(cm)	**SD**	7.2				
Weight	**X**	65.0	65.6	66.6	65.9	66.9
(kg)	**SD**	8.2	8.3	8.2	8.6	7.5
BMI	**X**	22.3	22.5	22.6	22.6	22.7
(kg/m^2)	**SD**	1.8	1.8	1.9	1.8	1.5
Waist	**X**	76.4	76.1	75.6	75.7	77.4
(cm)	**SD**	5.9	6.7	8.5	6.6	6.3
Hip	**X**	94.6	94.2	93.9	94.1	95.2
(cm)	**SD**	4.4	4.0	8.0	4.3	4.5
SBP	**X**	115	115	118	116	116
(mmHg)	**SD**	7	8	9	8	6
DBP	**X**	81	80	80	82	81
(mmHg)	**SD**	8	8	10	7	7
TP	**X**	81.9*	81.1**	79.7	75.9**	78.2
(g/L)	**SD**	6.5	6.2	6.4	8.4	5.2
Albumin	**X**	46.8*	45.3*	44.7	43.8**	45.4
(g/L)	**SD**	4.7	4.4	3.9	3.1	2.4
WBC	**X**	5.68	5.27	6.08	5.09	5.34
($x10^3/\mu L$)	**SD**	1.61	1.6	2.17	1.70	1.51
RBC	**X**	4.90	5.05	5.10	5.25	5.19
($x10^3/\mu L$)	**SD**	0.39	0.58	0.52	0.60	0.82
Hb	**X**	15.5	16.0	16.1	16.1	15.9
(g/dL)	**SD**	0.9	1.3	1.6	1.3	1.8
Haematocrit	**X**	43.7	45.1	45.0	46.0	45.9
(%)	**SD**	2.7	4.4	4.0	4.0	4.6
Platelets	**X**	273	285	299	297	298
($x10^3/\mu L$)	**SD**	53	60	45	67	81
CRP (mg/dL)	**X**	0.28	0.32	0.21	0.24	0.21
	SD	0.42	0.50	0.23	0.36	0.28

(X = mean; SD = standard deviation; BMI = Body Mass Index; SBP = Systolic Blood Pressure; DBP = Diastolic Blood Pressure; WBC = White Blood Cell Count; RBC = Red Blood Cell Count; CRP = C-reactive protein, * = differs significantly with $p<0.05$; ** = differs significantly with $p<0.01$)

No significant changes were measured in most of the general health indicators between day 8 (start of supplementation) and day 36 (end of supplementation) within the high acetate supplement group. Total Protein and albumin levels decresed significantly (Table 4.13).

4.3.3.2 Changes in the haemostatic profiles within the high acetate supplement study group

A statistically significant decrease in plasma factor VII activity ($p=0.018$), factor VIII activity ($p=0.011$), antithrombin III ($p<0.001$), fibrin monomer concentration ($p=0.002$) and percentage compaction of the fibrin networks ($p=0.008$) was measured between the start of

Table 4.14 Changes in the haemostatic profiles within the high acetate supplement group (n = 22)

VARIABLE		Day 1	Day 8	Day 22	Day 36	Day 43
FVII activity (%)	**X**	101	103*	101	101*	99
	SD	8	14	7	6	8
FVIII activity (%)	**X**	86	93*	90	88*	86
	SD	8	13	10	6	6
Fibrinogen (g/L)	**X**	2.59	3.48	3.16	2.80	2.71
	SD	0.57	1.61	1.20	0.84	0.84
Albumin/ Fibrinogen	**X**	18.0	15.2	16.3	16.9	16.2
	SD	3.8	6.4	4.3	4.7	5.0
ATIII (%)	**X**	110.6	109.2**	104.3	103**	105
	SD	12.4	16.0	8.6	10	13
D-Dimer (µg/dL)	**X**		82.5		111.8	
	SD		46.4		75.0	
Fibrin Monomer (mg/L)	**X**	14.3	13.9**	12.7	12.1**	12.9
	SD	2.6	2.2	2.7	3.6	3.9
Compaction (%)	**X**	14.4	14.2**	14.1	13.7**	13.7
	SD	5	5	4	4	5
µT ($x10^{12}$ Da/cm)	**X**	10.4	5.9	8.7	7.2	5.9
	SD	5.5	3.7	5.1	2.6	2.2
Network Fibrin Content (g/L)	**X**	2.0	3.1	2.3	2.3	3.2
	SD	0.4	1.2	1.3	0.7	2.2

(X = mean; SD = standard deviation; FVII = blood clotting factor VII; FVIII = blood clotting factor FVIII; ATIII= antithrombin III; FM = Fibrin Monomer; µT = mass/length-ratio of fibrin strands in fibrin networks; NFC = Fibrin Content of Fibrin Networks; * = differs significantly with $p<0.05$; ** = differs significantly with $p<0.01$)

supplementation (day 8) and end of supplementation (day 36) within the high acetate supplementation group. No other changes in any of the other haemostatic variables were significant (Table 4.14).

Figure 4.4 represents a graphic comparison between the changes in the fibrin network lysis rate during each study period, within the high acetate group. Turbidimetric changes showed a sharp increase in absorbance for the first 80 minutes and reached the plateau phase at 180 minutes, except for day 22 which reached it by 240 minutes. In comparison to the others, day 43 reached a much higher absorbance. The rate of lysis of day 8 and day 36 did not differ significantly.

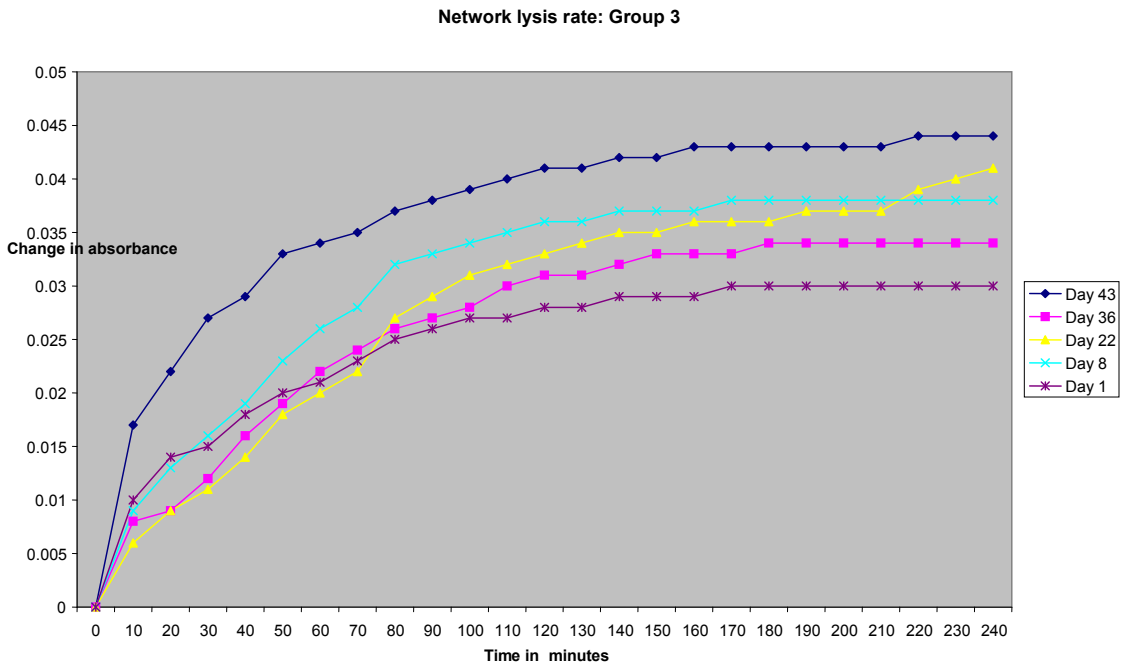

Figure 4.4 A turbidimetric presentation of the mean fibrin network lysis rate of the high acetate group for the entire study period

4.3.3.3 Changes in the lipid profiles within the high acetate supplement group

A marginal decrease in TC was measured from day 8 to day 36 within the high acetate supplement group. This group showed a statistically significant decrease in LDL-C ($p < 0.05$). HDL-C increased, however not significant and was accompanied by a statistical significant increase in the %HDL-C ($p=0.017$). NEFA decreaed signficanly ($p < 0.01$) (Table 4.15).

Table 4.15 Changes in the lipid profiles within the high acetate supplement study group (n = 22)

VARIABLE		Day 1	Day 8	Day 22	Day 36	Day 43
TC	**X**	4.70	4.95	4.96	4.57	4.74
(mmol/L)	**SD**	0.75	0.81	1.24	0.87	0.90
TG	**X**	1.04	1.16	1.07	1.26	1.04
(mmol/L)	**SD**	0.39	0.53	0.31	0.56	0.51
HDL-C	**X**	1.56	1.27	1.66	1.46	1.69
(mmol/L)	**SD**	0.65	0.28	0.70	0.66	0.83
LDL-C	**X**	2.39	3.10*	2.91	2.61*	2.61
(mmol/L)	**SD**	0.85	0.78	1.27	0.94	0.94
%HDL-C	**X**	34.90	26.30*	33.50	32.30*	34.9
(%)	**SD**	11.90	6.50	13.40	12.40	14.00
NEFA	**X**	3.60*	3.58	3.83	2.87**	3.10
(mmol/L)	**SD**	1.90	1.85	2.18	1.46	1.70

(X = mean; SD = standard deviation;TC = serum-Total Cholesterol; TG = serum-Triglycerides; HDL-C = serum-High-Density Lipoprotein Cholesterol; LDL-C = serum-Low-Density Lipoprotein Cholesterol; %HDL-C = HDL-C/TC * 100; NEFA = Non-Esterified Fatty Acids; * = differs significantly with $p<0.05$; ** = differs significantly with $p<0.01$)

4.3.3.4 Mean changes in the glycometabolic profiles within the high acetate supplement group

The mean fasting serum-glucose levels decreased significantly during supplementation (from day 8 – day 36). The circulating serum-insulin levels also showed a concomitant decrease during the supplementation ($p<0.001$) (Table 4.16).

Table 4.16 Mean changes in the glycometabolic profiles within the high acetate supplement study group (n = 22)

VARIABLE		Day 1	Day 8	Day 22	Day 36	Day 43
Glucose (mmol/L)	**X**	5.43	5.41*	5.46	4.96*	5.72
	SD	1.37	0.52	0.96	0.85	0.73
Insulin (μIU/mL)	**X**	6.70	5.80*	10.90	1.70*	15.80
	SD	3.60	0.77	4.90	0.36	9.90

(X = mean; SD = standard deviation; * = differs significantly with $p<0.05$)

4.4 COLLECTIVE SUMMARY

The group of individuals used for this study were exposed to almost identical environmental conditions for the duration of the study. They followed exactly the same diet, as they were eating at the same mess, performed the same physical activities and in some cases, even shared a living space. The general health profile indicates that the group of volunteers experienced no apparent health threat. The aim of this study was to measure the effect of two different combinations of short-chain fatty acids on metabolic risk markers in the group of volunteers. An additional placebo supplement was used for control purposes. Laboratory analyses show that during the study there are variations in the metabolic risk markers of the subjects. It is safe to assume that the changes in metabolic risk markers in these volunteers are at least in part related to the intake of the respective supplements. In Chapter 5 these changes will be discussed and also compared to the results reported in available literature that could lead towards the elucidation of possible mechanisms to better understand the proposed effects of short-chain fatty acids on human health.

