THE EFFECT OF A COMBINATION OF SHORT-CHAIN FATTY ACIDS ON THE LIPID PROFILES OF WESTERNISED BLACK MEN

HENRY GLEIMIUS

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THE EFFECT OF A COMBINATION OF SHORT-CHAIN FATTY ACIDS ON THE LIPID PROFILES OF WESTERNISED BLACK MEN

By

Henry Gleimius

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DECLARATION OF INDEPENDENT WORK

I, HENRY CHARLES LEIGTON GLEIMIUS, identity number [redacted] and student number [redacted], do hereby declare that this research project submitted to the Central University of Technology, Free State for the degree MAGISTER 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 myself or any other person in fulfilment of the requirements for the attainment of any qualification.

SIGNATURE OF STUDENT

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SUMMARY

South Africa is in the process of Westernisation, especially in the Black male population, with an adaptation of a westernised diet. Morbidity and mortality rates have increased because of the higher incidence of cerebrovascular and coronary heart disease (CHD) in this population group. Dietary fibre is used as one of the pillars in the prevention and treatment of CHD. Previous studies have shown that the addition of dietary fibre to the diet can significantly improve metabolic profiles, including lipid profiles, of human subjects. A decrease, especially in the serum total cholesterol levels, is beneficial and is associated with a decreased risk of the development of CHD and atherosclerosis. It is believed that some of these beneficial effects may be mediated in part by the SCFAs produced during the microbial fermentation of dietary fibre in the large intestine. These SCFAs include acetate, propionate and butyrate.

The main objective of this study was to examine the hypothesis that exposure to two different combinations of short-chain fatty acids (SCFAs), orally administered to a group of local westernised Black men will have beneficial effects on their lipid profiles. The concentration of SCFAs used was equivalent to that generated by the fermentation of 15g of mixed fibre. 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

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50% acetate and 50% propionate. It is believed that the increase of colonic acetate will have a positive effect on the lipid profile.

The study was that of a randomised, placebo-controlled, double-blinded clinical trial. Voluntary members of the South African National Defence Force, Tempe Military Base, were recruited for this study, using a very strict set of inclusion criteria. All subjects received a placebo for a period of one week following the collection of baseline blood samples. A second baseline blood sample was collected from each individual at the end of this period to ensure a stable baseline. Subjects were randomly assigned to three different intervention groups. Supplementation with the placebo, acetate-propionate-butyrate and acetate-propionate mixtures was sustained for a period of four weeks following the second baseline blood collection. Lipid profiles (including the serum-TC, -HDL-C, -LDL-C and non-esterified fatty acids (NEFA)), anthropometry, as well as some other general health markers were measured at each visit. Also, a 'wash-out' period of one week followed the supplementation phase.

No significant changes in any of the lipid variables were observed in the placebo group. The acetate/propionate/butyrate-supplement caused a non-significant decrease in the serum TC of approximately 8% after four weeks of supplementation. In contrast, the serum-LDL-C levels significantly decreased (-16%; from 3.10 mmol/L ± 0.78 to 2.61 ± 0.94 mmol/L) during the same period of time. In the study group receiving a combination of acetate and propionate salts, a beneficial statistically significant increase of the serum

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HDL-C (from 1.21 ± 0.24 mmol/L to 1.39 ± 0.34 mmol/L) was observed, without any changes in the other lipid fractions.

From the results it was evident that the inclusion of butyrate to the supplement was more beneficial in terms of lipid-lowering effects. These profile changes, associated with the additional intake of butyrate does reflect a decrease in risk for the future development of CHD.

It can therefore be concluded that short-chain fatty acid supplements could be used to the benefit of those individuals suffering from lifestyle diseases: but that the most appropriate ratio of acetate, propionate and butyrate supplementation needs further research.

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TABLE OF CONTENTS

		PAGE
DECLARATION		
ACKNOWLEDGEMENTS		
SUMMARY		
LIST OF APPENDICES		x
LIST OF TABLES		xi
LIST OF FIGURES		xiii
LIST OF ABBREVIATIONS		xiv
CHAPTER 1		1
INTRODUCTION		1
1.1	BACKGROUND	1
1.2	MOTIVATION	4
1.3	OBJECTIVES	6
1.4	STRUCTURE OF THE THESIS	7
CHAPTER 2		8
LITRATURE REVIEW		8

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2.1 INTRODUCTION 8

2.2 TERMINOLOGY 10

2.2.1 Atherosclerosis 10

2.2.2 Cardiovascular disease (CVD) 10

2.2.3 Coronary heart disease (CHD) 10

2.2.4 Non-starch polysaccharides (NSP) 10

2.2.5 Risk factor 10

2.2.6 Risk marker 11

2.2.7 Thrombosis 11

2.2.8 Stroke 11

2.2.9 Urbanisation 13

2.2.10 Westernisation 13

2.3 CHOLESTEROL METABOLISM 13

2.3.1 Classification and metabolism of the major blood lipids 13

2.3.1.1 Introduction 13

2.3.1.2 Triglycerides and fatty acids 15

2.3.1.3 Apolipoproteins 17

2.1.3.4 Low-density lipoprotein (LDL) 18

2.3.1.5 High-density lipoprotein (HDL) 20

2.3.1.6 Cholesterol biosynthesis and excretion 21

2.3.1.7 Atherosclerosis 24

2.4 LIPIDAEMIA 25

2.4.1 Introduction 25

2.4.2 Classification and diagnosis 26

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2.4.2.1 Secondary lipidaemia 26

2.4.3 Management of hyperlipidaemia 28

2.4.3.1 Cessation of tobacco use 28

2.4.3.2 Regular aerobic exercise 28

2.4.3.3 Prudent diets 29

2.5 DIETARY FIBRE 31

2.5.1 Introduction 31

2.5.2 Classification, chemistry and sources of dietary fibre 31

2.6 SHORT-CHAIN FATTY ACIDS 32

2.6.1 Characteristics of SCFAs 32

2.6.2 Production of SCFAs 33

2.6.3 Absorption and the metabolism of SCFAs 36

2.6.3.1 Absorption 36

2.6.3.2 Metabolism 38

2.6.3.2.1 Acetate metabolism 38

2.6.3.2.2 Propionate metabolism 40

2.6.3.2.3 Butyrate metabolism 42

2.6.4 Effects of SCFAs on lipid metabolism 43

2.7 SUMMARY 47

CHAPTER 3 **48**

METHODS AND TECHNIQUES **48**

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3.1	INTRODUCTION	48
3.2	ETHICAL CONSIDERATIONS	48
3.2	STUDY DESIGN	49
3.3	SUBJECTS	51
	3.3.1 Subject recruitment	51
	3.3.2 Screening	51
	3.3.3 Inclusion criteria	52
	3.3.4 Sample size	52
3.4	MEASUREMENTS	53
	3.4.1 Metabolic parameters	53
	3.4.2 Anthropometric status	54
	3.4.3 Blood pressure	55
	3.4.4 Dietary intake	56
	3.4.5 Blood sampling	56
	3.4.6 Phlebotomy	57
	3.4.7 Blood sample preparation	57
	3.4.8 Measurement of metabolic parameters	58
	3.4.9 Anthropometric measurements	65
3.5	QUESTIONNAIRES	68
	3.5.1 Screening questionnaire	68
	3.5.2 Food Frequency Questionnaire (FFQ)	68
	3.5.3 Tolerance questionnaire	69
	3.5.4 Standardisation of the FFQ	69
3.6	SUPPLEMENTS	69

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3.6.1 Capsules 69

3.6.1 Supplement 1 70

3.6.2 Supplement 2 70

3.6.3 Supplement 3 71

3.7 FIELDWORKERS AND STANDARDISATION OF TECHNIQUES 71

3.7.1 Fieldworkers 71

3.7.2 Standardisation of blood sampling 72

3.8 PILOT STUDY 73

3.9 MANAGEMENT OF THE STUDY 73

3.10 STATISTICAL ANALYSIS 76

3.11 LIMITATIONS OF THE STUDY 77

CHAPTER 4 **78**

RESULTS **78**

4.1 INTRODUCTION 78

4.2 BASELINE CHARACTERISTICS OF THE STUDY GROUP AS A WHOLE 79

4.2.1 Demographic characteristics of the study group as a whole 79

4.2.2 Anthropometric measurements of the study group as a whole 80

4.2.3 Lipid profiles of the study group as a whole 81

4.2.4 Glycometabolic indicators of the study group as a whole 82

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Central University of Technology, Free State

4.2.5 Other metabolic variables of the study group as a whole 83

4.3 RESULTS OF THE INTERVENTION STUDY 84

4.3.1 The placebo group 84

4.3.1.1 Changes in the anthropometrical measurements of the placebo group 84

4.3.1.2 Changes in lipid profiles of the placebo group 86

4.3.1.3 Changes in the glycometabolic indicators of the placebo group 86

4.3.1.4 Changes in other metabolic variables of the placebo group 87

4.3.2 The acetate/propionate/butyrate group 88

4.3.2.1 Changes in anthropometrical measurements of the acetate/propionate/butyrate-supplement group 86

4.3.2.2 Changes in the lipid profiles of the acetate/propionate butyrate-supplement group 89

4.3.2.3 Changes in the glycometabolic indicators of the acetate/propionate/butyrate-supplement group 91

4.3.2.4 Changes in the other metabolic variables of the acetate/propionate/butyrate-supplement group 91

4.3.3 The acetate/propionate group 93

4.3.3.1 Changes in the anthropometrical measurements of the acetate/propionate-supplement group 93

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4.3.3.2 Changes in the lipid profiles of the acetate/propionate-supplement group 95

4.3.3.3 Changes in glycometabolic indicators of the acetate/propionate-supplement group 95

4.3.3.4 Changes of other metabolic variables of the Acetate/Propionate-supplement group 96

4.4 A COMPARISON BETWEEN THE EFFECTS ON THE DIFFERENT SUPPLEMENT GROUPS (See table 4.16) 97

4.5 SUMMARY 101

CHAPTER 5 **103**

DISCUSSION **103**

5.1 INTRODUCTION 103

5.2 BASELINE CHARACTERISTICS OF THE GROUP AS A WHOLE 103

5.2.1 Demographic characteristics of the study group as a whole 103

5.2.2 Anthropometric measurements of the study group as a whole 103

5.2.3 Lipid profiles of the study group as a whole 103

5.2.4 Glycometabolic indicators of the study group as a whole 106

5.2.5 Other metabolic variables of the study group as a whole 107

5.3 CHANGES MEASURED DURING THE INTERVENTION PERIOD 108

5.3.1 Introduction 108

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5.3.2 The placebo group 109

5.3.2.1 Changes in the anthropometrical measurements of the placebo group 109

5.3.2.2 Changes in the lipid profiles of the placebo group 109

5.3.2.3 Changes in glycometabolic indicators of the placebo group. 109

5.3.2.4 Changes in other metabolic variables of the placebo group 110

5.3.3 The acetate/ propionate/ butyrate group 110

5.3.3.1 Changes in the anthropometrical measurements of the acetate/propionate/butyrate-supplement group 110

5.3.3.2 Changes in the lipid profiles of the acetate/propionate/ butyrate-supplement group 110

5.3.3.3 Changes in the glycometabolic Indicators of the acetate/propionate/butyrate-supplement group 112

5.3.3.4 Other metabolic variables of the acetate/propionate/ butyrate-supplement group 113

5.3.4 The acetate/propionate group 114

5.3.4.1 Changes in the anthropometrical measurements of the acetate/propionate supplement-group 114

5.3.4.2 Changes in the lipid profiles of the acetate/propionate supplement-group 115

5.3.4.3 Changes in the glycometabolic indicators of the acetate/propionate supplement-group 116

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5.3.4.4 Changes in the other metabolic variables of the acetate/propionate supplement-group 118