CHAPTER 5

DISCUSSION

5.1 INTRODUCTION

The aim of the study was to determine the possible effects of different combinations of SCFAs on coronary risk factors of westernised black men. Coronary heart disease (CHD) and stroke are considered as degenerative western diseases which in most cases can be prevented by a change in lifestyle, for example diet, physical activity and psychological well being. In this study, a group of westernised African male volunteers were recruited and supplemented with either one of two different combinations of short-chain fatty acids (SCFAs), as well as a placebo supplement, for an uninterrupted period of six weeks, with an additional one week placebo supplementation before and after the actual experimenetal phase. The main goal was to establish whether the specific combinations of SCFAs affect the coronary risk profiles of the respective study groups in a different manner. Distinction will be made between the following metabolic profiles:

- haemostatic,
- lipid, and
- glycometabolic.

The discussion of the baseline results of the entire study group will be presented first, followed by a discussion of the results of the separate intervals of the intervention study. These findings will then be compared to the results of other relevant studies. A short discussion on the limitations of the study is also included at the end of this chapter.

5.2 BASELINE RESULTS OF THE ENTIRE STUDY GROUP

It is important to provide a general summary of the baseline results of the study group as a whole. The section is used to identify the characteristics of the study population and possible factors that may affect the way in which they respond to the experimental intervention.

5.2.1 Baseline general health indicators of the entire study group

The strict criteria used for selection of the study group ensured that the makeup of the group was of a homogeneous nature. All the subjects were living on the same military base, where they received the same controlled westernised diet at a central mess. They also participated in exactly the same physical activities. Members of the study group had no history of previous or current cardiovascular events, diabetes, hypertension or a history of any other apparent familial diseases.

A comprehensive profile of metabolic markers, all of which reflect the general health status of the individuals, was analysed. It was therefore, not expected that the supplements should have an effect on all of the measured variables and will only those of significant interest be highlighted and discussed in this chapter.

Both the Red and White Blood Cell counts fell within the normal healthy range. It is important to mention that anaemic subjects or subjects with low platelets or white cell counts, which could be indicative of a wide variety of different pathological states, were excluded from the study. The blood cell count reference ranges used in this study were established for African males in the Free State region (unpublished data of the Department of Haematology and Cell Biology, University of the Free State, Bloemfontein, 1990), and thus very specific for the population. In general, the African population tends to have slightly lower red cell and white blood cell counts, compared to the local Caucasian population (Bain, 1995).

Serum albumin is acknowledged as an important indicator of nutritional status and longevity (Carlson, 2004). The serum-albumin levels of the study group fell within the normal reference range of between 35-52g/L for healthy adult males (Painter, Cope & Smith, 1999). Supported by this and other measured variables it is safe to conclude that the study population of this study presented no apparent metabolic abnormalities at the time of the study and could be classified as healthy.

Elevated plasma C-reactive protein (CRP) concentration has been reported in the majority of patients with unstable angina, myocardial infarction, and a history of unstable angina (Ford, Giles & Dietz, 2002; Devaraj, Rosenson & Jialal, 2004). CRP (an acute phase protein) in this study was measured to ensure that possible changes in other haematological parameters might not be due to inflammation or any other unknown acute phase response, including its association with

CHD and CVD. Ridker *et al*. (1997) suggested that the baseline plasma concentration of CRP in apparently healthy men could predict the risk of first myocardial infarction and ischemic stroke, independent of other risk factors.

There were no statistically significant changes in any of the general health indicators for both baseline one (day 1) and baseline two (day 8) of the entire study group. This indicates that the run-in phase with the placebo supplement managed to establish a stable baseline.

5.2.2 Dietary intake of the entire study group

Results from the dietary analysis show that there was no major change in die eating habits of the study population, as a whole, during the period of intervention, or supplementation with SCFAs. The diet of the group included higher intakes of total fat and dietary fibre. Protein intake of the group was in the recommended range and the total carbohydrate intake fell in the lower range. This is typical of a Westernised diet, which is high in fat and lower in carbohydrates. Other studies conducted on urban African men in the Free State (Mollentze *et al.*, 1995; Slabber *et al*., 1997) confirm that this is a general trend within the South African, African population and also believed to expose them to metabolic changes that promote the onset of CHD and CVD. The use of a FFQ was restricted to the purpose of control and no other analyses were performed in relation to diet, as part of this study. It can therefore be concluded that it is improbable that the measured metabolic changes can be ascribed to a change in dietary

intake, but rather that it is suggested to be the direct consequence of the respective SCFA supplements.

5.2.3 Anthropometric measurements of the entire study group

No overweight or obese subjects were included for the purpose of this study. This could also be ascribed to the strict inclusion criteria, and most importantly, the fact that the subjects were involved with physical activities most of the day, due to the nature of their occupation. The BMI and blood pressure measurements were similar to those reported by epidemiological studies performed on African population groups in South Africa, including Durban (Seedat *et al.*, 1992), the Cape Peninsula (Oelofse *et al.*, 1996), the Free State (De Wet, 2000) and North West Province (James *et al.*, 2000).

5.2.4 Haemostatic profile of the entire study group at baseline

Haemostatic risk factors associated with the development of CVD include plasma fibrinogen concentration, plasminogen activator inhibitor-1 (PAI-1), factor VII (Kannel, 2005) and factor VIII (Pan *et al.*, 1997) activities. Even though certain haemostatic risk markers are elevated in persons at risk of future cardiovascular events, data that assess the link between haemostatic risk markers and race/ethnicity is limited (Albert *et al.*, 2007). Fibrinogen seems the most fundamental haemostatic risk factor for CVD. Fibrinogen was reported to increase with age, smoking, the waist/hip ratio and LDL-C levels. Fibrinogen concentration decreases in relation to the level of education, physical activity, alcohol intake and HDL-C concentrations. Fibrinogen may also directly increase CVD risk because of its role in platelet aggregation,

plasma viscosity, and fibrin formation (Kannel, 2005). Normal values for plasma fibrinogen concentration in the African population group are a point of argument. The main reason for this being the different views regarding the strength of plasma fibrinogen as a predictor of stroke, compared to CVD and the prevalence of these two diseases within the different population groups.

The factor VII and VIII activities of the current study group were similar to those reported by De Wet (2000). The fibrinogen concentration of this study group was slightly lower when compared to that reported by James *et al.* (2000).

Both fibrin monomer and D-dimer levels are considered as potential risk markers for development of cardiovascular events. The thrombin-catalysed released of the fibrinopeptides from fibrinogen results in the formation of a transient intermediate, termed fibrin monomer. Increased fibrin monomer levels are present in patients with hypercoagulable disease states, especially after a cardiovascular event. The fibrin monomer concentrations in this study were higher than those reported by De Wet (2000). D-dimer is a product of clotted fibrin breakdown, and is usually found in increased levels in patients with hypercoagulable disease states occurring before and after cardiovascular events. It is an indication that fibrinolysis has occurred and therefore, it is increased in disseminated intravascular coagulation (Laffan & Manning, 2001).

Fibrin network architecture is believed to serve as a marker of the risk of developing coronary heart disease (Mills *et al*., 2002). The variables

included in such an analysis include the mass-length ratio from turbidity (as a measures of the thickness of the fibrin fibres in relation to its length), fibrin network compaction (as a measure of the degree of cross-linking of fibres within the overall network), as well as the network lysis rate. The fibrin network content reported in this study was similar to those reported by Pieters, Jerling & Weisel (2002) and Veldman *et al.* (1999). In relation, the mass-length ratio was lower, and the compaction higher in this study. Due to the lack of standardised control samples, it is impossible to conclude whether these differences are a result of variations in laboratory practice or an actual difference in metabolic profiles of our study group.

Gabriel, Muga & Boothroyd (1992) suggested that fibrin network architecture contributes to the regulation of the fibrinolytic rate. The fibrin network lysis rates of the two baseline measurements were very similar. Yet, the end-point absorbance of baseline one was lower when compared to that of baseline two. The network fibrin content at baseline two was slightly higher when compared to that of baseline one and may serve as an appropriate explanation for this phenomenon. The fibrin network content is a measure of the total amount of fibrin that is incorporated into the fibrin network.

There were no statistically significant changes in any of the haemostatic risk markers from baseline one (day 1) to baseline two (day 8). This indicates that the run-in phase with placebo managed to establish a stable baseline.

5.2.5 Baseline lipid variables of the entire study group

The predictive value of abnormal blood lipids has been studied for decades, with the focus shifting from the initial study of total-cholesterol (TC) alone, to that of the constituent components of TC (Norman *et al.*, 2007). In general, the dyslipidaemic patterns of concomitant high levels of LDL-C and low levels of HDL-C have been found to represent a high risk for developing CHD (NCEP, 2002). Total cholesterol and LDL-C levels are directly and HDL-C levels inversely related to CHD (NCEP, 2002). Only subjects with healthy lipid profiles were recruited for this study, which included individuals with TC levels in the higher range of the normal (4.10 – 5.50 mmol/L). Due to the nature of the study, it would be very difficult to show any metabolic response unless the risk marker in question is at a changeable level.

Mollentze *et al.* (1995) reported mean TC levels between 4.7 $\pm$ 1.2 mmol/L for black men in the age range 25-34 years, living in Mangaung, Free State Province. The authors also reported a concerning increased number of subjects in the moderate-risk hypercholesterolaemic category in this population. Subjects in the younger age groups had a higher prevalence of hypercholesterolaemia. This could possibly reflect the more powerful impact of urbanisation on the younger generation. Younger people may adopt a westernised diet to a greater extent than their elders (Mollentze *et al*., 1995). Most of the subjects of the current study originally come from a urban aea, Mangaung. Total cholesterol on its own is not an accurate predictor of cardiovascular events, and should be evaluated together with the three

other main serum-lipid fractions; namely High-Density Lipoprotein Cholesterol (HDL-C); Low-Density Lipoprotein Cholesterol (LDL-C) and Triglcerides (TG). The measured concentrations of these combined fractions predicted that the individuals used for this study were not at immediate high riks of cardiovascular events. The baseline serum-TC, -TG, -HDL-C & -LDL-C measured in this study are all comparable to those reported by James *et al.* (2000), Oelofse *et al.* (1996) and Seedat *et al.* (1992).

The measured HDL-C level of the group is high, when expressed as a percentage of the TC. Seftel, Raal & Joffe (1995) mentioned that almost all blacks have HDL-C levels above 20% of the TC, indicative of a protective effect against IHD. Oosthuizen *et al.* (2002) confirms that black Africans tend to have fasting lipid profiles that are less atherogenic when compared to that of the white population group, with lower TC and LDL-C levels and higher HDL-C concentrations. However, in comparison with the HDL-C, concomitant high LDL-C levels (up to 54% of the TC) were measured. In general, this is very common within the specific target group. According to Packard (2006) increased LDL-C levels appear to be a primary CHD risk factor. Even though the HDL-C level of our study group is high, it is also suggested that the high LDL-C levels put them at risk of future CHD events. The fasting baseline TG levels of the group fell below the high-risk cut-off level of 1.7 mmol/L (NCEP, 2002). Consequently, at first glance, the lipid profiles seem healthy.

No statistically significant changes were measured in any of the lipid variables from baseline one (day 1) to baseline two (day 8) of the study for the entire study group.

5.2.6 Glycometabolic indicators of the entire study group

Measures of obesity, hypertension, dyslipidaemia, and hyperglycaemia, together form what is known as the metabolic syndrome (MS), a well-recognised constellation of risk factors for type 2 diabetes mellitus and CVD in adults (NCEP, 2002). None of these clinical manifestations are expected to be present in the study group, as they formed part of the exclusion criteria (see section 3.6.1).

Furthermore, it is also suggested that the high level of physical activity, age and absence of obesity may further benefit glycometabolic control of the subjects used in this study. Neither did the actual fasting serum-glucose and -insulin levels of the entire study group reflect the presence of any apparent glycometabolic disorders. In general, glucose of healthy individuals are below 5.6 mmol/L (ADA, 2008). With a fasting mean of 5.32 ± 0.83 mmol/L at baseline one, and 5.47 ± 0.88 mmol/L at baseline two, for serum-glucose levels, the study group seems to have an apparently healthy glycometabolic state. In addition, insulin levels of healthy individuals should vary between 0-17 μIU/mL, depending on the time of day and the state of fasting (Painter *et al.*, 1999).

No significant changes were measured in any of the glycometabolic indicators from baseline one (day 1) to baseline two (day 8) of the entire study group.

5.3 INTERVENTION STUDY

Attention will now be given to the results of the intervention study and the individual supplements. Attention will mainly be given to the changes in metabolic profiles that occured between day 8 (start of experimental SCFA supplementation) and day 36 (end of experimental SCFA supplementation).

Studies show that the clinical effects of SCFA administration may be influenced by the method of administration (oral, rectal or intravenous) (Wolever *et al.* 1995). The capsules used for this study were coated with a thin layer of complex edible polymer, which prevented the acidity of the stomach from dissolving the capsules. The capsules were designed to release their contents only in the small gut. This ensured that maximum distribution of the supplements took place within the colon, resembling the SCFA distribution that takes place after dietary fibre fermentation.

5.3.1 The Placebo Supplement Study group

This group consumed placebo supplements for the entire period of the study.

5.3.1.1 Anthropometric and general health changes of the placebo supplement study group

No statistically significant changes in any of the anthropometric and general health indicators within the placebo group were measured during the study. It was not expected that the placebo supplement should cause any changes to the anthropometric and general health

indicators of this group. It is interesting to note that the plasma CRP concentration of day 8 was higher when compared to that of any of the other visits, even though not statistically significant. The change was of no clinical significance.

5.3.1.2 The haemostatic profiles of the placebo supplement study group

A statistically significant decrease was measured in the plasma fibrinogen concentration, for the period from day 8 to day 36. Yet, the change seems to be of no clinical significance. No statistically significant change were measured in any of the other haemostatic variables, between any of the study intervals.

Fibrin network lysis rate of day 1, 8, 36 and 43 showed the same pattern and similar lysis rates, with only day 22 that reached a lower end-point absorbance. It is important to note that the network fibrin content of day 22 was lower when compared to all the other visits, which could explain the difference in network lysis rate. Most importantly, there were no significant changes observed between any of the haemostatic risk markers measured on day 8 and 36.

5.3.1.3 The lipid profiles of the placebo supplement study group

No significant changes were measured in any of the lipid variables of the placebo group from baseline to the end of the experimental phase.

5.3.1.4 The glycometabolic indicators of the placebo supplement study group

No significant differences in any of the glycometabolic parameters of the placebo group were measured during the intervention period.

5.3.2 The Acetate/Propionate (50/50) Supplement Group

This study group was supplemented with placebo from day 1 to day 8, followed by supplementation with acetate-propionate (in the ratio of 50% to 50%) up to day 36, after which an additional one week supplementation with placebo followed.

5.3.2.1 Changes in anthropometric and general health indicators of the acetate-propionate (50/50) supplement study group

No significant changes were observed in any of the anthropometric and general health indicators of the acetate/propionate (50/50) supplemented group over the duration of the study.

5.3.2.2 The haemostatic profiles of the acetate-propionate (50/50) supplement study group

A statistically significant decrease in factor VII activity, antithrombin III and an associated increase in the plasma fibrinogen concentration were measured within this group from baseline to the end of supplementation (day 8 and day 36). This group also showed a slight non-significant decrease in fibrin monomers. Yet, even though of statistical significance, the changes were clinically insignificant. No statistically significant changes in any of the other haemostatic variables were measured.

In general, a decrease in factor VII activity is beneficial, because some studies have suggested that a low factor VII activity is associated with an increased risk of CHD (Meade *et al.*, 1980; Vorster *et al.*, 1988; Buzzard *et al*., 1996). The small decrease in AT III and increase in plasma fibrinogen concentration could render the blood

more coagulable and the individual at higher risk of development of cardiovascular complications.

No significant changes were measured in any of the fibrin network architecture variables, as reported by Veldman *et al*. (1999). Yet, in the current study, the amount of acetate in the supplements were significantly less. In addition, Veldman *et al*. (1999) did not employ propionate in their supplements.

The network lysis rates of day 8, 22, and 36 showed similar rates of lysis.

5.3.2.3 The lipid profiles of the acetate-propionate (50/50) supplement study group

A combination of acetate and propionate salts resulted in a significant increase in HDL-C from baseline two (day 8) to the end of the supplementation phase (day 36). LDL-C showed a marginal decrease during the supplemental phase. Todesco *et al*. (1991) reported that when sodium propionate is administered orally to healthy subjects, the LDL-C and HDL-C levels decreased without any effect on the TC concentration. In a study performed by Venter, Vorster & Cummings (1989) a sodium propionate supplement also increased circulating HDL-C in human subjects. Previous rectal infusion studies performed by Wolever *et al*. (1995) showed that colonic acetate was incorporated into TC and TG, and acutely raised the concentrations of serum lipids, and that these effects are blocked by propionate.

NEFA concentration in this group also decreased significantly during supplementation. As explained earlier, a decrease in NEFA levels is

associated with an increase in SCFA concentration and therefore serves as an indication that the SCFAs within the supplements were absorbed and had reached the peripheral circulation (Crouse *et al*., 1968; Scheppach *et al*., 1988, Veldman *et al*., 1999).

5.3.2.4 The glycometabolic indicators of the acetate-propionate (50/50) supplement group

A significant decrease in the fasting serum-glucose levels, with no change in the serum-insulin concentrations was measured from baseline to end of supplementation with a mixture of acetate-propionate (50/50). Berggren *et al.* (1996) also reported that when propionate was fed orally to hyperinsulinaemic rats, that the fasting plasma glucose levels decreased markedly, with no effects on the fasting insulin levels. Venter, Vorster & Cummings (1990) reported similar results for human subjects.

The U.S. Department of Agriculture (USDA) recommends dietary fibre intake of 14g fibre per 1000 kcal to reduce the risk of type 2 diabetes mellitus. It is suggested that the blood glucose lowering effect of soluble dietary fibres may be related, in part, to the metabolic effects of SCFAs generated during their fermentation (Thacker *et al*., 1981). However, the physical characteristics of the fibre itself, also play a role and should not be neglected.

Akanji & Sacks (1991) demonstrated a rise in blood acetate levels and an associated decrease in the fasting serum-glucose levels of diabetic patients after a high-fibre diet. It is suggested that acetate could reduce blood glucose levels in the long-term by reducing the

non-esterified fatty acid concentrations (Crouse *et al*., 1968), but propionate may have exactly the opposite effect. Propionate is an important gluconeogenic substrate in ruminants and horses (Judson *et al.,* 1968). Yet, Venter, Vorster & Cummings (1989) reported that a propionate supplement significantly decreased the fasting glucose levels as well as the maximum insulin increments during glucose tolerance tests. These authors conclude that the beneficial effects of dietary fibre may at least be partially mediated by propionate and its effect on hepatic carbohydrate metabolism.

5.3.3 The High Acetate Supplement Study Group Results

This study group was supplemented with placebo from day 1 to day 8, followed by supplementation with acetate-propionate (in the ratio of 70% acetate, 15% propionate and 15% butyrate) up to day 36, after which an additional one-week supplementation with placebo followed.

5.3.3.1 Anthropometric and general health indicators of the high acetate supplement study group

No significant differences in any of the anthropometric and general health indicators of the high acetate supplemented study group were measured, between any of the sampling intervals.

5.3.3.2 Haemostatic profiles of the high acetate supplement study group

A beneficial decrease in factor VII activity, factor VIII activity, ATIII, compaction and fibrin monomer concentration were measured during the experimental period (day 8 to 36). The decrease in factor VII and factor VIII activity may affect the CVD risk profile positively, while a

decrease in ATIII is unknown, compaction's decrease affects the risk profile negatively.

Highly compact networks have a high degree of cross-linking. A more compact the network would have a high degree of cross-linking, this will not allow easy access for fibrinolytic enzymes (Nair & Shats, 1997; Blombäck *et al.*, 1992). A high degree of cross-linking renders the clot more rigid and less lysable. It is believed that these structures are more atherogenic in nature, when compared to the soft, less rigid networks (Collet *et al*., 2000). The clinical significance of these changes is difficult to interpret, since not all changes are in the same direction. Yet, it is evident that the supplement had little or no effect on the overall structure of the fibrin networks. In contrast with the results from this study, De Wet (2000) and Veldman *et al*. (1999) reported beneficial changes in fibrin network structure after SCFA supplementation. It is important to note that the supplement of this study is a different combination of SCFAs than used by De Wet (2000) and pectin used by Veldman *et al.* (1999).

Network lysis rate of all the visits showed a similar rate of lysis with no statistically significant changes amongst them. The change in compaction was small and therefore was not reflected in the network lysis rate.

The significant results within haemostatic variables of this group receiving a higher percentage of acetate, less propionate and added butyrate are difficult to interpret. Some changes were beneficial (such as the decrease in the circulating Fibrin Monomer levels), and others

less beneficial (such as the decrease in the fibrin network compaction). After supplementation with SCFAs (65% acetate, 19% propionate and 16% butyrate) De Wet (2000) also reported a decrease in factor VII and VIII activity, and Fibrin Monomer concentration. However, the higher acetate group in the present study showed more changes in the haemostatic variables when compared to that of the acetate-propionate supplement group.

5.3.3.3 Lipid profiles of the high acetate supplement study group

The SCFA combination of acetate, propionate and butyrate significantly decreased serum LDL-C levels with 16% during the four-week supplementation. The TC levels also decreased slightly in this group. However, this decrease of TC levels was a small non-significant change and it may be due to the decrease in LDL-C levels. Added to the beneficial decrease of LDL-C levels , this group also had a significant increase in % HDL-C levels.

The dyslipidaedemic patterns of high levels of LDL-C associated with or without low levels of HDL-C, have been found to impart high risk for developing IHD (NCEP, 2002). The CDC (2001) estimated that a 10% reduction in serum-TC decreased CHD incidence by at least 30%. The approximately 8% decrease in serum-TC levels seen in this group could therefore, although of no significance, and together with the significant decrease of LDL-C and increase in %HDL-C levels as beneficial in decreasing the risk of CHD.

The specific effect of acetate intake on cholesterol metabolism is controversial. Wolever *et al.* (1989) suggest that acetate may reduce

cholesterol synthesis by reducing the circulating free fatty acid (NEFA) concentration. In this study group, circulating NEFA concentrations decreased significantly. In general, a decrease in NEFA levels is associated with an increase in circulating blood short-chain fatty acid concentrations and often used as a confirmation that a given oral SCFAs supplement was absorbed successfully (Scheppach *et al.*, 1988; Akanji & Sacks, 1991).