5.4 A COMPARISON BETWEEN THE EFFECTS OF THE DIFFERENT SUPPLEMENT GROUPS 118

CHAPTER 6 **123**

CONCLUSION AND RECOMMENDATIONS **123**

6.1 INTRODUCTION 123

6.2 CONCLUSION 124

6.3 RECOMMENDATIONS 126

6.3.1 Further research 126

6.3.2 Dietary recommendations and possible clinical use 126

BIBLIOGRAPHY 128

© Central University of Technology, Free State

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LIST OF APPENDICES

Appendix 1	Consent Form
Appendix 2	Recruitment Questionnaire
Appendix 3	Food Frequency Questionnaire
Appendix 4	Questionnaire for Subjects Who Completed the Study

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LIST OF TABLES

		Page
Table 2.1	Classification of lipidaemia (Berger and Marais, 2000)	26
Table 2.2	Short chain fatty acids (Morensen and Clausen, 1996)	33
Table 2.3	After a 24hr fermentation of different dietary fibres *in vitro* the following SCFA molar percents were found (Bugaut and Bentéjac, 1993)	35
Table 3.1	Normal ranges for metabolic indicators used in this study	54
Table 4.1	Anthropometric measurements of the study group as a whole	81
Table 4.2	Lipid profiles of the study group as a whole	82
Table 4.3	Glycometabolic indicators of the study group as a whole	83
Table 4.4	Other metabolic variables of the study group as a whole	84
Table 4.5	Changes in the anthropometrical measurements of the placebo group	85
Table 4.6	Changes in the lipid profiles of the placebo group	86
Table 4.7	Changes in glycometabolic indicators of the placebo group	87

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Table 4.8 Changes in other metabolic variables of the placebo group 88

Table 4.9 Changes in the anthropometrical measurements of the acetate/propionate/butyrate-supplement group 89

Table 4.10 Changes in the lipid profiles of the acetate/propionate/butyrate-supplement group 90

Table 4.11 Changes in the glycometabolic indicators of the acetate/propionate/butyrate-supplement group 91

Table 4.12 Changes in other metabolic variables of the acetate/propionate/butyrate-supplement group 92

Table 4.13 Changes in anthropometrical measurements of the acetate/propionate supplement group 93

Table 4.14 Changes in the lipid profiles of the acetate/propionate supplement group 95

Table 4.15 Changes in glycometabolic indicators of the acetate/propionate-supplement group 96

Table 4.16 Other metabolic variables of the acetate/propionate supplement group 97

Table 4.17 Comparisons between mean (±SD) changes in measured variables from day 8 to the end of supplementation (day 36) within the three different supplement groups 100

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LIST OF FIGURES

Page

Figure 3.1 Schematic representation of the study design **50**

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LIST OF ABBREVIATIONS

%	percent
µmol/ L	micromole per litre
<	smaller
>	larger
µIU/mL	micro international units per millilitre
µL	microlitre
a.m.	*ante meridium* before noon
Acetyl-CoA	acetyl coenzyme A
AHA	American Heart Association
ADA	American Dietetic Assosiation
ART	antiretroviral therapy
ATP	adenosine triphosphate
BCG	Bromocresol-green
BMI	body mass index
cat. no.	catalogue number
CH_4	methane
CHD	coronary heart disease
Cl^-	ionic chloride
cm	centimetre
CO_2	carbon dioxide

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CV	coefficient of variation
CVD	cardiovascular disease
EDTA	Ethylenediaminetetraacetic acid
et al.	*et alii*
FFQ	food frequency questionnaire
g	relative centrifugal force
g	gram
g/dl	gram per decilitre
g/L	gram per litre
h	hour
H^+	ionic hydrogen
H_2O_2	hydroperoxides
HCO_3^-	hydrogen carbonate
HDL	high-density lipoprotein
HDL-C	high-density lipoprotein cholesterol
HIV	human immunodeficiency virus
HL	hepatic lipase
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
hr	hour
IPP	isopentenyl pyrophosphate
K^+	ionic potassium
kg	kilogram
kg/m^2	kilograms per square metre

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LASSA	Lipid and Atherosclerosis Society of Southern Africa
LDL	low density lipoprotein
LDL-C	low density lipoprotein-cholesterol
mL	millilitre
mmHg	millimetre mercury
mmol/L	millimole per litre
n	number
Na^+	ionic sodium
NEFA	non-esterified fatty acid
NSP	non-starch polysaccharides
°C	degrees Celsius
p	probability
PCO^2	partial pressure carbon dioxide
pH	percentage hydrogen
p*K*	negative logarithm of ionizing constant
POD	peroxidase
SAMA	South African Medical Association
SANDF	South African National Defence Force
SCFA	short-chain fatty acids
SD	standard deviation
TC	total cholesterol

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TP	total protein
UFS	University of the Free State
vitamin B_{12}	cyanocobalamin
VLDL	very low density lipoprotein
VLDL-C	very low density lipoprotein cholesterol
WHO	World Health Organisation
X	mean

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CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Cerebrovascular disease 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**., 1995). Steyn **et al**. (1992) found that 7.2 percent of all deaths in 1988 in South Africa were attributed to cerebrovascular disease and 8.7 percent to ischemic heart disease. Furthermore, the incidence of the western diseases, atherosclerosis, CHD and cerebrovascular disease is progressively rising in black populations in South Africa (Khan and Tollman, 1999; Mollentze **et al**., 1995).

The risk factor prevalence for stroke and CHD alters as changes in lifestyle and diet occur during the process of urbanisation. Westernisation and migration to an urban environment contribute even further to the rise in the risk for the development of stroke and CHD (Bourne **et al**., 1993; Mollentze **et al**., 1995; Seedat **et al**., 1992; Solomoms and Gross, 1995; Steyn **et al**., 1991).

Based on the 1985 census figures for the South African population, and

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the current prevalence data, it is estimated that at least 4.8 million and 3.1 million people suffer from hypercholesterolaemia and raised low-density lipoprotein cholesterol levels, respectively. The South African Medical Association (SAMA) and the Lipid and Atherosclerosis Society of Southern Africa (LASSA) recognised hypercholesterolaemia as the most common and clinically significant lipid abnormality in South Africa (Berger and Marais, 2000). It is therefore important to investigate all possible factors that may influence lipid metabolism, including diet.

Prevention and cost-effective management of even moderately dyslipidaemia patients requires an appropriate modification of life-style: avoidance of tobacco smoking, participating in regular exercise, and a health-promoting diet (Hubbard **et al**., 1994; Mollentze **et al**., 1995; Steyn **et al**., 1987; Steyn **et al**., 1992; Walker, 1995). The correct choice for drug treatment is a therapeutic decision, and is best done only after full lifestyle modification (Berger and Marais, 2000).

The prudent low fat, high-fibre diet is regarded as one of the few controllable risk factors in the prevention of degenerative disease, and is therefore also effective in controlling known coronary risk factors (hyperinsulinaemia, hyperlipidaemia, hypertension, obesity, etc.) as well as raised clotting factors (Hubbard **et al**., 1994; Vorster **et al**., 1988; Vorster **et al**., 1997).

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The physiological effects of dietary fibre in humans are significantly influenced by the degree to which the fibre is fermented in the colon (Bourquin **et al.**, 1992; Cummings, 1983). Colonic fermentation results in the production of the short-chain fatty acids (SCFAs) acetate, propionate and butyrate (Bugaut and Bentéjac, 1993; Muir **et al.**, 1995). Previous studies have shown effects of SCFAs on the lipid metabolism (Topping and Wong, 1994), haemostasis (Veldman **et al.**, 1999), Factor VII activity (Marckmann and Jesperson, 1996), fibrinogen metabolism (Vorster **et al.**, 1988) and carbohydrate metabolism (Hubbard **et al.**, 1994).

Both acetate and propionate concentrations are believed to be related to serum cholesterol levels in men (Wolever **et al.**,1996), and their ratio may predict the risk of cardiovascular disease. In a previous study, a 4-week acetate supplementation caused a significant reduction in serum cholesterol levels in hypercholesterolaemic subjects (Veldman **et al.**, 1999). These results strongly suggest that the effect of soluble dietary fibre could be partially mediated by the production of acetate. It is evident that acetate supplementation can therefore be useful during the treatment or the prevention of some clinical abnormalities, especially those associated with raised total cholesterol, and possibly also plasma fibrinogen.

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1.2 MOTIVATION

Previous studies have shown that the addition of dietary fibre to the diet can significantly improve the metabolic profiles, including the lipid profiles, of human subjects. A decrease, especially in the serum total cholesterol levels, is beneficial and is associated with a decreased risk of the development of CHD and atherosclerosis. It is believed that some of these beneficial effects may be mediated in part by the SCFAs produced during the microbial fermentation of dietary fibre in the large intestine.

The black population of the Free State is already in the advanced stages of urbanisation and westernisation (Mollentze **et al**., 1995). Urbanisation of black populations in the Cape Peninsula represents a phase towards a progressively atherogenic Western diet (Bourne **et al**., 1993). It is expected that this phenomenon may be similar in other ethnic groups of the South African population (Steyn **et al**., 1987). Slabber **et al**. (1997) have already indicated that urban African men in the Free State Province show a tendency towards an atherogenic Westernised diet, characterised by a low-fibre, high-fat dietary intake.

The effect of different combinations of SCFAs on the metabolic parameters in human subjects has been the topic of investigation for many years. The intake of dietary fibre is beneficial to human health, especially

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in those individuals with a westernised lifestyle. However, the mechanisms involved in promoting these health effects in human subjects are not fully understood and remain unclear. At this stage it seems highly likely that the effect of dietary fibre intake is mediated by a combination of physical characteristics, chemical structure, as well as the metabolic products formed during its breakdown (Mortensen and Clausen, 1996).

The role of SCFAs as partial mediators of reducing cardiovascular risk received widespread interest from as long ago as 1831 (Wrong,1995). Study results are controversial and no two specific studies duplicate each other. To date, no conclusive evidence exists that explains the specific role of SCFA production during fibre fermentation, and the role it may play in human health (Wolever **et al**., 1995).

Living in a society in the process of transforming from a prudent lifestyle to an increasingly unhealthy westernised lifestyle, makes it important to investigate any mechanisms that could improve the health status of those individuals at risk of developing lifestyle associated diseases. Lifestyle changes are much more cost-effective than medication, and in most cases, much less invasive.