Previous studies performed on humans and animals have shown a definite relation between propionate and acetate and cholesterol metabolism. It is known that both acetate and propionate influence cholesterol synthesis (Chen, Anderson & Jennings, 1984; Nishina & Freeland, 1990; Wright, Anderson & Bridges, 1990).

Several studies reported that consumption of propionate could reduce serum cholesterol in both animal and human models (Thacker *et al.*, 1981; Illman *et al.*, 1988; Stephan, 1994). Yet, Venter, Vorster & Cummings (1990) showed that dietary supplementation with propionate increased the serum HDL-C concentrations in baboons, whereas no effects were observed on TC concentrations.

However, the collective metabolic effect of a combination of acetate and propionate is not well documented and requires further research. Very little information is available on the effect of butyrate on cholesterol metabolism in human subjects (Topping & Pant, 1995).

5.3.3.4 Glycometabolic profiles of the high acetate supplement study group

The combination of acetate, propionate and butyrate caused a beneficial and statistically significant decrease in the serum-glucose

levels of the study group. A concomitant significant decrease in the circulating insulin levels was also measured. This change indicates that the body requires less insulin to keep the glucose levels in circulation at a constant concentration. Limited data is available on the effect of a combination of acetate, propionate and butyrate on glucose metabolism. De Wet (2000) also found that when taken orally a combination of acetate, propionate and butyrate orally that glucose levels decrease significantly. Although other authors (Anderson & Bridges, 1984; Jenkins *et al*., 1990) found that SCFA might influence glucose levels, Alamowitch *et al.* (1996) reported that acute ileal perfusion of a combination of acetate, propionate and butyrate did not significantly reduce serum-glucose levels in human subjects. Yet, when propionate was consumed with bread, postprandial blood glucose and insulin responses were significantly reduced in healthy subjects (Todesco *et al*., 1991; Liljeberg, Lonner & Bjorck, 1995).

5.4 COLLECTIVE DISCUSSION

It is well known that the consumption of dietary fibre may introduce metabolic changes that reduce the risk of cardiovascular disease (Anderson & Hanna, 1999; Krummel, 2008). It is proposed that the SCFAs (acetate, propionate and butyrate), produced from colonic fermentation of dietary fibre, may at least in part, be responsible for these changes (Anderson & Bridges, 1984; Wright, Anderson & Bridges, 1990; Krummel, 2008). Results from the present study

confirm the findings that SCFAs may influence risk factors for cardiovascular disease. However, the findings from this study indicate that there was a definite and clear difference in response to the different SCFA mixtures. For this study, one of the supplements contained a mixture of acetate (70%), propionate (15%) and butyrate (15%) and the other supplement contained a mixture of acetate (50%) and propionate (50%) only. In order to explain these differences, it was necessary to focus on the different contents of the two experimental supplements. Only those differences that are of clinical significance will be discussed.

It is important to mention that literature available on the long-term effect of short-chain fatty acids is limited. It should also be noted that most other studies applied either rectal infusions or intravenous injections of SCFAs, which differ from the encapsulated supplements used in this study. It is widely reported in the literature that a definite difference exists in response to the different routes of administration when supplementing humans with combinations of SCFAs (Wolever, *et al*., 1995).

Both experimental groups experienced statistically significant changes in their haemostatic risk profiles, from the beginning to the end of the study. In both groups, factor VII activity and the ATIII concentration decreased significantly, while in the high acetate group a decrease was also measured in the factor VIII activity and fibrin monomer concentration. Although the high acetate group showed little or no effect on the overall structure of the fibrin networks, some other

beneficial changes, such as a decrease in the circulating fibrin monomer concentration, were measured. In contrast, authors such as Veldman *et al.* (1999) reported beneficial effects after oral acetate supplementation. De Wet (2000) also reported that supplementation with SCFAs (65% acetate, 19% propionate and 16% butyrate) caused a significant beneficial decrease in both the fibrin monomer concentration and factor VII activity. In all the above instances, SCFA supplements were given to human subjects to investigate the possibility that some of the beneficial effects associated with the intake of dietary fibre, may at least in part, be mediated by the production of SCFAs in the large gut. It is very important to realize that not all dietary fibres result in the production of the same combination of SFCAs.

The supplement in this study is of a unique combination of SCFAs and cannot be compared to the supplements used by other authors, such as Veldman *et al.* (1999). Yet, the beneficial haemostatic changes observed in the high acetate supplement group, agrees with the results from another study by De Wet (2000), where exactly the same SCFAs were used in a different ratio. Although no other studies are available to support this finding, it is suggested that the presence of butyrate in the high-acetate supplement may also evoke a response on the haemostatic risk profiles. The addition of butyrate to SCFA supplements requires further investigation.

The present study confirms the favourable and independent effect of the oral consumption of different combinations of SCFAs on lipid profiles. The work of Cummings & Macfairlane (1991) support these

results. The precise metabolic mechanism of the action of SCFAs in reducing plasma cholesterol concentrations is unknown (Wolever *et al.*, 1995). Yet, it is also known that the physical structure of the fibre itself may exert certain physiological changes that reflect in the chemical composition of blood, such as its beneficial effect on the total cholesterol content. All of the subjects in this study were normolipidaemic. The acetate-propionate (50/50) supplement had a small albeit favourable, effect on the lipid profiles of the group. Yet, it seems as if the high-acetate group, with the addition of butyrate, had an even more favourable effect on lipid metabolism, when compared to that of the acetate-propionate (50/50) supplement. This suggests that either increased acetate production relative to propionate production, or reduced propionate production relative to acetate production, may directly affect cholesterol production (Wolever *et al.,* 1995). The concomitant decrease in TC concentration and increase in the %HDL-C in the high-acetate group are in congruence with a study performed by Veldman *et al.* (1999) during which acetate supplementation alone caused a significant increase in %HDL-C, as well as a statistically significant decrease in the serum-TC concentration. A study performed by Venter, Vorster & Cummings, (1990) supports our findings in the acetate/propionate group where the supplementation resulted in increased HDL-C levels. Venter, Vorster & Cummings (1990) also reported an increase in the HDL-C concentration after oral administration of propionate for seven weeks.

In this study, both the acetate-propionate (50/50) and the high-acetate supplement resulted in favourable changes to the glycometabolic indicators. Glucose levels decreased in both groups, while the serum-insulin levels did not change in the acetate-propionate group, but did decrease in the high-acetate supplement group. De Wet (2000) also reported a decrease in the serum-glucose concentrations of westernised black men, after four weeks of supplementation with a combination of acetate, propionate and butyrate. Available information also suggests long-term metabolic effects in humans, and dietary supplementation with propionate for 7 weeks decreased fasting serum-glucose and reduced maximum insulin increments during a subsequent oral glucose tolerance test (Venter, Vorster & Cummings, 1990).

Considering the results from the present study, it is important to acknowledge that the study group was of a healthy nature, with no apparent clinical manifestations of cardiovascular disease. Previous studies by Molentze *et al.* (1995) and Slabber *et al*. (1997) demonstrated a trend towards the adoption of a westernised diet amongst this population group in the Free State Province. Although the subjects from this study were physically healthy, active and normolipidaemic, adoption of a westernised lifestyle would increase their risk for the development of cardiovascular disease. The current study provides evidence to show that improvements in the haemostatic, lipid and glycometabolic risk profiles are possible, and that it would be to their own benefit to sustain such a healthy lifestyle, which could be improved even further by the intake of SCFA supplements.

It is well documented that dietary fibre plays a protective role against the development of CHD. It is suggested that one possible mechanism through which the intake of dietary fibre benefits health, is based on the metabolic effect of the SCFAs produced during fermentation in the colon (Krummel, 2008). From the results presented in the present study it is clear that different combinations of SCFAs may evoke different metabolic responses. From this study, it seems highly likely that the intake of a combination of acetate, propionate and butyrate induces metabolic changes that are more beneficial when compared to that of a supplement that contains only acetate and propionate. This effct may also be a consequence of the higer acetate alone. The acetate/propionate/butyrate supplement induced a significant and beneficial decrease in the factor VII and VIII activity, ATIII and fibrin monomer concentration, LDL-C concentration, as well as the serum-glucose and -insulin levels. Added to these beneficial effects the %HDL-C also increased significantly. The acetate/propionate supplement induced only beneficial effects on the factor VII activity, ATIII concentration, as well as HDL-C and serum-glucose levels. It is possible that both the addition of butyrate and higher acetate content to this supplement may collectively alter the physiological response, but it cannot be said with certainty. De Wet (2000) showed similar overall results with a SCFA supplement that also contained a higher percentage of acetate, as well as butyrate. SCFAs produced during fermentation do not exist in isolation, and the proportion of acetate produced is always greater when compared to that of propionate

(Mortensen, Holtug, Rasmussen, 1988). The production of butyrate during the fermentation of dietary fibre may then also have additional beneficial metabolic effects, even though studies suggest that butyrate has only a limited metabolic importance. Wolever *et al*. (1996) reported that there was no definite relationship between butyrate and lipid production in the fasting state.

It is concluded that the results from this study show that supplementation with different combinations of SCFAs affect human metabolism, and more specific, the metabolism of those markers that are generally associated with the development of cardiovascular disease, even though it is clear that the changes are small. Yet, it is important to acknowledge that within a clinical environment we investigate each marker separately, but that in reality the cardiovascular risk depends on the collective risk all these markers. It is evident though that the specific combination (and respective ratios) in which these SCFAs as a supplement were used, plays a significant role in the way it affects human metabolism. It therefore becomes clear that it is not only the presence of a single one of the SCFAs that mediate the overall metabolic effects of dietary fibre on human metabolism, but rather the ratio in which they occur relative to each other, that collectively affect human metabolism.

5.5 LIMITATIONS OF THE STUDY

The healthy nature of the study population made it very difficult to show any improvements in those metabolic risk markers that we generally associate with CHD. It is unknown whether the metabolic effect of the supplements would be different in those that do suffer from a raised risk for the development of CHD. In addition, the long-term effects of SCFAs on the risk factors associated with CHD may differ from the short-term effects. Even though a four-week trial is considered long-term, when compared relative to acute effects, it is not sure whether the short-chain fatty acids may indice effects that are only metabolically visible after a much longer time of intervention.

In the following chapter 6, the author provides a conclusion, based on the findings of this study.

CHAPTER 6

CONCLUSION

6.1 INTRODUCTION

Urbanisation is accompanied by a rise in the rate of CHD and stroke. Mortality rates from CVD indicate that in urban black South Africans, stroke represents the highest mortality rate (Vorster, 2002). This epidemiological trend goes hand in hand with changes in lifestyle, especially an increase in consumption of processed, energy-dense food, which are high in saturated fat and low in fibre, and a concomitant decrease in physical activity. CHD and stroke are degenerative western diseases, which in most cases can be prevented by a change in lifestyle. Abundant data since World War II shows how dietary intervention can significantly reduce the risk of CHD and stroke. Especially a prudent low-fat, high fibre diet plays an important role in the prevention of CHD. It is postulated that dietary fibre plays a central role within the mechanism, but the exact action is unknown. It is suggested that the production of SCFAs as a result of fibre digestion in the large gut, may mediate some of the beneficial effects associated with fibre intake. Dietary fibre is fermented in the large gut to the short-chain fatty acids acetate, propionate and butyrate. These SCFA are absorbed and enter the hepatic blood circulation, where it is finally metabolised in the liver. A small percentage of these acids may reach the peripheral circulation. In the liver,

acetate, propionate and butyrate are used as a source for many different metabolic pathways, such as that of cholesterol production.

The aim of this study was to test the possible metabolic effects associated with the intake of different combinations of SCFAs on haemostatic and other metabolic risk markers associated with the development of CHD in westernised African men. A group of westernised African male volunteers were recruited and supplementedwith two different combinations of short-chain fatty acids, as well as a placebo for an experimental period of six weeks, with an additional one week placebo supplementation before and after this period. Blood was drawn at different intervals and analysed to assess metabolic changes induced by the different supplements. Focus was mainly on the differences between start of supplementation (day 8) and end of supplementation (day 36). The conclusion of the baseline results of the entire study group will be presented first, followed by the intervention study results, where the conclusion of the different study groups will be presented. The chapter will be concluded with recommendations.

6.2 BASELINE CHARACTERISTICS OF THE ENTIRE STUDY GROUP

The characteristics of this group of subjects reflect a group of apparently healthy individuals without any current metabolic abnormalities. This could be ascribed to the strict inclusion criteria adhered to during the recruitment of subjects. The anthropometrical measurements reflect that this group of subjects were physically healthy. Both the blood pressure, lipid profile and full blood count measurements support this finding.

The more specific haemostatic profiles were also within the normal reference ranges for the specific subject group. In addition, the lipid profile of this group reflected that of a normolipidaemic group with a slight change to the westernised lipid profile, though not pronounced.

It is therefore safe to conclude that the overall characteristics of this study group reflect that of an assumingly healthy population. Yet, this by no means implies that they could not benefit from intervention, or that it is impossible to show the benefit of such intervention on their risk of developing CHD in the future.

There were no significant changes, statistical or clinical, observed between the two baselines measurements during which all subjects consumed placebo supplements. The placebo supplement therefore, was successful in establishing a stable baseline.

6.3 INTERVENTION STUDY

6.3.1 The Placebo Supplement Study group

No significant changes occurred in any of the measured variables of this group of subjects, apart from a significant decrease in the plasma fibrinogen levels from baseline two (Day 8) to the end of experimental supplementation (Day 36). Yet, this decrease is of no clinical significance. Plasma fibrinogen levels of Day 1 and 36 were almost identical and remained so for the duration of the study.

6.3.2 The Acetate-Propionate (50/50) Supplement Study Group

This group received a supplement containing a mixture of acetate and propionate in a ratio of 50% acetate and 50% propionate. As expected the general health indicators, which were only used as a measure of general health, were not affected by the supplements. As for the haemostatic variables a beneficial decrease in the factor VIII activity was measured, with a concomitant increase in the fibrinogen concentration, perceived as a detrimental change. Though statistically significant, these changes were so small that they are of no clinical significance.

The supplement had no effect on the fibrin network architecture. The acetate-propionate supplement induced a slight non-significant decrease in LDL-C combined with a statistically significant increase in HDL-C.

The supplement showed benefit in terms of glycometabolic control of the subjects, with a statistically significant decrease in the blood glucose levels of the study group, from Day 8 to 36.

6.3.3 The High Acetate Supplement Study Group

This supplement contained a mixture of acetate, propionate and butyrate in a ratio of 70% acetate, 15% propionate and 15% butyrate. As with the acetate-propionate supplement group, the general health indicators were not affected by the supplement. A statistically significant decrease in the factor VII and VIII activity and fibrin monomer concentration lower the individual risk of developing CHD.

The concomitant small decrease in fibrin network compaction is believed to be of no clinical significance. In addition, it is uncertain what the exact clinical value of fibrin network compaction is, due to a lack of both evidence and research in this regard. The high-acetate supplement induced a beneficial, significant decrease in the LDL-C concentration and significant increase in %HDL-C. The combination of acetate, propionate and butyrate caused a statistically significant decrease in serum-glucose levels, with a concomitant significant decrease in the circulating insulin levels.

6.3.4 Conclusion

It is evident from this study that the intake SCFAs exert some metabolic effects on those risk markers that we associate with the development of CHD. It is believed that these same acids, as a by-product of microbial fermentation of dietary fibre in the large gut, does contribute, in part, to the metabolic changes associated with the intake of dietary fibre. Yet, results from this study could not confirm this, even though small metabolic changes were induced as a result of the different SCFA supplements. However, what this study does show is that the metabolic effects of SCFAs depend on the specific combination of SCFAs, which could also be the reason why different types of fibre induce different metabolic responses. Fermentation of dietary fibre in the large gut produces SCFAs of a specific quantity, but the ratios in which the different acids are produced are not the same for all fibres. It is very

important to acknowledge that the use of SCFA supplements should, based on the results of this study, not replace the intake of dietary fibre, as the physical characteristics of any dietary fibre also exert an additional effect independent of that of its metabolic by-products, which is lost when giving short-chain fatty acid supplements. At this stage it seems highly likely that the physical characteristics contribute significantly towards its metabolic effects.

In terms of the original hypothesis of this study, it is concluded that the addition of propionate and butyrate in different quantities to colonic release acetate supplements significantly alters the effect of the supplement on haemostatic, lipid and glycomatebolic profiles of westernised African men. It is therefore suggested from this study that the ratio in which short-chain fatty acids are produced, as a result of microbial fermentation in the large gut, could partially explain why not all dietary fibres exert exactly the same metabolic response in human subjects, and requires further investigation.

6.4 RECOMMENDATIONS

6.4.1 General recommendations

This study show that the long-term intake of two specific combinations of SCFA supplements exert metabolic effects that can be measured in the lipid, carbohydrate and haemostatic profiles of human subjects. Yet, it is very important to acknowledge that the use of SCFA supplements should not be promoted as a replacement for fibre in the

diet. The physical characteristics of any dietary fibre also exert an effect over and above that of its metabolic SCFA by-products. People should therefore, primarily be educated to eat fruit and vegetables, oats, legumes, psyllium seeds and other foods high in fibre to experience the full benefit from the products just mentioned. An increased intake of fruit, vegetables and high fibre foods provide additional benefits to health, such as micronutrients, which also include antioxidant vitamins and minerals. Migration from a rural to urban environment is associated with a change in lifestyle that includes the adoption of westernised eating habits. In addition, over nutrition becomes the burden of an affluent lifestyle. In contrast to a prudent diet, the westernised diet provides little protection against those diseases that are associated, in general, with lifestyle: obesity, insulin resistance, hypercholesterolaemia, hypertension, etc. Several studies report that urbanisation is associated with metabolic profiles, which are believed to promote the development of CHD and CVD. Diet stays the sure way to counteract these changes. Public health intervention should therefore, address these issues by means of appropriate health education interventionprograms.

6.4.2 Further research

Results from this study show that the intake of SCFAs exerts a metabolic effect that can alter the level of those markers that are used to predict the risk for development of CHD. In general, lifestyle influences the development of CHD. Raised CHD risk associated with the Metabolic Syndrome, a by-product of our modern lifestyle,

constitutes a growing problem worldwide. In this study, the study population was apparently healthy. Clinical trials are required to investigate the possible additional beneficial effects, if any, of SCFA supplementation on subjects diagnosed with the Metabolic Syndrome (abdominal obesity, dyslipidaemia, hypertension, insulin resistance or glucose intolerance, etc.).

It s clear from this study that different combinations of SCFAs exerts different metabolic effects in human subjects. Additional research is therefore required to establish the appropriate ratio in which these SCFAs should be present in any given supplement. It is suggested that the supplement that contained a ratio of acetate, propionate and butyrate, similar to the ratio of SCFAs produced during natural fermentation of fibre in the large gut, benefits health more so than an acetate- and propionate-containing supplement. Furthermore, previous studies also showed how the route or method of administration might influence the metabolic outcome of SCFA supplementation. The route or method of administration determines the location of the gastro-intestinal tract where absorption will take place, which in turn, determines the site where these acids are metabolised. SCFAs released in the colon enter the hepatic circulation, where it can take part in lipid and carbohydrate metabolism in the liver. Intravenous injections of SCFAs reach the cells of the periphery, where it can partake in cellular metabolism of other body tissue. SCFAs derived from the microbial fermentation of dietary fibre enter the hepatic blood circulation via the colon. It is important to know whether the beneficial

effects associated with the intake of dietary fibre are related to the specific region of SCFA absorption.

Before making available supplements that contain acetate, propionate and butyrate, it would be necessary to evaluate the degree to which the most appropriate combination of SCFAs can contribute to the prevention and onset of CHD. The results of this study open new possibilities that could be used to the benefit of understanding the relationship between food and disease and ~~h~~opefully" stimulate further research.

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APPENDIX: A

CONSENT FORM

THE EFFECTS OF SHORT-CHAIN FATTY ACIDS ON HAEMOSTATIC RISK FACTORS FOR CARDIOVASCULAR HEART DISEASES IN WESTERNISED BLACK MEN

DECLARATION BY OR ON BEHALF OF THE PARTICIPANT

I, undersigned,
..... of
.....
.....
... (Address)
Identity number: .....

A I confirm that:

1. I have been asked to participate in the above-mentioned research project, carried out by the Fibrinogen Unit, Technikon Free State and University of the Orange Free State.

2. The information including the purpose of the study, advantages and disadvantages have been completely explained to me.

3. I give my permission for the use of the results obtained in this research project for publication purpose, thus making other scientists aware of the findings as long as my anonymity is protected at all times. The information obtained will be confidential.

4. It was clearly explained to me that I can refuse to participate in this study or I can withdraw my permission to participate at any time. If I refuse or withdraw, I will not be disadvantaged in any way and it will not be held against me.

5. The information was explained to me by ..... (name of interviewer) in ..... (language) and I confirm that I have good command of this language and understood the explanations. I was also given the opportunity to ask questions on things I did not understand, and I can also ask further questions at any time during the project.

6. No pressure was applied on me to take part in this research project.

B I hereby agree voluntarily to take part in this research project.

Signed/confirmed at ..... on 200

..... (signature) .....
Participant Witness

DECLARATION BY OR ON BEHALF OF RESEARCHER

I, ……., declare that:

1. I have explained the information in this document and about the project to ……
……..…….. (name of participant).

2 I asked the participant to ask any questions if something was not clear.

3 That the interview was conducted in English, Afrikaans, or …….. (language) OR that the interview was conducted in …… (language) with the help of a translator, ……(name of translator).

Signed/confirmed at ……..... on ……...200

…….. (signature) ……
Researcher or his representative Witness

DECLARATION BY TRANSLATOR

I, ……., declare that:

1 I have translated the content of this document from English/Afrikaans into …….. (language) to ……..…… (name of participant) and that I have translated the participant's questions to the researcher or his/her representative as well as the answers of the latter to the participant.

2 That the information that I have so translated was a correct interpretation of what I was asked to translate

Signed/confirmed at ……..... on ……...200

…….. (signature) ……
Translator Witness

FOROMO YA TUMELLO

FATTY ACIDS TSA KETANE TSE KHOTSWANE DI TSHWENYA JWANG MATSHWAO A LEFU LA PELO MO BANNENG BA BATHO BANTSHO

BOIKANO KA, KAPA MO SEBAKENG SA MO NKA KAROLO

Nna, ... mo nka karolo, wa .....
.....
.....
.... (aterese)
Nomoro ya boitsibiso: .....