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1.3 OBJECTIVES

The main objective of this study is to examine the hypothesis that exposure to two different combinations of short-chain fatty acids (SCFAs), orally administered in a group of local westernised Black men, will have beneficial effects on their lipid profiles. The concentration of SCFAs used will be equivalent to that generated by fermentation of 15g mixed fibre.

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. It was believed that the increase of colonic acetate would have a positive effect on the lipid profile. Differences in metabolic response to the two different supplements could be ascribed to the following mechanisms:

- The ratio of acetate to propionate, as well as the absence or presence of butyrate which may each evoke a collaborative physiological response.

Hopefully the results from this study will provide enough information to help develop an inexpensive but effective way of controlling the increasing rise in the incidence of cardiovascular disease in the South African Black population group.

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1.4 STRUCTURE OF THE THESIS

The structure of the thesis is as follows:

Chapter 2 is 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. Chapter 3 explains and gives the motivation behind experimental methods used during the intervention phase. The results of the intervention study are given in Chapter 4. In Chapter 5 the results of the study are discussed and compared to previous studies. Finally, in Chapter 6, the results, conclusions, and recommendations made from the intervention studies are discussed. The possible application of the findings to contribute to the lowering of cardiovascular disease is highlighted and discussed in depth.

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CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

Diet is a strong factor in the control of atherosclerosis, which relates to general vascular disease, coronary heart disease and stroke (Hubbard **et al.**, 1994). There is an especially strong relation between dietary intake and the prevalence of coronary heart disease in westernised cultures (Hubbart **et al.**, 1994). It is estimated that approximately 75% of South Africa's population were urbanised by the turn of the century (Mollentze **et al.,** (1995). The process of urbanisation affects mainly the black population of Southern Africa. Morbidity and mortality from cardiovascular diseases in the black population is expected to increase with the adoption of Western lifestyles and associated dietary habits (Steyn **et al.,** 1987; Gross and Monteiro , 1989; Le Roux and Le Roux, 1991; Steyn, Fourie and Bradshaw, 1992; Mollentze **et al.**, 1995; Jenkins **et al.**, 2001). With the introduction of antiretroviral therapy (ART) to the public sector, a rise in CHD is expected, due to cardiovascular side-effects associated with the long-term use of these drugs (Hadigan, 2001).

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Coronary heart disease is preventable. Both the prevention and treatment of CHD depend on intervention aimed at altering its known risk factors, especially those associated with lifestyle, such as raised serum total cholesterol levels, exercise, smoking, high blood pressure, etc. (Kannel and Wilson, 1995). The prudent low fat, high fibre diet is regarded as one of the most effective tools used for the prevention of degenerative westernised diseases. It is therefore also believed to be effective in controlling the known coronary risk factors (Hubbard **et al.**, 1994; Jenkins **et al.**, 2002; Vorster **et al.**, 1988; Vorster **et al.**, 1997).

The high fibre diet has been the subject of many investigations, and is believed to play a significant role in promoting human health on different physiological levels. A variety of specific studies has investigated especially the breakdown process (i.e. fermentation process) of dietary fibre as mediator of its effect on human metabolism. Colonic fibre fermentation results in the production of the short-chain fatty acids (SCFA), acetate, propionate and butyrate (Bugaut and Bentéjac, 1993; Muir **et al.**, 1995), each of which are believed to have a specific metabolic effect.

The role of dietary fibre as a cholesterol-lowering agent has received widespread attention. Furthermore, it was also shown that the SCFAs produced after fibre ingestion have an effect on lipid metabolism (Topping

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and Wong, 1994). The specific effect of propionate on cholesterol metabolism has not yet been clarified (Beauliew and McBurney 1992; Breggren **et al.**, 1996; Kishimoto **et al.**, 1995). It is believed that a combination of the three major SCFAs (acetate, propionate and butyrate) in different ratios may be more effective in lowering blood cholesterol levels (Wolever **et al.,** 1991; Demignè **et al.**, 1994; De Wet, 1999; Veldman **et al.**, 1996).

The literature relevant to this study will be reviewed in this chapter. The literature should supply the reader with sufficient information to be able to interpret and understand the results of this study without referral to other sources.

2.2 TERMINOLOGY

To understand some of the terminology in this chapter, it will be useful to provide some definitions and criteria as a framework.

2.2.1 Atherosclerosis: Atherosclerosis is an aggregated inflammatory response to injury of the endothelial and the smooth muscle cells of the arterial wall. It can also be defined as a complex process of thickening and narrowing of the arterial walls of the large- and medium-sized blood

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vessels by the accumulation of lipids, primarily cholesterol, in the intimal or inner layer in combination with connective tissue and calcification (Meydani, 2001).

2.2.2 Cardiovascular disease (CVD): Any disease that causes damage to the heart or to arteries that carry blood to and from the heart muscle (De Bono and Boon, 1991)

2.2.3 Coronary heart disease (CHD): CHD is a multicausal disease manifested by arthrosclerosis and/or thrombosis, which involves the network of blood vessels surrounding and serving the heart; manifested in clinical end-points of myocardial infarction and sudden death (Hubbard **et al.**, 1994; Krummel, 2004).

2.2.4 Non-starch polysaccharides (NSP): All dietary fibre, except lignin, is plant polysaccharides and is termed non-starch polysaccharides (WHO, 1998). NSP can be classified by solubility in water, since water-soluble (pectin) and water-insoluble (cereal) fibres have distinct physiological effects (Slavin, 1987).

2.2.5 Risk factor: An aspect of personal behaviour or lifestyle, an environmental exposure, or an inborn or inherited characteristic that, on the basis of epidemiological evidence, is associated with a health-related

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condition or conditions. It is not necessarily the cause of a disease but is an attribute or exposure that increases the probability of the occurrence of a disease or other specified outcome. By knowing the risk factor or factors of a disease, one can possibly decrease or eliminate the incidence of the disease by eliminating or modifying the risk factor (Last, 1988).

2.2.6 Risk marker: A non-causal factor associated sufficiently well with a risk factor that it can be used as a possible reliable marker, or indicator, of the risk factor's presence (Last, 1988).

2.2.7 Thrombosis: Thrombosis is the formation of a solid mass from the constituents of blood (platelets, fibrin and entrapped red blood cells) within the heart or vascular system of a living organism. Arterial thrombosis is common, and typically occurs after endothelial damage and local turbulence caused by atherosclerosis. The combination of thrombosis and atherosclerosis in an artery can obstruct the normal flow of blood and may lead to degeneration of a specific body part or organ if left untreated (Chandrasoma and Taylor,1991).

2.2.8 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 (Chandrasoma and Taylor, 1991).

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2.2.9 Urbanisation: The social process whereby cities grow and societies become more urban. The process of urbanisation is taking place in both less and more developed countries, but at a much higher rate in the former (Le Roux and 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).

2.3 CHOLESTEROL METABOLISM

2.3.1 Classification and metabolism of the major blood lipids

2.3.1.1 Introduction

The presence of cholesterol in blood was described for the first time by Chevreul in 1815, who named it *cholesterine* (Vance and Van den Bosch, 2000). It was only later discovered that cholesterol consists of a conglomerate of lipid fractions, each having its own structure and function

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within the human body (Willett, 1998). Research into the anabolic and catabolic pathways of cholesterol gave new insights into the role that each of these fractions plays. In most cases cholesterol is treated as a "risk molecule" (Willett, 1998). However, it has been shown that cholesterol plays a very important role in normal body function, and is essential for maintaining life (Garrett and Grisham, 1999).

Virtually all cells and body fluids contain some cholesterol (Warnick **et al.**, 1996). Cholesterol serves many critical functions in the brain and in the body. It is required for the brain to form "synapses" that permit nerve cells to communicate, leading to learning and memory. Cholesterol is also a structural component of cell membranes (Davidson, 1991). The ratio of cholesterol to polar lipids affects the stability, permeability and the protein mobility of a membrane. Cholesterol is stored in the adrenals, testes and ovaries, chiefly as the fatty acid ester and converted to steroid hormones. These hormones include the male and the female sex hormones (androgens and estrogens) as well as the adrenal corticoids (cortisol, corticosterone, aldosterone and others). In the liver, cholesterol acts as the precursor of bile acids (Marieb, 2001).

A short summary of the metabolic pathways and physiological importance of the major blood lipid fractions will now be given in order to make the results of the study clear and understandable to the reader.

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2.3.1.2 Triglycerides and fatty acids

Fatty acids of dietary origin are oxidised by most tissues after a meal, and the excess stored away in adipose tissues in the form of triglycerides. The stored triglycerides are degraded in the fasting state, when released fatty acids are the main energy source for the body (Meisenberg and Simmons, 1998).

Fatty acids consist of an alkyl chain with a terminal carboxyl group; and the simplest configuration is a completely saturated straight chain. The basic formula is R-COOH where 'R' stands for an alkyl chain (Rifai **et al**., 1999). Most naturally occurring fatty acids have an even number of carbon atoms that form an unbranched chain. Fatty chains that contain only carbon-carbon single bonds are referred to as saturated. Those molecules that contain one or more double bonds are said to be unsaturated (McKee and McKee, 1996). Monounsaturated fatty acids are fatty acids that contain one double bond. When two or more double bonds are present, the fatty acids are referred to as polyunsaturated (Garrett and Grisham, 1999).

Fatty acids that are synthesised within the human body are known as nonessential fatty acids. Because mammals do not posses the enzymes

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required to synthesise some fatty acids, and these fatty acids must be obtained from dietary sources, they are called essential fatty acids (McKee and McKee, 1996). In general, the fatty acids derived from plant seeds or fish lipids are more unsaturated and contain more of the essential fatty acids required by humans (Kaplan **et al.**, 1995).

Triglycerides are esters of glycerol containing three fatty acid molecules. Up to 95% of dietary lipids consist of triglycerides (Mann and Skeaff, 2000; McKee and McKee, 1996). The physical and biological properties of triglycerides are determined by the nature of the constituent fatty acid (Mann and Skeaff, 2000). Triglycerides have no charge and are sometimes referred to as neutral fats. Most triglyceride molecules contain fatty acids of various lengths, which may be either saturated, unsaturated, or a combination thereof (McKee and McKee, 1996).

Dietary triglycerides are a major source of metabolic energy, accounting for close to 40% of the total calories in the typical Western diet. Triglycerides are also important as the principal storage form of energy in the body. Most people carry between five and twenty kilograms of triglycerides in their adipose tissue. These triglycerides are synthesised in the well-fed state, and degraded during the fasting state (Meisenberg and Simmons, 1998). Stored triglycerides in adipose tissue can also provide

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insulation against cold temperatures, because fat is a bad conductor of heat (McKee and McKee 1996).