A Ke dumela hore:

1. Ke kopilwe hore ke nke karolo resetjheng eo ho buiwang ka yona hodimo, e etswa ke Fibrinogen Unit, Technikon ya Foreisetata le University ya Foreisetata.

2. Ke hlaloseditswe maikemisetso, melemo le dikotsi tsa resetjhe ena ka botlalo.

3. Ke fana ka tumello ya ka hore diphetho tsa resetjhe ena di sebedisetswe ho ngola atikele etla tsibisa bo rra saense ba bang ka diphitlhelelo tsena, empa dipheto di tla ba sephiri, feela di tla sebediswa fa lebitso la ka le sa hlahe ka nako tsohle.

4. Ke hlaloseditswe hore nka hanna ho nka karolo, kapa nka hula tumello ya ka ya ho nka karolo ka nako e ngwee le e ngwee, mme sena ha se na ho sebediswa kgahlano le nna.

5. Ke hlaloseditswe ka resetjhe ke ..... (lebitso la mohlalosi) ka .....(puo) ke a dumella hore puo ena ke a e utlwisisa le hona ho e tseba. Ke fuwe sebaka sa ho botsa dipotso ka dintho tseo ke neng ke sa di utlwisise, ke utlwisisa hore nka botsa ka nnako e ngwee le e ngwee ha resetjhe e tsweletse.

6. Ha ke a bewa ka tlasa khatelelo hore ke nke karolo mo resetjheng ena.

B. Ke dumella ka ho rata ha ka ho nka karolo mo resetjheng ena.

E saennwe ..... . (sebaka), ka la ..... 20(

....
Mo nka karolo Paki

BOIKANO KA KAPA MO SEBAKENG SA MORESETJHI

Nna, ..., ke ikana hore:

1. Ke hlaloseditse ..... (lebitso la mo nka karolo) ka resetjhe le foromo ena.

2. Ke bolleletse mo nka karolo ho botsa dipotso ha e ba a sa utlwisise

3. Dipotso le thlahiso lesedi di ne di nne di le ka English, Afrikaans, kapa .....(puo); kapa dipotso di ne di botswa ka .....(puo) ka thuso ya mofetoledi, ...
.....(lebitso la mofetoledi).

E saennwe ..... . (sebaka), ka la 200

....
Moresetjhi Paki

BOIKANO KA MOFETOLEDI

Nna, ..., ke ikana hore:

1. Ke fetoletse ntshetso lesedi le dipotso ho tswa ho English/Afrikaans ho ya ho ..... (puo) ho .....(lebitso la mo nka karolo), le dikarabo ho tswa ho moresetjhi ho ya ho mo nka karolo.

2. Ke fetoletse di ntho tseo ho neng ho thwe ke di fetolele hantlhe

E saennwe ..... . (sebaka), ka la 200

....
Mofetoledi Paki

APPENDIX: B

TECHNIKON FREE STATE FIBRINOGEN UNIT

SCFA PROJECT: RECRUITMENT QUESTIONNAIRE

DATE: _____/_____/_____

INTERVIEWER: _____

SURNAME **AND** **INITIALS:**

_____

HOUSE **DOCTOR:**

_____

PATIENT:
NAME:_____

ADDRESS:_____

_____

_____

SECTION/DEPARTMENT:_____

TEL: _____

AGE: _____years_____months

SMOKING HABITS:

YES

NO

BODY MASS: _____cm

LENGTH: _____kg

BLOOD PRESSURE: _____/_____

ACTIVITY LEVEL:

INACTIVE

MEDIUM ACTIVE

ACTIVE

FAMILY HISTORY:

CORONARY HEART DISEASE _____
DIABETES MELLITUS _____
HYPERCHOLESTEROLAEMIA _____
OTHER (specify):

_____

MEDICAL HISTORY:

ANGINA/CORONARY HEART DIASEASE _____
MYOCARDIAL INFARCTION _____
STROKE _____
BYPASS _____
BLOOD CLOTS _____
HIGH BLOOD PRESSURE _____
DIABETES TYPE I/II _____
FAMILIAL CHOLESTEROL _____

ANY CHRONIC DISEASES MEDICATION:

YES

NO

SPECIFY:_____

HOW OFTEN DO YOU USE ALCOHOL:

DAILY

WEEKLY

MONTHLY

OTHER: _____

BLOOD TESTS

BLOOD CHOLESTEROL: _____

FASTING GLUCOSE: _____

PLASMA FIBRINOGEN: _____

FOR OFFICE USE ONLY:

SUBJECT: APPROVED REJECTED

ASSIGNED SUBJECT NO: _____

APPENDIX:C

FOOD FREQUENCY QUESTIONAIRE

Name: _____ Respondent number: [ ][ ] 1-2

Force number : _____

Interviewer: _____ [ ][ ] 3-4

QUANTITATIVE FOOD FREQUENCY QUESTIONNAIRE

Thank you for giving your time to participate in this survey We would like to find out if short-chain fatty acids, which is a fermentation product of dietary fiber, have a effect on blood sugar and insulin levels in men The short-chain fatty acids will be given to you in the form of a capsule. The information about your eating habits is important as it will tell us if this supplement has any effect on the eating habits of people taking it

Please think carefully about the food and drinks you have consumed during the past 6 months. I will now go through a list of foods and drinks with you and I would like you to tell me:

- if you eat these particular foods.
- how the food is prepared (by the mess or elsewhere)
- how much of the food you eat at a time, and
- how many times a day you eat it and if you do not eat it every day, how many times a week or a month it is eaten"

To help you describe the amount of food, I will show you models of different amounts of food. Please say which model is the closest to the amount eaten, or if it is smaller, between sizes or bigger than the models Amounts must be reported as cups (c) , tablespoons (T), serving spoons (SP) or teaspoons (t).

- THERE ARE NO RIGHT OR WRONG ANSWERS.
- EVERYTHING YOU TELL ME IS CONFIDENTIAL
- IS THERE ANYTHING YOU WANT TO ASK NOW?
- ARE YOU WILLING TO GO ON WITH THE QUESTIONS?

- ENCIRCLE APPROPRIATE ANSWER

Do you follow any special diet? Yes (1) No (2) [ ] 5

If yes, please specify (encircle appropriate answer) [ ] 6

1. Diabetic diet
2. Slimming diet
3. Cholesterol diet
4. Allergies
5. Other, (specify) _____

- Do you use salt in your food? Yes (1) NO(2) Don't know (3) [ ] 7

- Do you use any dietary supplements? Yes (1) NO(2) Don't know (3) [ ] 8

- If yes, please specify the type (name), how often , and how much:

Vitamins: _____ [ ][ ][ ] 9-11
Minerals: _____ [ ][ ][ ] 12-14
Protein: _____ [ ][ ][ ] 15-17
Energy: _____ [ ][ ][ ] 18-20
Other: _____ [ ][ ][ ] 21-23

EATING PATTERNS: (FREQUENCY OF EATING)

PLEASE INDICATE WHICH OF THE FOLLOWING BEST DESCRIBES THE EATING PATTERN YOU USUALLY FOLLOW (MARK ONLY ONE): [ ] 24

- 1. More than three meals with eating between meals
- 2. Three meals with eating between meals
- 3 Three meals with no eating between meals
- 4 Two meals with eating between meals
- 5 Two meals with no eating between meals
- 6 One meal with eating between meals
- 7 One meal with no eating between meals
- 8 Nibble the whole day, no specific meals
- 9 Others (please specify) _____

DO YOU EAT BREAKFAST? [ ] 25

- 1 Regularly (> 4 times a week)
- 2 Sometimes (1 - 3 times a week)
- 3 Never

HOW OFTEN DO YOU EAT AT THE FOLLOWING PLACES AWAY FROM HOME?

Family	1. Never	2. > once/week	3. Weekly	4. Monthly	5. > once/month	[ ] 26
Friends	1. Never	2. > once/week	3. Weekly	4. Monthly	5. > once/month	[ ] 27
Café	1. Never	2. > once/week	3. Weekly	4. Monthly	5. > once/month	[ ] 28
Restaurant, Fast food	1. Never	2. > once/week	3. Weekly	4. Monthly	5. > once/month	[ ] 29
Other specify _____	1. Never	2. > once/week	3. Weekly	4. Monthly	5. > once/month	[ ] 30

Do you drink coffee with your meals? [ ] 31

- 1. Yes
- 2. No

If yes, at which meals

Breakfast	1. Yes	2. No	[ ] 32
Lunch	1. Yes	2. No	[ ] 33
Supper	1. Yes	2. No	[ ] 34
Snacks	1. Yes	2. No	[ ] 35

Do you drink tea (except Rooibos) with your meals?

- 1. Yes
- 2. No

[ ] 36

If yes, at which meals

Breakfast	1. Yes	2. No	[ ] 37
Lunch	1. Yes	2. No	[ ] 38
Supper	1. Yes	2. No	[ ] 39
Snacks	1. Yes	2. No	[ ] 40

With how many meals per day do you eat meat fish, or poultry?

[ ] 41

- 1. One meal
- 2. Two meals
- 3. All meals
- 4. None

Do you eat fresh fruit and/or vegetables with the following meals?

Breakfast	1. Yes	2. No	[ ] 42
Lunch	1. Yes	2 No	[ ] 43
Supper	1. Yes	2. No	[ ] 44
Snacks	1. Yes	2. No	[ ] 45

How often do you usually drink alcohol?

[ ] 46

1. Every day
2. 5-6 days / week
3. 3-4 days / week
4. 1-2 days / week
5. Weekends
6. Less than once a week
7. Never

On a weekday when you do drink alcohol how many drinks do you usually have ?

	per day	
Beer (how many bottles/cans)		[ ] [ ] 47-48
Brandy/whisky (how many tots)		[ ] [ ] 49-50
Vodka/gin (how many tots)		[ ] [ ] 51-52
Sjerrie/ sweetwine (how many glasses)		[ ] [ ] 53-54
Table wine (how many glases)		[ ] [ ] 55-56
Other, specify:		[ ] [ ] 57-58

On a weekend day when you do drink alcohol how many drinks do you usually have ?

	per day	
Beer (how many bottles/cans)		59-60
Brandy/whisky (how many tots)		61-62
Vodka/gin (how many tots)		63-64
Sjerrie/ sweetwine (how many glasses)		65-66
Table wine (how many glases)		67-68
Other, specify:		69-70

Do you smoke? 71

1. Never smoked
2. Smoked previously, but not currently
3. Currently smoking

If currently smoking, how many sigarettes do you smoke per day ? _____ 72 -

If you smoked previously, how many years did you smoke? _____ 74 -

How many cicarettes did you smoke a day? _____ 76 -

Are you a living-in member ? 1. Yes 2. No 78

SUMMARY OF FOOD FREQUENCY QUESTIONNAIRE

FOOD	CALCULATIONS	CODE				AMOUNT/DAY (g)				
										1-8
										9-16
										17-24
										25-32
										33-40
										41-48
										49-56
										57-64
										65-72
										73-80
										1-8
										9-16
										17-24
										25-32
										33-40
										41-48
										49-56
										57-64
										65-72
										73-80
										1-8
										9-16
										17-24
										25-32
										33-40
										41-48
										49-56
										57-64
										65-72
										73-80
										1-8
										9-16
										17-24
										25-32
										33-40
										41-48
										49-56
										57-64
										65-72
										73-80
										1-8
										9-16
										17-24
										25-32
										33-40
										41-48
										49-56
										57-64
										65-72
										73-80

FOOD	DESCRIPTION	AMOUNT USUALLY EATEN	TIMES EATEN				CODE	AMOUNT/DAY
			Per day	Per week	Per month	Seldom/never		
Maize-meal porridge	Stiff (pap)						3400	
Maize-meal porridge	Soft (slappap)						3399	
Maize-meal porridge	Crumbly (phutu)						3401	
Sour porridge	Specify ratio Mabella/Maize						3399	
Mabella porridge	Stiff, coarse, fine						3437	
Oats porridge	Brand name:						3239	
Breakfast cereals	Puffed Wheat, plain						3325	
	Puffed wheat, sweet							
	Corn Flakes, plain						3243	
	Weet Bix						3244	
	Puffed Rice, sweet						3372	
	Rice crispies						3252	
	Pronutro, plain						3245	
	Pronutro, High fibre						3436	
	Muesli						3303	
	Specify types usually eaten _____ _____ Brand names of cereals available at home now: _____							
Milk on porridge or cereal: Circle type usually used:	None							
	Whole/fresh						2718	
	sour						2787	
	25 fat						2772	
	Fat free/ skimmed						2775	
	Milk blend						2771	
	Soy milk						2737	
	Condensed (whole, sweet)						2714	
	Condensed (skim, sweet)						2744	
	Evaporated whole						2715	
	Evaporated low fat						2827	
	Non-dairy creamer						2751	
Is sugar added to porridge or cereal? (tick box)	None							
	White						3989	
	Brown						4005	
	syrup						3988	
	Honey						3984	
	Sweetner: type _____							
Is fat added to porridge or cereal? (tick box)	None							
	Animal fat (butter)						3479	
	Hard margarine						3484	
	Soft margarine						3496	
	Oil						3507	
	Peanut butter						3485	
Samp/ Maize rice	Bought						3250	
	Self ground						3725	
Samp and beans	Specify ratio (1:1)						3402	
Samp and peanuts	specify ratio							

FOOD	DESCRIPTION	AMOUNT USUALLY EATEN	TIMES EATEN				CODE	AMOUNT/DAY
			Per day	Per week	Per month	Seldom/never		
Rice: specify brands	White						3247	
Names:	Brown						3315	
	Sorghum rice						3437	
Stamped wheat							3249	
Pastas	Macaroni						3262	
	spaghetti						3262	
	Spaghetti in tomato sauce						3258	
	Other:							
Bread/Bread rolls	White						3210	
Bread slices: thin	Brown						3211	
Medium, thick	whole wheat						3212	
Other breads	Specify types, e.g.							
	Raisin						3214	
	Maize Meal						3278	
	Sweetcorn						3379	
	Rye						3213	
	Other							
Pizza (specify toppings)	Cheese, tomato & onion						3353	
Hot dogs (specify sausage)								
Hamburger (specify meat)								
Are any of the following spreads used on bread? (tick box)	Butter						3479	
	Butro						3523	
	Animal fat (beef tallow)						3494	
	Lard						3495	
	Hard margarine (brick)						3484	
	Soft margarine (light)						3496	
	Cooking fat						3516	
Peanut butter							3485	
Sweet spreads	Jam						3985	
	Syrup						3988	
	honey						3984	
Marmite/							4030	
OXO/							4029	
Bovril							4029	
Fish paste							3109	
Meat paste							2917	
Cheese	Specify types:							
	Cottage low-fat cheese						2760	
	Cream cheese						2725	
	Gouda						2723	
	Cheddar						2722	
	Other:							
Cheese spreads	Low fat						4310	
	Full fat						2730	
	Specify types:							

FOOD	DESCRIPTION	AMOUNT USUALLY EATEN	TIMES EATEN				CODE	AMOUNT/DAY
			Per day	Per week	Per month	Seldom/never		
Atchar							3117	
Other spreads: (Specify types)								
Dumpling							3210	
Vetkoek							3257	
Provita							3235	
Crackers	Refined						3331	
	Whole wheat						3391	
Rusks - *Commercial*	Bran						3330	
	Buttermilk						3329	
	White						3364	
	Boerebeskuit, white						3364	
Home-made	All-bran						3380	
	Raisins						3380	
	Buttermilk, white						3215	
	Buttermilk, whole wheat						3255	
	Other, specify							
Scones							3237	
Muffins	Plain						3408	
	Bran						3407	

HOW MANY TIMES A DAY DO YOU EAT BREAD? _____

FOOD	DESCRIPTION	AMOUNT USUALLY EATEN	TIMES EATEN				CODE	AMOUNT/DAY
			Per day	Per week	Per month	Seldom/never		
Chicken	Boiled with skin						2926	
	Without skin						2963	
Do you eat the chicken with the skin? Yes ☐ No ☐	Fried: in batter/crumbs						3018	
	Fried but not coated						2925	
	Roasted/grilled - with skin						2925	
	Roasted/grilled - without skin						2950	
Chicken bones stew							A003	
Chicken heads, raw							2999	
Chicken stew, with veg & skin							3005	
Chicken offal	Giblets						2998	
Chicken pie	Commercial						2954	
	Home-made						2954	
Red meat: Beef	Fried/grilled: with fat						2908	
	without fat						2959	
	Stewed/boiled: with fat						3006	
	without fat						2909	
	Mince with tomato & onion						2987	
	Mince - curry						3015	
	Meatball regular						2966	
	lean						3034	
Red meat: Mutton	Fried/grilled: with fat						2927	
	without fat						2934	
	Stewed/boiled: with fat						3040	

FOOD	DESCRIPTION	AMOUNT USUALLY EATEN	TIMES EATEN				CODE	AMOUNT/DAY
			Per day	Per week	Per month	Seldom/never		
	without fat						2916	
Red meat: Pork	Fried/grilled: with fat						2930	
	without fat						2977	
	Stewed/boiled: with fat						3046	
	without fat						3045	
	Crumbed/Schnitzel						2992	
Red meat: Goat	Fried/grilled: with fat						4281	
	without fat							
	Stewed/boiled: plain						4281	
	with veg						4282	
Offal:	Intestines: boiled, nothing added						3003	
Specify type:	"Velderm" fried						3003	
	Stewed with vegetables							
	Liver						2955	
	Kidney						2956	
	Tripe "pens" trotters, head						3003	
	Pluck (lungs, heart, gullet)						3019	
Specify vegetables used in meat stews (only if not mentioned elsewhere)								
Wors/sausage	Fried						2931	
Bacon							2906	
Cold meats	Polony						2919	
	Ham						2967	
	Vienna's canned						2936	
	Russian						2948	
	Frankfutter						2937	
	Other (specify)							
Canned meat	Bully beef						2940	
	Other (specify)							
Meat pie	Bought						2939	
Biltong	Beef with fat						2911	
	fat trimmed						3021	
	"Droë wors"						2949	
Legumes:	Stews & curries (specify)							
specify dried beans/ peas/lentils	Soups						3157	
	Salads						3174	
Baked beans							3176	
Soya products e.g.	Brands at home now Don't know							
Toppers	Show examples						3196	
Imana								
Fried fish (fresh or frozen fried in sun oil)	With batter/crumbs						3072	
	Without batter/crumbs						3060	
Fresh water fish Specify type	Specify cooking method Medium fat, batter, fried						3094	

FOOD	DESCRIPTION	AMOUNT USUALLY EATEN	TIMES EATEN				CODE	AMOUNT/DAY
			Per day	Per week	Per month	Seldom/never		
Canned fish:								
Pilchards	In brine						3055	
	In tomato sauce						3102	
	Mashed with fried onion						A005	
Sardines	In oil						3087	
	In tomato sauce						3087	
Tuna	In oil						3093	
	In brine						3054	
Mackerel							3113	
Salmon							3101	
Pickled fish/curried							3076	
Fish cakes Specify canned or other	Fried: oil/butter/margarine, commercial						3080	
Salted dried fish							2867	
Eggs	Boiled/poached						2876	
	Scrambled: in oil						2889	
	in butter						2886	
	in margarine						2887	
	Fried: in oil						2869	
	in butter						2868	
	in margarine						2877	
	in bacon fat						2870	
	Curried						2902	

HOW MANY TIMES A WEEK DO YOU EAT:

MEAT _____
BEANS _____
CHICKEN _____
FISH _____ AND
EGGS _____ ?

FOOD	DESCRIPTION	AMOUNT USUALLY EATEN	TIMES EATEN				CODE	AMOUNT/DAY
			Per day	Per week	Per month	Seldom/never		
CABBAGE	Boiled, nothing added						3756	
	Boiled with potato, onion, fat						3813	
	Fried, in margarine (nothing added)						3810	
	Fried, in oil (nothing added)						3912	
	Boiled, then fried with potato and onion						A006	
	Other:							
Spinach/morogo/ imfino/ other greem leafy vegetables: List names:	Boiled, nothing added						3913	
	Boiled fat added (margarine)						3898	
	Boiled with onion/tomato, fat						A011	
	- onion & potato (margarine)						3901	
	- onion, tomato & potato							
	- with peanuts							
	Other:							
Tomato and onion gravy'/relish/chow	Home made with fat						3910	
	without fat						3925	

FOOD	DESCRIPTION	AMOUNT USUALLY EATEN	TIMES EATEN				CODE	AMOUNT/DAY
			Per day	Per week	Per month	Seldom/n ever		
	Canned						4129	
Pumkin	Cooked in fat and sugar						3893	
Specify Type:	Boiled, little sugar and fat							
	Boiled						4164	
	Other:							
Carrots	Boiled sugar & fat						3819	
	Boiled nothing added						3757	
	Boiled, potato, onion, no fat						3934	
	Boiled, potato, onion, margarine						3822	
	Boiled, with sugar						3818	
	Raw, salad (orange juice)						3711	
	Chakalaka							
	Other:							
Mealies/	On cob						3725	
Sweet corn	Off cob - creamed sweetcorn						3726	
	Off cob - whole kernel						3942	
Beetroot	Cooked						3698	
	Salad (bought or home made)						3699	
Potatoes	Boiled with skin						4155	
	without skin						3737	
	Baked in skin (flesh and skin)						3736	
	Baked in skin (flesh only)						3970	
	Mashed - skim milk, margarine						3875	
	Mashed - whole milk, margarine						3878	
	Roasted in beef fat						3878	
	French fries/potato chips (oil)						3740	
	Salad (mayonnaise and egg)						3928	
	Other:							
Sweet potatoes	Boiled with skin						3748	
	without skin						3903	
	Baked with skin (flesh only)						3748	
	without skin						3903	
	Mashed						3903	
	Mashed with fat & sugar						3749	
	Other:							
Peas	Green, frozen						4146	
	Green, frozen with sugar						3720	
	With sugar and butter						3859	
	Tinned peas						4149	
Green peppers	Raw						3733	
	Cooked (stew with oil)						3865	
Brinjal/egg plant	Cooked						3700	
	Fried in oil						3802	
	Stew (oil, tomato, onion)						3798	
Mushrooms	Raw						3842	
	Sautéed in brick margarine						3839	
	Sautéed in oil						3841	
Onions	Sautéed in sun oil						3730	
	Sautéed in margarine						3844	
Salad vegetables	Raw tomato						3750	
	Lettuce						3723	