2.3.1.3 Apolipoproteins

A major characteristic of all lipids is the fact that they are not soluble in water. Transportation of lipids on their own in blood, which is a water-based substance, is therefore problematic. In circulation, lipids associate with specific proteins, also known as the apolipoproteins, which form water-miscible complexes and have the ability to carry lipid-soluble material to the different parts of the body (Meisenberg and Simmons, 1998). These complexes consist of a core of neutral lipids (triglyceride and cholesterol esters) surrounded by a single surface layer of polar lipids (phospholipids and cholesterol). Coiled chains of apolipoproteins extend over the surface (Meisenberg and Simmons, 1998). There are five classes of lipoproteins which are identified according to their densities: high-density lipoproteins (HDL), low density lipoproteins (LDL), very low density lipoproteins (VLDL) and chylomicrons (Garrett and Grisham, 1999). Each has a distinct physiological role, and when present in inappropriate amounts (too high or too low), each has different adverse health consequences (Mann and Skeaff, 2000). Free fatty acids, which make up only about 2% of the total plasma lipid fraction, are transported in

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the blood as complexes with circulating albumin (Montgomery, Conway and Spector, 1990).

2.1.3.4 Low-density lipoprotein (LDL)

VLDL acts as the main transporter of triglycerides from the liver to the tissues, including the muscles, the heart and the adipose tissue (Kaplan **et al**., 1995). VLDL is generated in the liver for transportation of the 25 to 50g of triglycerides, and smaller amounts of other lipids that are synthesised daily in the liver. These lipoproteins are released from the liver into the sinusoidal blood (Marieb, 2001).

Approximately half of the VLDL remnants are taken up by the liver, and the rest transforms into intermediate-density lipoproteins (IDL). IDL are precursors of low-density lipoprotein (LDL), which will be discussed in the next section (Mann and Skeaff, 2000).

IDL is remodelled to LDL-C in plasma (Kaplan **et al.,** 1995). Hepatic lipase (HL) hydrolyses the excess triglycerides and phospholipids in the IDL. HL is also responsible for the transfer of excess apolipoproteins to the HDL complex (Meisenberg and Simmons, 1998).

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LDL-C has a well-defined structure. Its lipid component includes a high proportion of free cholesterol (8.6%) and cholesterol esters (41.9%) (Kaplan **et al.**, 1995).

LDL-C delivers cholesterol either to the liver for bile acid formation, or to other tissues for metabolic breakdown. Receptors on the surface of the cell membranes of the liver and the tissues recognise and attach to the apolipoprotein of the LDL-C (Meisenberg and Simmons, 1998). The LDL-C is then internalised by endocytosis, and degraded by liposome enzymes (Rawn, 1989). The non-esterified cholesterol may be used as a structural component of the membranes of dividing cells; they may be converted to steroid hormones by the adrenal or gonad cells, or may be esterified and stored in the cell as esters (Kaplan **et al.**, 1995).

LDL receptors are essential for the normal metabolism of cholesterol. LDL receptors in the peripheral tissues bind the remnants of VLDL and degrade those (Meisenberg and Simmons, 1998). If the receptors are missing, the VLDL remnants are converted to cholesterol-rich LDL. The circulating concentration of LDL-C in the serum is thus greatly elevated (Meisenberg and Simmons, 1998). These receptor proteins play an integral part in cholesterol metabolism by maintaining healthy circulating cholesterol concentrations (Mathews and Holde, 1990)

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The metabolism of LDL is intimately connected to the cholesterol metabolism (Kaplan **et al.**, 1995). Cholesterol is a necessary component of mammalian cell membranes, but the synthesis of excess cholesterol results in a greatly increased risk of cardiovascular disease. It is hypothesised that the accumulation of cholesterol ester deposits in the intimae of arteries is the main cause of atherosclerosis (Meisenberg and Simmons, 1998), and this will be discussed later in this section.

It is hypothesised that the risk for coronary artery disease increases with rising plasma concentrations of LDL-C. However, it is believed that the risk associated with LDL-C is diminished when associated with high HDL-C concentrations. For this reason, some laboratories calculate the ratio of LDL-C: HDL-C for estimating the degree of risk; a ratio of less than 3:1 is considered desirable (Kaplan **et al.**, 1995; Nader **et al.**, 1999).

2.3.1.5 High-density lipoprotein (HDL)

HDL is synthesised and secreted by both the liver and intestine and consist of phospholipids (24%), cholesteryl esters (15%), free cholesterol (2%), triglyceride (4%) and apolipoproteins (Rawn, 1989).

HDL transports cholesterol from tissues to the liver for catabolism (conversion to bile salts), whereas LDL transports cholesterol from sites of

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origin to deposition in tissues, including blood vessels (Warnick **et al**., 1996). The HDL competes with LDL for binding to tissue receptors and may thus reduce cholesterol accumulation in the blood vessel walls. The concentration of the HDL cholesterol appears to be inversely related to the risk of cardiovascular disease (Kaplan **et al**., 1995). A low HDL level (below 0.91 mmol/L) is predictive of cardiovascular disease, even if the total cholesterol level is in the desirable range. The relative amount of HDL to total cholesterol is also important; the amount of HDL should be at least one quarter of the total cholesterol level (Kaplan **et al**., 1995). The Framingham study shows that people with ratios of TC/HDL-C greater than 4.5 have more cardiovascular problems, and if the ratio is above 5.0, the risk is even greater (Garrison **et al**., 1980).

2.3.1.6 Cholesterol biosynthesis and excretion

Slightly less than half of the cholesterol in the body is derived from biosynthesis. The rest is derived from dietary sources, such as meat, dairy products, or eggs (Kaplan **et al**., 1995). Biosynthesis in the liver accounts for approximately 10%, and in the intestines approximately 15%, of the amount of biosynthesised cholesterol produced each day in the body (Montgomery, Conway and Spector, 1990). Cholesterol synthesis occurs in the cytoplasm and microbes where Acetyl-CoA is utilised for cholesterol biosynthesis (Nader **et al**.,1999).

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The biosynthesis of cholesterol entails the following five major steps:

- Acetyl-CoA is converted to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA)
- HMG-CoA is converted to mevalonate
- Conversion of mevalonate to an isoprene based molecule, isopentenyl pyrophosphate(IPP), with the concomitant loss of CO_2
- IPP is converted to squalene and
- Squalene is converted to cholesterol (Kaplan **et al.,** 1989; Mathews and Van Holde, 1990; Nader **et al**., 1999; Rawn, 1989; Montgomery, Conway and Spector, 1990).

Approximately 70% of plasma cholesterol is in the acyl-ester form. The esterification takes place almost exclusively within the HDL-complex. One third of the daily production of cholesterol is categorised by the shortening of the cholesterol side chain, and hydroxylation of the sterol nucleus. This results in the formation of bile acids that conjugate and are secreted into the gastro-intestinal tract with glycine or taurine before secretion into bile (Nader **et al**., 1999). These bile acid-conjugates act as emulsifying agents that are essential for the digestion and absorption of dietary fats. Some cholesterol is also secreted into the bile. Both bile acids and biliary cholesterol are reabsorbed to some extent by the intestines, from where it enters the enterohepatic circulation (Kaplan **et al.,** 1995).

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Normal healthy adults synthesise cholesterol at a rate of approximately 0.5 – 1.0g/day (Meisenberg and Simmons, 1998). It is also estimated that the average adult consumes approximately 0.3 – 1.0g dietary cholesterol per day (Nader **et al**., 1999). This applies especially to foods such as meat, dairy products and eggs that are rich in dietary cholesterol (Block **et al**., 1985). A relatively constant level of cholesterol in the body (<5.18 mmol/L) is maintained primarily by controlling the level of *de novo* synthesis in the liver (Kaplan **et al**., 1995). It is known that the dietary intake of cholesterol also regulates the level of cholesterol synthesis (Nader **et al**., 1999). Cholesterol from both the diet (exogenous) and synthesis (endogenous) is utilised in the formation of cell membranes, the synthesis of the steroid hormones, as well as in the synthesis of bile acids. The greatest proportion of cholesterol is used for bile acid synthesis (Kaplan **et al**., 1989).

The synthesis and utilisation of cholesterol must be tightly regulated in order to prevent over-accumulation and an abnormal deposition within the body. Of particular clinical importance is the abnormal deposition of cholesterol-rich lipoproteins in the coronary arteries. These depositions, eventually lead to atherosclerosis (Nader **et al**., 1999).

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2.3.1.7 Atherosclerosis

Atherosclerosis is defined by the presence of athermanous plaques, with characteristic lesions in the intima of large arteries (De Bono and Boon 1991).

The typical athermanous plaque contains a core of cholesterol esters surrounded by an area of fibrosis. The plaque impairs blood flow by narrowing the lumen of the artery, and it can lead to such complications as calcification, haemorrhage into the plaque and thrombus formation (Woolf 1998). This process takes place over a long period of time and causes thickening of the arterial wall, eventually leading to closure of the artery, in turn leading to tissue damage within the body part/organ supplied with oxygenated blood by the relevant artery (Chandrasoma and Taylor, 1991). The resulting partial blockage of a coronary artery may cause chest pains (angina) (Davidson, 1991). A complete blockage of a heart artery, results in necrosis of the affected muscle area and eventually heart attack (Edwards and Bouchier, 1991). Ideally, circulating serum-LDL-C levels should be less than 3.4 mmol/L and ideally less than 2.6 mol/L (Kaplan **et al.**, 1995).

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2.4 LIPIDAEMIA

2.4.1 Introduction

Lipidaemia is defined as a clinically alteration in the circulating lipids and lipoproteins predisposing to CHD and related disorders. In practice, the most important and common is hypercholesterolaemia (Berger and Marais, 2000). The term hyperlipidaemia emphasises the importance of excess lipid in generating the adverse consequences of the common lipid (lipoprotein) disorders (Ahmed **et al**., 1998). The chief exception is a low HDL concentration with its independently atherogenic effect: a high HDL level (>1.5 mmol/L) is generally protective (Berger and Marais, 2000).

Lipidaemia is common in westernised, industrialised communities. In South Africa, CHD is most widespread in the Indian and White groups, with a somewhat lower incidence in the Coloured community (Wyndham, 1982). CHD in the black population is still relatively uncommon, but risk factors for the possible emergence of future CHD are already apparent, including hypertension, diabetes, hyperlipidaemia, obesity and tobacco smoking (Steyn **et al.,** 1991).

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2.4.2 Classification and diagnosis

The lipoprotein phenotype is most conveniently classified in terms of hypercholesterolaemia, hypertriglyceridaemia or as a mixed picture with cholesterol and triglyceride levels more or less equally elevated (Warnick **et al.,** 1996).

Table 2.1 Classification of lipidaemia (Berger and Marais, 2000).

	Desirable lipid profile1	Hypercholesterol-emia		Mixed Hyperlipid-aemia	Hypertriglyceridaemia	
		Moderate	Severe		Moderate	Severe
TG	<or = 1.5	< 1.5	< 1.5	1.5 - 5.0	5 - 15	> 15
TC	<or = 5.0	5 - 7.5	> 7.5	> 5.0	<5 -increased	> 5.0
LDLC	<or = 3.0	3.0 - 5.0	> 5.0	Variable	Variable	Variable
HDLC	>or = 1.2	Variable	Variable	Low	Low	Low

Concentrations are expressed in mmol/L

2.4.2.1 Secondary lipidaemia

In secondary lipidaemia the genetic constitution of a person is normal or contains only minor gene defects, both the environment and the underlying incidental disease brings out the lipidaemia. Causes of secondary lipidaemia may be the following:

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- **An unhealthy diet** (high saturated fat, high cholesterol, low fibre and high-energy intakes) can directly or indirectly bring about a moderate lipidaemia, often contributing to the primary disorders mentioned above (Meisenberg and Simmons, 1998).

- **Diseases** that cause hypercholesterolemia include hypothyroidism, nephrotic syndrome and obstructive jaundice. Diseases that predispose to hypertriglyceridaemia include truncal obesity, diabetes and Cushing's syndrome (Berger and Marais, 2000).

- **Pregnancy** causes a rise in plasma cholesterol concentration and may induce severe hypertriglyceridaemia in susceptible women (Punnonen, 1977).

- **Drugs** that influence the lipid profile in deleterious ways include steroids, some beta-blockers, diuretics at high doses, retinoic acid derivatives and protease inhibitors used in HIV treatment (Amprenavir, Agenerase) (Pronsky, 2001).

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2.4.3 Management of hyperlipidaemia

The two key elements in the management of hyperlipidaemia are:

- Lifestyle modification, which is desirable in virtually all patients and,
- the use of appropriate lipid modifying drugs in those patients at high risk (Gibbons **et al**., 2003). However, this will not be discussed in this chapter.

The main targets of lifestyle or behavioural modification are: cessation of tobacco use, a healthy diet, regular aerobic exercise and, where indicated, loss of weight (Russel, 1998).

2.4.3.1 Cessation of tobacco use

This is the single most important therapeutic action in patients with hyperlipidaemia (Ahmed **et al**., 1998; Berger and Marias, 2000).

2.4.3.2 Regular aerobic exercise

The minimum effective target is 30 minutes of brisk walking, 3 times weekly. A preferable minimum target would be 4 hours of moderate exercise per week, spread over 5-6 sessions. A planned exercise programme should be appropriate to age, gender and clinical status – a

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cardiology opinion should be obtained where clinical CHD is present or suspected. The exercise should elevate heart rate to about 75% of age-related maximum heart rate (220 beats/minute minus age in years) (Ahmed **et al**., 1998; Berger and Marais, 2000).

2.4.3.3 Prudent diets

Dietary intervention should be the first step in the treatment of dyslipidaemia and the main purpose of a prudent diet is to maintain or achieve a desirable body mass and optimal health, as well as to lower raised TC, LDL-C and TG levels and raise the HDL-C level (Wolmarans, 2004:Online). As discussed, LDL cholesterol is conclusively linked to CHD development. Consequently, LDL cholesterol is the primary target for intervention efforts (Krummel, 2004). It is recommended that a moderate hypercholesterolaemia without CHD receives dietary treatment for at least 4-12 weeks before drug treatment is introduced. In those with hypercholesterolaemia and CHD, drug treatment should be introduced earlier (Wolmarans, 2004). Berger and Marais (2000) recommend that a person with a cholesterol level between 5.0 - 7.5 mmol/L, in the absence of additional risk factors, be treated with a prudent diet.

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The main elements of a prudent or anti-coronary diet are: a reduction of saturated fat, cholesterol and energy intake relative to the typical Westernised intake (Berger and Marais, 2000).

Current dietary guidelines recommend a total daily intake of at least 20 to 30g for adults, with 25 percent of the fibre being soluble fibre (National Cholesterol Education Program [NCEP], 2001). 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. Adding 3g per day of soluble fibre from oat bran can reduce total cholesterol by 0.13 mmol/L (Blake and Triplett, 1995). A higher daily intake of soluble fibre promotes a further modest reduction of the cholesterol values. A high-fibre, low fat diet also provides other beneficial effects, including improved glycaemic control, weight reduction and the prevention of diverticular disease, and possibly, prevention of colorectal cancer (Anderson and Gustafson, 1987).

In addition to lowering total fat intake, intake of alcohol should be reduced if clinically indicated, and in hypertriglyceridaemia states, also simple carbohydrates. Ideal weight may be defined by a BMI < 25 kg/m^2 in the general population (Berger and Marias, 2000).

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2.5 DIETARY FIBRE

2.5.1 Introduction

The specific role of dietary fibre on cholesterol metabolism will now be discussed.

2.5.2 Classification, chemistry and sources of dietary fibre

Dietary fibre is generally defined as plant material, mainly derived from plant cell walls, that is resistant to digestion by human gastrointestinal enzymes (Hunt **et al.**, 1993). Food chemists prefer to define fibre as "lignin and non-starch polysaccharides (NSP), where NSP includes celluloses, hemicelluloses, pectin gums and mucilage, found in food" (Hunt **et al.**, 1993). All dietary fibres, except for lignin, are plant polysaccharides and are therefore termed NSP (Vorster **et al.**, 1990). NSP are not hydrolysed by the small-intestinal enzymes and are a suitable substrate for the bacterial production of the short-chain fatty acids (SCFAs) acetate, butyrate and propionate in the large bowel, together with incompletely digested starch, lactose and proteins (Cummings **et al.**, 1987; Camp **et al.**, 2003). The fermentability of NSP is related to important clinical effects (Camp **et al.**, 2003)

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Soluble fibres (pectins, gums) are largely fermented, while insoluble fibres are more difficult to degrade The gases CO_2, H_2, CH_4 and water are produced as by-products (Brand-Miller, 2000).

2.6 SHORT-CHAIN FATTY ACIDS

2.6.1 Characteristics of SCFAs

SCFAs can be described as saturated unbranched alkyl monocarboxylic acids of 2-4 carbon atoms as shown in Table 2.2 (Wrong, 1995). SCFAs are biochemically more closely related to carbohydrates than to fats. Some of them are not constituents of natural fats, and they are not "fatty", as the layman envisages the term, as they are completely miscible in water (Wong, 1995). The three major SCFAs (acetate, propionate and butyrate) are moderately strong acids, with p*K* values of about 4.8. Intestinal contents are more alkaline than this, so SCFAs are predominantly present within the intestine as negatively charged anions and not as free acids (Wrong, 1995), and create a slightly acidic pH level (Fleming **et al**., 1991). In all regions of the colon, acetate, propionate and butyrate account for 90-95 present of the total SCFAs, with acetate being the principal anion (Mortenson and Clausen, 1996). Serum acetate is derived primary from colonic fermentation, serum butyrate primarily from

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endogenous fatty acid metabolism, and serum propionate from both exogenous and endogenous sources (Wolever **et al**., 1997).

Table 2.2 Short chain fatty acids (Morensen and Clausen, 1996)

Chemical formula	Trivial name
CH_3-COOH	Acetate
CH_3-CH_2-COOH	Propionate
CH_3-$(CH_2)_2$-COOH	Butyrate

2.6.2 Production of SCFAs

NSPs, defined as indigestible complex carbohydrates, remain in the ileum but are partly hydrolysed by bacteria in the colon (Hubbard **et al**., 1994). Much of the research into the benefits of NSP consumption on human health has focused on the actions of fibre in the large intestine. These actions depend largely on the extent to which fibre is fermented by the residing population of anaerobic bacteria, and on the physical characteristics of unfermented fibrous material (Bourquin **et al**., 1992). The main substrates for the colonic fermentation in healthy individuals are NSP (cellulose, hemicelluloses and pectin) (Mortensen and Clausen,

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1996; Camp **et al**., 2003). Bacterial fermentation of one gram of monosaccharide yields approximately 10 mmol of organic acid (Scheppach **et al**., 1992). Colonic fermentation leads to the production of SCFAs, certain gases (carbon dioxide, methane, hydrogen), and microbial cell mass (Bourquin **et al**., 1992). Furthermore, total SCFA production from fermentation is the greatest for oat bran (Bourquin **et al**., 1992). Anderson (1995) also supports this in demonstrating that serum acetate levels produced from oat bran peak twice as high as those observed with intakes of beans or wheat bran. The SCFAs vary widely in their relative proportions, depending upon the fibre source in the diet (Bugaut and Bentéjac, 1993). Table 2.3 indicates the SCFA molar percents from 24-hour fermentation of dietary fibres in, *in vitro* incubation systems inoculated with fresh human faecal flora (Bugaut and Bentéjac, 1993). After absorption, each of the primary SCFAs produced (acetate and butyrate) is metabolised by the body and many biological effects of SCFAs have been reported (Bourquin **et al**., 1992). The SCFAs that escape colonic metallisation enter the hepatic portal blood, where their concentration varies in a wide range, depending on the production rates, (Cheng **et al**., 1987). 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 and Trowell, 1986; Wolever **et al**., 1991). SCFAs also contribute to the energy needs of the body and play a potential role in protection

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against the development of colonic disorders (Mortensen and Clausen, 1996). SCFAs in human faeces, following consumption of different defined polysaccharides, have been measured on the average, in the molar ratio of acetate to propionate of 53:27:20 (Bugaut and Bentéjac; 1993; Savage, 1987). Levels of all three SCFAs varied significantly during the day, tending to decrease after breakfast, and to increase transiently after lunch and dinner. Both the time of day and glucose tolerance status affect SCFA levels in non-diabetic humans (Wolever **et al.**, 1997).

Table 2.3 After a 24hr fermentation of different dietary fibres *in vitro* the following SCFA molar percents were found (Bugaut and Bentéjac, 1993).

Substrate	Acetate	Propionate	Butyrate
Pectin	81	11	8
Gum arabic	68	23	9
Oat bran[a]	65	19	16
Wheat bran[a]	63	16	21
Cellulose[b]	53	21	26

[a] ∝-cellulose and hemicelluloses are 7% and 19% dry total dietary fibre, respectively, in oat bran and 19% and 38%, respectively, in wheat bran.

[b] 48-h fermentation.

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2.6.3 Absorption and the metabolism of SCFAs

SCFAs produced in the large intestine in substantial amounts are absorbed and subsequently utilised by the animal as a substrate of energy metabolism (Engelhardt, 1995). According to Mortensen and Clausen (1996), an increase of SCFA absorption is equal to 150-360 Kcal/day of metabolised energy. Western diets, where SCFAs are absorbed in the gut, provide three to nine percent of their total energy requirements (Green 2000). SCFAs, especially butyrate, are metabolised to different extents in the mucosa of the large intestine (Engelhardt, 1995).

2.6.3.1 Absorption

SCFAs *in vivo* appear to be absorbed rapidly and nearly completely. Clearance rates of SCFAs have generally been observed to increase with chain length, even though there are differences between the rates in the distal and proximal colon (Bugaut and Bentéjac, 1993; Engelhardt, 1995). Studies done on the mechanism for SCFA absorption supported trans-cellular absorption as a major pathway (Mortensen and Clausen, 1996). A suggested model for trans-cellular transport can be either through para-cellular or cellular transport (Engelhardt, 1995, Mortensen and Clausen, 1996). This also accounts for the large number of observations describing the dependence of SCFA absorption rates on luminal pH and PCO^2, as

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well as on the fluxes of water, protons and inorganic ions (Cl^-, HCO_3^-, Na^+ and K^+) through the colonic mucosa (Engelhardt, 1995; Mortensen and Clausen, 1996).