FOOD	DESCRIPTION	AMOUNT USUALLY EATEN	TIMES EATEN				CODE	AMOUNT/DAY
			Per day	Per week	Per month	Seldom/never		
	Cucumber						3718	
	Avocado's						3656	
Green Beans	Boiled nothing added						3696	
	Cooked, potato, onion, margarine						3792	
	Cooked, potato, onion, no fat						3933	
Cauliflower	Boiled						3716	
Other vegetables specify								
If you fry veg or add fat specify type of fat usually used (tick box)	Butter						3479	
	Butro						3523	
	Animal fat (beef tallow)						3494	
	Lard						3495	
	Hard margarine (brick)						3484	
	Soft margarine (tub)						3496	
	Soft margarine (light)						3524	
	Sunflower oil						3507	

HOW MANY TIMES A WEEK DO YOU EAT VEGETABLES? _____

FOOD	DESCRIPTION	AMOUNT USUALLY EATEN	TIMES EATEN				CODE	AMOUNT/DAY
			Per day	Per week	Per month	Seldom/never		
Mayonnaise/	Mayonnaise: Bought						3488	
	home-made						3506	
Salad dressing	Cooked sald dressing						3503	
	Salad dressing low-oil						3505	
	Salad dressing French						3487	
	Oil: Olive						3509	
	Oil: Sunflower						3507	
	Oil: Canola						4280	
Apples	Fresh						3532	
	Canned, unsweetened						4216	
Pears	Fresh						3582	
	Canned, in syrup						3583	
Bananas							3540	
Oranges							3560	
Naartjies							3558	
Grapes							3550	
Peaches	Fresh						3565	
	Canned, in syrup						3567	
Apricots	Fresh						3534	
	Canned, in syrup						3535	
Mangoes	Fresh						3556	
Pawpaw	Raw						3563	
Pineapple	Raw						3581	
	Canned, in syrup						3648	
Guavas	Fresh						3551	
	Canned, in syrup						3553	
Watermelon							3576	

FOOD	DESCRIPTION	AMOUNT USUALLY EATEN	TIMES EATEN				CODE	AMOUNT/DAY
			Per day	Per week	Per month	Seldom/never		
Spanspek	Orange flesh						3541	
	Green flesh						3575	
wild fruit/berries (Specify types)								
Dried fruit (also as snacks)	Raisins						3552	
	Prunes (raw)						3596	
	Prunes (cooked with sugar)						3564	
	Peaches (raw)						3568	
	Peaches (cooked with sugar)						3569	
	Apples (raw)						3600	
	Dried fruit sweets						3995	
	Other							
Other fruit								

HOW MANY TIMES A WEEK DO YOU EAT FRUITS? _____

WE NOW WILL ASK YOU QUESTIONS ABOUT WHAT YOU USUALLY DRINK

FOOD	DESCRIPTION	AMOUNT USUALLY EATEN	TIMES EATEN				CODE	AMOUNT/DAY
			Per day	Per week	Per month	Seldom/never		
Water							4042	
Tea	Ceylon						4038	
	Rooibos						4054	
Coffee							4037	
Sugar per cup of tea or coffee	White						3989	
	Brown						4005	
Milk per cup of tea or coffee	Fresh/Longlife						2718	
	Fresh Longlife 2%						2772	
What ype of milk do you put in tea	Goat						2738	
	Fresh/Longlife/Fat free (skimmed milk)						2775	
	Whole milk powder						2831	
	Reconstituted Specify brand							

FOOD	DESCRIPTION	AMOUNT USUALLY EATEN	TIMES EATEN				CODE	AMOUNT/DAY
			Per day	Per week	Per month	Seldom/never		
	Skimmed milk powder Reconstituted Specify brand _____						2719	
	Milk blend, reconstituted Specify brand						2771	
	Whitener/non-dairy creamer Specify brand _____						2751	
	Condensed milk (whole)						2714	
	Condensed milk (skim)						2744	
	Evaporated milk (whole)						2715	
	Evaporated milk (low fat)						2827	
	None							
Milk a such: What type of milk do you drink as such?	Fresh/long life/ whole						2718	
	Fresh/long life/ 2%						2772	
	Fresh/longlife/fat free(skimmed)						2775	
	Goat						2738	
	Sour / Maas						2787	
	Buttermilk						2713	
Milk drinks Specify brands, including milk Supplements and type of milk used	Nestlé - nesquik _____						4287	
	Milo						2735	
	Flavoured milk _____						2774	
	Other							
Yoghurt	Drinking yoghurt						2756	
	Thick yoghurt, plain, fruit						2732	
Squash	SixO						3990	
	Oros						3982	
	Lecol with sugar						3982	
	Lecol with artificial sweetner						3990	
	Kool Aid						3982	
	Other: _____							
Fruit juice	Fresh/ Liquifruit/Ceres/						2866	
	"Tropica"/mixtures with milk						2791	
Fruit syrups	Average						2865	
	Guava syrup						2864	
Fizzy drink Coke, Fanta	Sweetened						3981	
	Diet						3990	
Mageu/Motogo	Sorghum beer						4056	
Alcoholic beverages such as sorghum beer	Specify: _____						4039	
Other, specify:	Beer average						4031	
	Wine						4033	
	Cider						4057	

FOOD	DESCRIPTION	AMOUNT USUALLY EATEN	TIMES EATEN				CODE	AMOUNT/DAY
			Per day	Per week	Per month	Seldom/never		
	Spiritus, e.g brandy, whisky, gin, vodka						4035	
	Liqueur						4055	
	Other: _____							

PLEASE INDICATE WHAT TYPES AND AMOUNTS OF SNACKS, PUDDINGS AND SWEETS YOU EAT:

FOOD	DESCRIPTION	AMOUNT USUALLY EATEN	TIMES EATEN				CODE	AMOUNT/DAY
			Per day	Per week	Per month	Seldom/never		
Potato crisps/chips							3417	
Peanuts	Roasted, unsalted						3452	
	Roasted, salted						3458	
Cheese curls:	Average						3267	
Niknaks, etc.	Savoury						3418	
Popcorn	Plain (no salt and butter)						3332	
	Plain (salt and butter added)							
	Sugar coated						3359	
Raisins (seeds)							4231	
Chocolates	Milk						3987	
	Kit Kat						4024	
	Peppermint crisp						3997	
	Specify types and names:							
Candies	Sugus, gums, hard sweets (specify)						3986	
	Peppermint						4004	
Sweets	Toffees						3991	
	Hard boiled						3986	
	Fudge, caramels (specify)						3991	
Biscuits/cookies	Specify type: _____							
	Home made plain						3233	
	shortbread, butter						3296	
	Commercial, plain						3216	
	Commercial, with filling						3217	
Cakes and tarts	Chocolate, plain						3419	
Pancakes/crumpets							3344	
Koeksisters							3231	
Savouries	Sausage rolls						2939	
	Samousas - vegetable						3414	
	Samousas - mutton						3355	
	Biscuits e.g. bacon kips						3331	
	Other: _____							
Pudding: jelly							3983	

FOOD	DESCRIPTION	AMOUNT USUALLY EATEN	TIMES EATEN				CODE	AMOUNT/DAY
			Per day	Per week	Per month	Seldom/never		
Baked pudding	Plain batter						3429	
Instant pudding	Skim milk						3314	
	Whole milk						3266	
Ice cream	Commercial regular						3483	
	Commercial rich						3519	
	Soft serve						3518	
	Sorbet						3491	
	Ice lollies						3982	
	Chocolate coated individual ice creams (e.g. Magnum)							
Custard	Home made, whole milk						2716	
	Ultramel						2716	
Cream	Fresh						3520/ 3480	
Other puddings (Specify)								

HOW MANY TIMES A WEEK DO YOU EAT SNACK FOODS? _____

SAUCES / GRAVIES / CONDIMENTS

FOOD	DESCRIPTION	AMOUNT USUALLY EATEN	TIMES EATEN				CODE	AMOUNT/DAY
			Per day	Per week	Per month	Seldom/never		
Tomato sauce							3139	
Worcester sauce							4309	
Chutney	Fruit						3168	
	Tomato						3114	
Pickles							3866	
Packet soups							3158	
Beef stock							4029	
Chicken stock							4029	
Others:								

WILD BIRDS, ANIMALS, INSECTS OR FRUITS AND BERRIES (hunted or collected in rural areas or on farm, specify)

PLEASE MENTION ANY OTHER FOODS YOU EAT MORE THAN ONCE EVERY TWO WEEKS WHICH WE HAVE NOT TALKED ABOUT AND OR FOODS EATEN IN OTHER HOMES OR PLACES DURING THE PAST WEEK

FOOD	DESCRIPTION	AMOUNT USUALLY EATEN	TIMES EATEN				CODE	AMOUNT/DAY
			Per day	Per week	Per month	Seldom/never		

FOOD	DESCRIPTION	AMOUNT USUALLY EATEN	TIMES EATEN				CODE	AMOUNT/DAY
			Per day	Per week	Per month	Seldom/never		

ARE THERE ANY FOODS THAT YOU EAT WHICH WE HAVEN'T TALKED ABOUT? PLEASE LIST THEM.

FOOD	DESCRIPTION	AMOUNT USUALLY EATEN	TIMES EATEN				CODE	AMOUNT/DAY
			Per day	Per week	Per month	Seldom/never		

THANK YOU FOR YOUR CO-OPERATION AND PATIENCE

GOOD BYE !

APPENDIX:D

QUESTIONNAIRE FOR SUBJECTS WHO COMPLETED THE STUDY

Please answer the following questions regarding your participation in the research study:

Respondent number: _____

Employee Number: _____

1. Did you experience any vomiting during the study?

1. Yes 2. No

If yes, how many times?

1. After each meal 2. Once a day 3. Once a week

4. Other

2. Did you experience any flatulence during the study?

1. Yes 2. No

If yes, how many times?

1. After each meal 2. Once a day 3. Once a week

4. Other

3. Did you experience any stomach cramps during the study?

1. Yes 2. No

If yes, how many times?

1. After each meal 2. Once a day 3. Once a week

4. Other

4. Did you experience any changes in your lifestyle during the study?

1. Yes 2. No

If yes, describe the changes:

5. Did you experience any constipation during the study?

1. Yes 2. No

If yes, how frequent?

1. After each meal 2. Once a day 3. Once a week

4. Other

6. Did you experience an increased appetite during the study?

1. Yes 2. No

If yes, explain:

7. Did you experience any changes in your alcohol consumption during the study?

1. Yes 2. No

If yes, to what extent?

8. Did you use any medication/supplements during the study?

1. Yes 2. No

If yes, what is the name of the medication/supplement you used?

If yes, how many times did you use this medication/supplement?

1. After each meal 2. Once a day 3. Once a week

4. Other

and for how long (days)?

9. Did you consume all of the experimental capsules every day?

1. Yes 2. No

10. Was the amount of capsules consumed acceptable?

1. Yes 2. No

11. Would you be willing to consume these capsules daily if they are considered as healthy?

1. Yes 2. No

12. Did you experience any other side-effects of the supplement during the study?

1. Yes 2. No

If yes, please specify?

If yes, how frequent?

1. After each meal 2. Once a day 3. Once a week

4. Other

13. Did you experience any positive effects on your health during the study?

1. Yes 2. No

If yes, explain.

14. Do you have any other comments you would like to make regarding the study?

The research team would like to thank you for your co-operation during the study. The project is very important for gaining new scientific knowledge.