Para-cellular transport is defined as non-active transport that should depend on the trans-epithelial difference. However, the para-cellular pathway is not a major site for SCFA absorption (Mortensen and Clausen, 1996). The possible mechanism of cellular uptake can be through passive diffusion or facilitated diffusion (Mortenson and Clausen, 1996). The trans-mural movement of SCFA is a concentration-dependent, passive diffusion process, whereby SCFAs are, at least in part, transported in the protonated form. Hydrogen ions, which are needed for SCFA protonation may be available from Na^+/H^+ exchange and from hydration of luminal CO_2 to HCO_3^- and H^+ (Bugaut and Bentéjac, 1993). A relationship between SCFA absorption and bicarbonate secretion has frequently been observed (Engelhardt, 1995). SCFAs may also be transported in the ionised form via a SCFA-HCO_3^- exchange mechanism, also known as the facilitated diffusion process (Bugaut and Bentéjac, 1993; Mortensen and Clausen, 1996). The precise mechanism for the absorption process of SCFAs is still unknown.

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2.6.3.2 Metabolism

Several studies have shown that SCFAs, once taken up, are metabolised at a high rate inside the ceacal and colonic mucosal cells (Bugaut and Bentéjac, 1993). 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 relative proportions of the three major acids in the portal blood reflect the relative proportions of those found in the intestinal contents (Bugaut and Bentéjac, 1993). After absorption, the body metabolises each of the primary SCFAs differently.

2.6.3.2.1 Acetate metabolism

For metabolism, acetate requires activation with coenzyme-A (CoA). This process requires the equivalent of two molecules of ATP. This process may even start as early as within the colonocytes of the gut. Mitochondrial acetyl-CoA is used in the citric acid cycle (Krebs cycle) for ATP production, for the synthesis of ketone bodies (Henning and Hird, 1972) and, after transfer to the cytosol, for the synthesis of lipids (Dietschy and Spady, 1983).

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Based on the concentration in the peripheral and portal blood, approximately 75% of the acetate is extracted during a single pass of blood through the human liver (Dankert **et al**., 1981; Peters **et al**., 1992). However, not all SCFAs taken up by the liver are metabolised there. Also, after ethanol administration, studies show the liver to be a net producer of acetate (Lundquist **et al**., 1962). Under these conditions, a variety of human tissues, including skeletal muscle (Lindeneg **et al**., 1964) and brain (Juhlin-Dannfelt, 1977) utilise considerable quantities of acetate. Changes in the blood acetate reflect, at least qualitatively, changes in colonic acetate production. Short-term starvation reduces serum acetate, whereas infusion of acetate into the rectum causes a dose-dependent increase in blood acetate (Wolever **et al**., 2002).

In humans, only acetate of the SCFAs reaches the circulation beyond the liver in appreciable quantities. The venous plasma concentration of acetate in normal humans, as measured by gas-liquid chromatography, is about 50 μmol/ L in the fasting state (Scheppach **et al**., 1991). Acetate in the peripheral blood is not entirely derived from the colon, since several tissues both produce and consume acetate simultaneously (Bleiberg **et al**., 1992).

The utilisation and the metabolic effects of SCFA may depend on the rate and the route of administration. For example, acetate given orally appears

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to have no effect on the plasma concentration of the pancreatic glucagons, but given rectally, it has repeatedly been shown to increase the concentration of this peptide hormone in the circulation (Scheppach **et al**., 1988; Stephen **et al**., 1989). Rectal, oral and intravenous acetate each lowered the concentration of plasma free fatty acids (FFA) (Scheppach **et al**., 1988). This observation is consistent with the effect of SCFAs in sparing long-chain fatty acids from oxidation in humans (Lundquist **et al**., 1962) and in reducing lipolysis in the rat adipocytes *in vitro* (Nilsson and Belfrage, 1978). The antilipolytic effect may be mediated by the same mechanism that occurs when ketone bodies reduce lipolysis in adipose tissue (Robinson and Williamson, 1980).

2.6.3.2.2 Propionate metabolism

Propionate metabolism has been extensively studied in ruminants where it is a major glucose precursor. Much less is known of its role in man. 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 utilised primarily by the liver, where it is used as substrate for gluconeogenisis (Bugaut and Bentéjac, 1993) and it has also been suggested as a potential modulator for cholesterol synthesis (Chen **et al**.,

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1984). Under normal conditions, propionate is completely metabolised by the liver (Rémésy **et al**., 1995). This process is certainly favoured by facilitated diffusion, which is efficient even in the presence of relatively low propionate concentrations (Fafounoux, Rémésy and Demignnè, 1985). Propionate metabolism depends on the bioavailability of vitamin B_{12} or biotin (Chiang and Mistry, 1974). If propionate metabolism increases, the requirement for vitamin B_{12} increases (Cullen and Oace, 1989).

Propionate supplemented diets have been shown to lower blood cholesterol in rats (Chen **et al**., 1984, Illman **et al**., 1988) and pigs (Boila **et al**., 1981), but in humans the effects are less clear. In a double-bind placebo controlled study of ten female volunteers fed with 7.5g sodium propionate daily for seven weeks, there was no change in serum cholesterol, but HDL cholesterol increased, as did triglycerides (Venter **et al**., 1989). As discussed earlier in this chapter, an increase in HDL-C is of beneficial value. In a similar study, lasting one week, no effect on cholesterol was seen but glucose levels were lowered (Todesco **et al**., 1991). In another study, nine healthy males, whose initial cholesterols were all > 5.5 mmol/L, were given 5.4g propionate daily for 15 days and showed a decrease in cholesterol and in LDL cholesterol levels, whilst acetate was without effect (Stephen, 1994).

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To overcome the objections that oral propionate does not reflect the true physiology of propionate produced by fermentation in the hindgut, two studies using rectal or colonic infusions of propionate have been undertaken (Wolever **et al.**, 1989; Wolever **et al.**, 1991). Propionate had no effect on slightly raised serum cholesterol in short-term studies. Other studies have however shown that propionate inhibits hepatic cholesterol synthesis by the redistribution of cholesterol from plasma to the liver (Chen **et al.**, 1984; Illman **et al.**, 1988).

2.6.3.2.3 Butyrate metabolism

Hepatic uptake of butyrate is considered to be practically complete under any physiological conditions. Butyrate is the preferred energy source for colonocytes and is thus extensively metabolised by the colon (Roediger, 1982). Butyrate uptake could be facilitated by the presence of a butyrate binding protein in the cytosol (Rémésy **et al.**, 1995). Butyrate is exclusively metabolised in the mitochondria (carnitine-independent source of acetyl-CoA) and it is a potentially ketogenic substrate during the post absorptive period. Butyrate activation is probably mediated by medium-chain acyl-CoA synthetase(s) (Rémésy **et al.**, 1995). Furthermore, butyrate leads to ketone body production and could be used as an important respiratory fuel in preference to acetate, propionate, glutamine, glucose and ketone bodies (Bugaut and Bentéjac, 1993). High

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concentrations of butyrate inhibit propionate utilisation. It is therefore suggested that butyrate probably thwarts some of the inhibitory effects of propionate (Demignnè **et al**., 1986).

2.6.4 Effects of SCFAs on lipid metabolism

Dietary intervention studies show that the greatest reduction in the percentage of blood lipids and lipoproteins are seen in a high fibre vegetable diet with reductions in total cholesterol, LDL cholesterol and the ratios of total to HDL cholesterol, as well as LDL to HDL cholesterol. The high fibre vegetable diet is also associated with a significantly higher output of total SCFAs, when compared to other diets containing more fats and proteins (Jenkins **et al**. 2001).

It is known that water-soluble fibres such as psyllium, pectin, guar gum and oat gum have potent hypocholesterolaemic effects. Early research has led to the suggestion that SCFAs might contribute to the hypocholesterolaemic effects of these water-soluble fibres (Anderson and Chen, 1979).

Two popular hypotheses explaining the possible mechanisms though which these fibres exert hypocholesterolaemic effects were developed over time, and are:

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- Soluble fibres bind bile acids in the intestine, alter lipid and bile–acid absorption and increase faecal loss of the bile-acids (Anderson and Siesel, 1990; Jenkins **et al**., 2001).

- Soluble fibres are fermented in the colon to SCFAs, which are absorbed into the portal vein and attenuate hepatic cholesterol synthesis (Anderson and Siesel, 1990). This would then cause the liver to convert more cholesterol to bile acids, thereby reducing body cholesterol (Anderson and Siesel, 1990).

Hara **et al**. (1999) examined possible mechanisms involved in the cholesterol-lowering effects of SCFAs in rats. They found that SCFAs produced by caecal fermentation were possibly involved in lowering plasma cholesterol levels by negating the counteractive induction of hepatic cholesterol synthesis caused by an increase in bile excretion.

The liver is the principal site of the propionate metabolism and cholesterol synthesis. Methylcellulose, a modified polysaccharide resistant to microbial metabolism, and which, therefore, is not fermented in the colon to SCFAs, had no effect on the plasma cholesterol, hepatic cholesterol synthesis and faecal excretion of bile acids (Topping **et al**., 1988). However, with guar gum, a fermentable fibre, hydrolysis had the same decreasing effects on cholesterol levels, as did intact guar gum. A tenfold

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increase of caecal SCFAs contents was observed with both kinds of substrates. Veldman **et al**. (1999) found that supplementation with acetate alone caused significant decreases in the total cholesterol, and LDL cholesterol. Such results suggest that action of absorbed SCFAs on the hepatic and peripheral metabolism of the cholesterol may be an alternative mechanism for the effect of fermentable carbohydrates on the bile cholesterol concentrations.

It has been suggested that propionic acid, and possibly even acetate, may reduce plasma cholesterol levels (Briges **et al**., 1992). Wolever **et al**. (1989) suggests that acetate might reduce cholesterol synthesis by reducing circulating free fatty acids (FFA). Veldman **et al**. (1999) found that both acetate and pectin supplementation decreased FFA significantly. If cholesterol synthesis is decreased through a decrease in FFA, it is evident that both acetate and pectin could decrease total cholesterol by the same mechanism. Strong associations between changes in levels of LDL cholesterol and changes in the levels of serum acetate also support the hypothesis that changes in the SCFAs contribute to the hypocholesterolaemic effects of soluble fibres (Anderson, 1995). Wolever **et al**. (1995), however, found that acetate is incorporated into the plasma lipids, and that propionate resulting from colonic fermentation inhibits this process.

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Wolever **et al**. (2001) found that although acetate is the preferred substrate for hepatic fatty acid and cholesterol synthesis, it does not increase the overall rate of lipid synthesis. Nevertheless, the incorporation of acetate into cholesterol and fatty acids is inhibited by propionate, and the addition of propionate to rectal infusions of acetate reduces acetate incorporation into serum lipids, especially triacylglycerols, in humans. Thus the ratio of acetate to propionate produced in the colon may be an important determinant of the effect of colonic SCFAs on blood lipids.

In another study during which propionate capsules were given to healthy subjects for a period of seven weeks (Venter **et al**.,1990), propionate use did not affect serum cholesterol levels, but significantly increased HDL cholesterol by 11 percent when compared to the control changes.

Todesco **et al**. (1991) showed a significant difference in the serum lipids in subjects who consumed bread containing 9.9g of propionate per day to the subjects who consumed white bread without propionate. Total cholesterol, LDL cholesterol, LDL cholesterol tended to be lower while triglycerides tended to be higher after propionate-containing bread consumption.

Wolever **et al**. (2002) suggest that the adaptation of blood lipid responses occurs partly in response to high fibre diet, and to a long-term adaptation

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in SCFA production from colonic fermentation of unabsorbed dietary carbohydrates, but is cautious in attributing the changes in the blood lipids over time to true physiologic adaptation. Most dietary studies suggest that fasting blood lipids respond maximally to dietary changes within 2-4 weeks (Garg **et al**., 1994; Jenkins **et al**., 1993).

2.7 Summary

It is evident that urbanisation and the adoption of a westernised diet is responsible for the rise in cardiovascular disease in the black population of South Africa.

Diet is one of the controllable factors in the treatment and the prevention of westernised diseases such as CHD. On the basis of current understandings, it is apparent that western diets do not contain enough NSP, and that the average intake falls below the recommended level. Fermentation of NSP results in production of SCFAs, which have beneficial effects on coronary risk factors (lipid profiles).

SCFAs fermented in the colon can be successfully substituted with the same results. There is, however, a controversy surrounding SCFA supplementation.

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CHAPTER 3

METHODS AND TECHNIQUES

3.1 INTRODUCTION

The main objective of this study is to determine the effect of a combination and ratio difference of short-chain fatty acids on the lipid profile in men of the South African National Defence Force (SANDF). To achieve this objective, the study design, selection of subjects, measurements, and statistical analyses, as well as the limitations of the study, will be discussed in this chapter.

3.2 ETHICAL CONSIDERATIONS

The study was approved by the Ethics Committee of the Faculty of Health Sciences of the University of the Free State (UFS) (Ethics Number: 227/98). All subjects participating in the study gave their written consent (Appendix 1).

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3.2 STUDY DESIGN

The study design was that of a randomised, placebo-controlled, double-blinded clinical trial as represented in Figure 3.1.

Subjects falling within a predetermined set of inclusion criteria (see 3.3.1 below) were included in the study. Two baseline blood samples and other information were obtained (day 0 and day 8) in order to ensure an accurate reflection of the variables, and a stable baseline. All subjects received equal amounts of the placebo for a period of one week prior to the intervention period of the study, in order to obtain a stable baseline (day 8). After baseline two, subjects were randomised into three equal groups with 25 subjects in each group. Group 1 received a placebo supplement and group 2 (experimental 1) and 3 (experimental 2) were supplemented with various combinations of SCFAs. Supplementation with the various combinations of short-chain fatty acids and the placebo was then sustained for 4 weeks (day 8 – 36). A washout period followed the intervention period, during which all subjects again received the placebo supplement for a period of one week (day 43).

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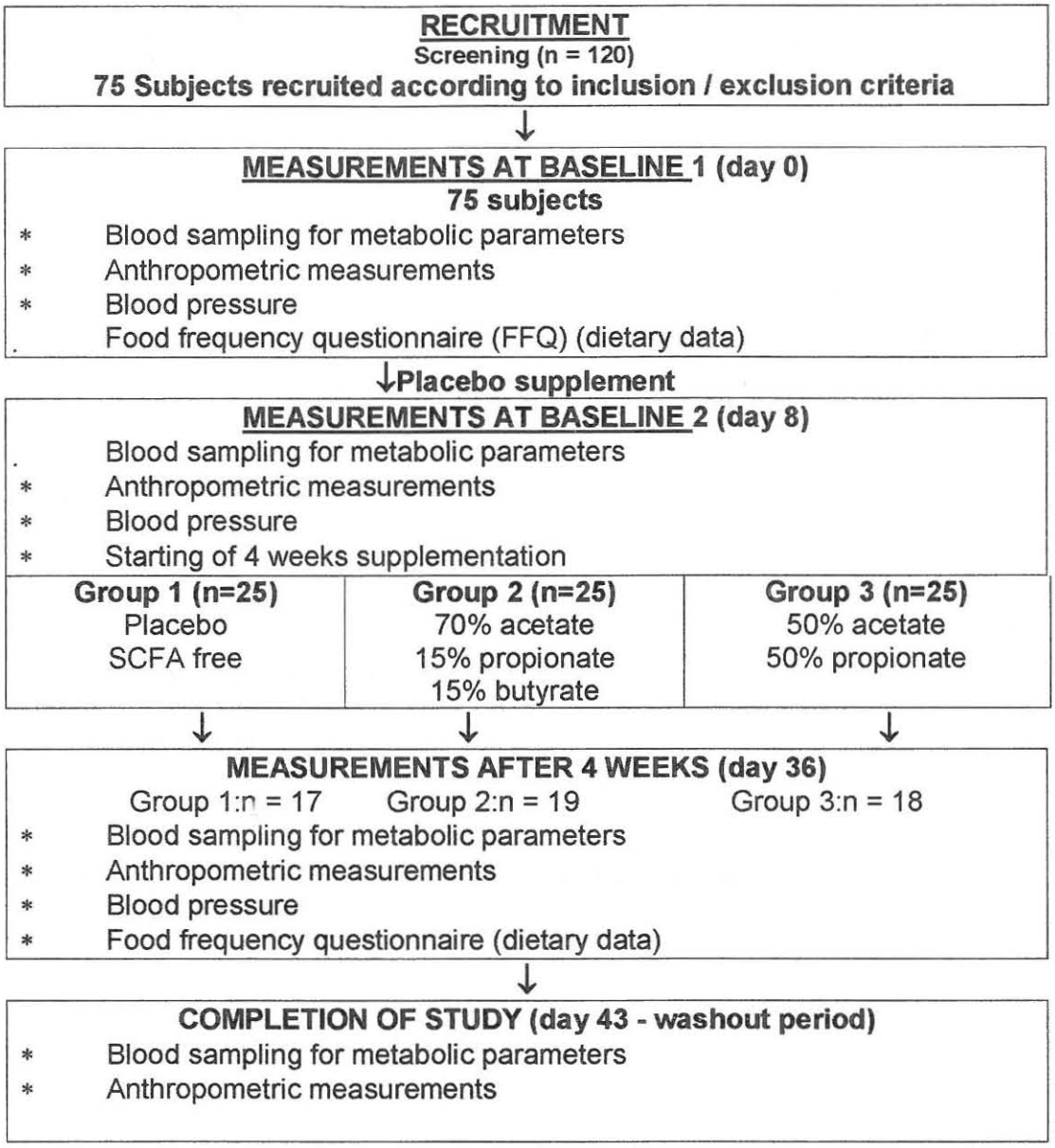

Figure 3.1 Schematic representation of the study design

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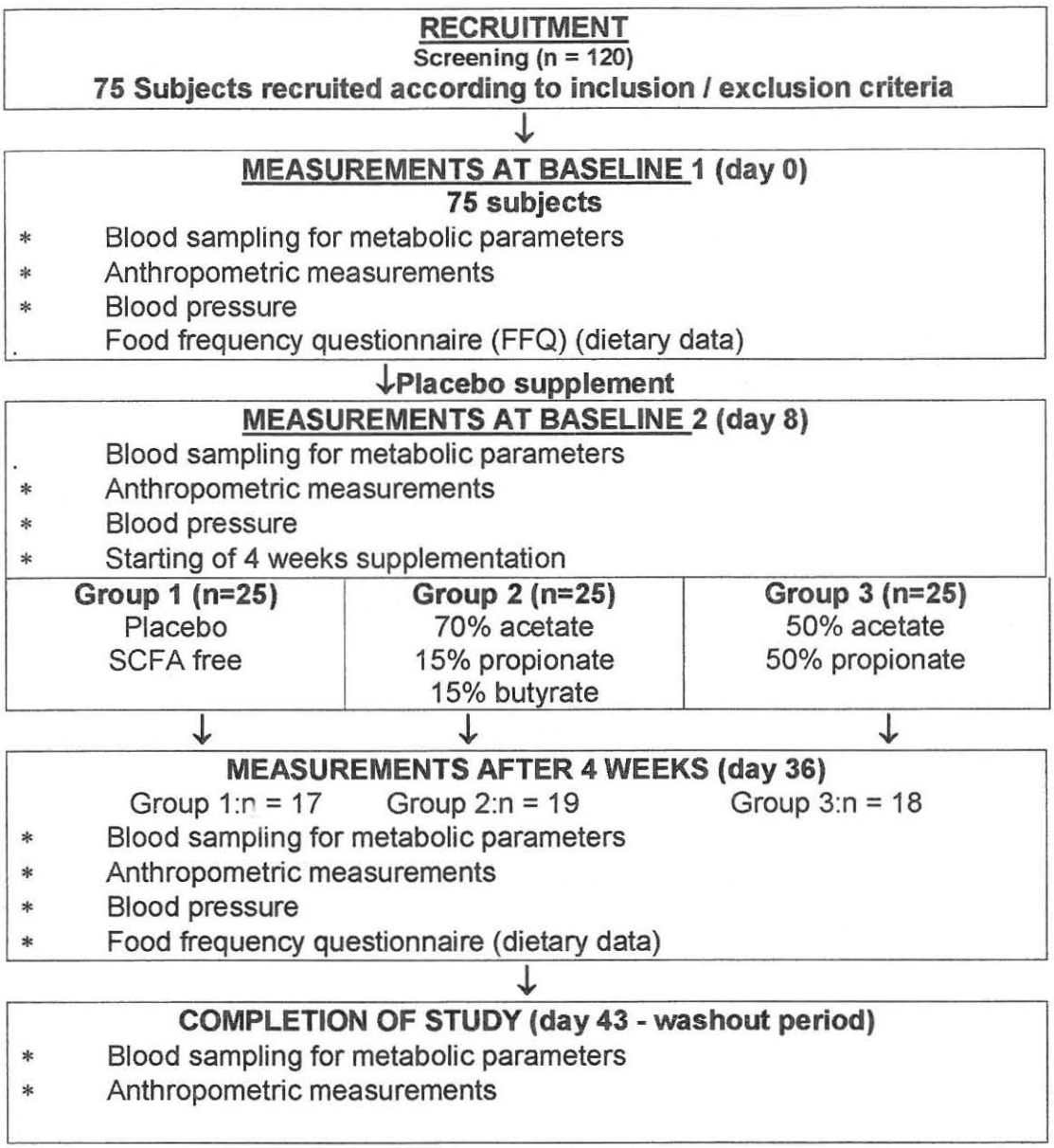

Figure 3.1 Schematic representation of the study design

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Food frequency questionnaires (FFQ- **Appendix 3**) were used to determine dietary intake at baseline 1 (day 0), and after the intervention period (day 36). The study was undertaken at a time specifically designed to minimise possible seasonal effects on human metabolism.

3.3 SUBJECTS

3.3.1 Subject recruitment

Approximately one hundred and twenty members of the SANDF were recruited to participate in the study voluntarily. Subjects were recruited in the following manner:

- Subjects had to volunteer
- A recruitment/screening questionnaire was completed (**Appendix 2**)
- Blood pressure, glucose and cholesterol levels above a specified level, as discussed later in this section, were taken.

3.3.2 Screening

Screening ensures that participants meet eligibility criteria and comply with the requirements of the study (Dennis and Kris-Etherton, 1991). Screening for this study took place during an individual interview, using a

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questionnaire (**Appendix 2**). A trained fieldworker was present during the interview to translate any issues which the subjects might not have understood.

3.3.3 Inclusion criteria

Subjects for this study were included/chosen according to the following inclusion criteria.

- males aged between 18 and 45
- blood glucose levels below 5.8 mmol/L
- no alcohol abusers (< 3 drinks / day or <28g alcohol/day)
- no subjects using medication for chronic diseases
- subjects had to have been permanent staff of the SANDF for at least one year prior to the study.

3.3.4 Sample size

75 Volunteers were recruited to participate voluntarily in the study. The total sample size (3 x n) was determined by the Department of Biostatistics at the University of the Free State (UFS). Power calculations showed that 15 individuals per group would be sufficient for measuring significant changes in cholesterol levels. A number of

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25 individuals per group would be sufficient to allow for a drop-out rate of 10 per group. This estimate is realistic.

3.4 MEASUREMENTS

3.4.1 Metabolic parameters

The following metabolic parameters were measured during this study:

- Serum total cholesterol, serum low density lipoprotein-cholesterol (LDL-C), serum high density lipoprotein-cholesterol (HDL-C), serum triglycerides and non-esterified fatty acid (NEFA), as markers for the lipid profile (Willet 1998).

- total serum protein, serum albumin, a full blood count plasma fibrinogen, and haemoglobin as markers for health and nutritional status (Lindsey, 1996)

- fasting serum glucose and insulin levels as indicators of glycometabolic control (Donnelly, 1996; Franz, 2004).

- Normal accepted ranges for the metabolic variables for black populations, according to the Department of Chemical Pathology

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and Haematology at the University of the Free State, are summarised in Table 3.1.

Table 3.1 Normal ranges for metabolic indicators used in this study

Metabolic indicators	Normal range
Total cholesterol	3.0 – 5.2 mmol/L
Triglycerides	0.3 – 2.0 mmol/L
NEFA	0.1 – 0.9 mmol/L
LDL-C	2.02 – 3.37 mmol/L
HDL-C	0.98 – 1.83 mmol/L
Glucose	3.6 – 5.6 mmol/L
Insulin	5.0 –25 µIU/mL
Total protein	64 – 84 g/L
Albumin	34 – 52 g/L

3.4.2 Anthropometric status

Height, weight, hip and waist circumference were measured to determine the anthropometric status of volunteers. Height and weight were used to calculate the body mass index (BMI). BMI can be used as an indicator of obesity, and is associated with an increased risk of developing health problems (Earl and Borra, 2000). BMI is classified as follows:

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• 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

(adapted from Laquatra, 2004).

3.4.3 Blood pressure

Blood pressure is a dynamic variable, and was measured as an indicator of the subjects' physical and emotional state at the time of the measurement (De Bono and Boon, 1991). Hypertension, which refers to high arterial blood pressure and is represented by raised systolic and diastolic blood pressure (Seedat **et al.,** 1993) is classified as follows:

• Normal blood pressure	< 140/90. mmHg
• Borderline hypertension	> 40/90 mmHg < 160/95 mmHg
• Hypertension	> 160/95 mm Hg

(adapted from De Bono and Boon, 1991).

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3.4.4 Dietary intake

The dietary intake of subjects was measured by means of a standardised food frequency questionnaire (**Appendix 3**). The prudent diet guidelines were used as a reference for macronutrient intake (Wolmarans **et al.**, 1988).

3.4.5 Blood sampling

Biochemical information was obtained using the appropriate blood. The analysis was performed in the laboratory of the School of Health Technology at the Central University of Technology, Free State, using standardised procedures.

The coefficient of variation (CV) of the methods was determined for each set of measurements for the control of accuracy and reproducibility of the methods. The CV for each method was calculated as follows:

CV = [standard deviation]/ [mean] x 100

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3.4.6 Phlebotomy

Venipuncture was done by trained personnel using a plastic tube holder (bulldog), a tourniquet, Precision Glide needles™ (Becton Dickinson, 0.8 x 38mm), and 70% alcohol swabs (Medi-Swab*) into pre-vacutained tubes. The subject was seated, his/her arm supported and venipucture was performed in the antecubital area of the arm where the medial cubital, cephalic and basilica veins are close to the surface.

3.4.7 Blood sample preparation

a. Serum

5 ml of whole blood was left to clot at room temperature. These samples were centrifuged at 2800 x g for 20 minutes in order for the serum to separate. Serum samples were frozen at –72°C in Eppendorf® vials (Safelock micro test tubes cat. no. 0030120.094 US no.1,404,655, Canadian no.315.460)

b. EDTA blood

5mL blood was obtained in pre-treated K_3EDTA-tubes (VAC-U-TEST). After using some of the sample for determining full blood counts, the remainder of the sample was centrifuged for 15 minutes at 2800 x g and

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the plasma stored at –72°C in Eppendorf® vials (Safelock micro test tubes cat. no. 0030120.094 US no.1,404,655, Canadian no.315.460).

c. Citrate plasma

Citrated blood (1 mmol/L citrate, pH 4.5-4.8 plus 9 ml venous blood) was centrifuged at 2800 x g for ten minutes to prepare citrated plasma and stored at –72°C in Eppendorf® vials (Safelock micro test tubes cat. no. 0030120.094 US no.1,404,655, Canadian no.315.460). Citrate acts as an inhibitor of early activation of factor V and VII. Citrate plasma was used for the analyses of clotting factors (plasma fibrinogen).

3.4.8 Measurement of metabolic parameters

The methods used to measure the above-mentioned metabolic parameters will be discussed in the next section.

a. Serum glucose

Serum glucose was determined in duplicate by using an enzymatic colorimetric assay (Glucose GDO-PAP; Roche Diagnostics GmbH, Mannheim, Germany; Cat. no. 1448668) on the Roche/Hitachi 902 auto analyser. This method is based on the oxidation of glucose to gluconolactone in the presence of atmospheric oxygen. The resultant hydrogen peroxide oxidises 4-aminophenazone and phenol to 4-(p-benzoquinone-monoimino)-phenazone in the presence of peroxidase

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(POD). The colour intensity of red dye is directly proportional to the glucose concentration, and can be measured photometrically. This method was calibrated against the Calibrator for Automated Systems (cat. no. 759 350, Roche Diagnostics GmbH, Mannheim, Germany). Precinorm U/normal values (cat. no. 171 735, Roche Diagnostics GmbH, Mannheim, Germany) and Precipath U/abnormal values (cat. no. 171 760, Roche Diagnostics GmbH, Mannheim, Germany) were used as control serum. The CV for the method was 1.41 %.

b. Serum insulin

Serum insulin was determined in duplicate by means of an enzyme immunoassay for the quantitative measurement of insulin in human serum (Cat no. EIA-2935, manufactured by DRG Instruments GmbH Germany). The DRG insulin enzyme-linked immunosorbent assay (ELISA) is a solid phase two-site enzyme immunoassay. It is based on the direct sandwich technique, in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During incubation insulin in the sample reacts with biotin-conjugated anti-insulin antibodies and anti-insulin antibodies bound to the inside of a microtitration well. A simple washing step removes the unbound biotin labelled antibody.

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During the second incubation step streptavidin peroxidase enzyme complex binds to the biotin-anti-insulin antibody. The bound horseradish peroxidase complex is detected by its reaction with 3,3`,5,5`-tetramethylbenzidine. The reaction is stopped by adding acid to the sample to give a colorimetric endpoint that is read spectrophotometrically. The CV for the method was 6.8 %.

c. Serum-total protein (TP)

Total protein was determined using a colorimetric assay supplied by Roche Diagnostics GmbH, Mannheim, Germany (cat. no. 1929917). Divalent copper reacts in an alkaline solution with protein peptide and bonds to form the characteristic purple-coloured buiret complex. The colour intensity is directly proportional to the protein concentration which can be determined photometrically. This method was calibrated against the Calibrator for Automated Systems (cat. no. 759 350, Roche Diagnostics GmbH, Mannheim, Germany). Precinorm U/normal values (cat. no. 171 735, Roche Diagnostics GmbH, Mannheim, Germany) and Precipath U/abnormal values (cat. no. 171 760, Roche Diagnostics GmbH, Mannheim, Germany) were used as control serum. The CV for this method was 1.5 %.

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d. Serum-total albumin

Serum albumin was determined in duplicate using the colorimetric Bromocresol-green (BCG) method supplied by Roche Diagnostics GmbH, Mannheim, Germany (cat. no 197 0909) on the Boehringer Mannheim Hitachi 902 chemistry analyser. Albumin complexates with bromocresol-green at a pH of 4.2. The intensity of the coloured complex is directly proportional to the albumin concentration in the sample. The method was calibrated against the Calibrator for Automated Systems (cat. no. 759 350, Roche Diagnostics GmbH, Mannheim, Germany). Precinorm U/normal values (cat. no. 171 735, Roche Diagnostics GmbH, Mannheim, Germany) and Precipath U/abnormal values (cat. no. 171 760, Roche Diagnostics GmbH, Mannheim, Germany) were used as control serum. The CV for this method was 2.1 %.

e. Serum total cholesterol

Serum total cholesterol was determined using an enzymatic colorimetric test. The method was performed using reagents supplied by Roche Diagnostics GmbH, Mannheim, Germany (cat. no 1491458). Cholesterol is determined enzymatically using cholesterol esterase and cholesterol oxidase. Cholesterol esters are cleaved by the action of cholesterol esterase to yield free cholesterol and fatty acids. Cholesterol is converted by oxygen with the aid of cholesterol oxidase to choles-4-4-one and hydrogen peroxide. Hydrogen peroxide created forms of a red

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dye by reacting with 4-aminophenazone and phenol under the catalytic action of peroxidase. The colour intensity is directly proportional to the concentration of cholesterol, and can be determined photometrically. This method was calibrated against the Calibrator for Automated Systems (cat. no. 759 350, Roche Diagnostics GmbH, Mannheim, Germany). Precinorm U /normal values (cat. no. 171 735, Roche Diagnostics GmbH, Mannheim, Germany) and Precipath U /abnormal values (cat. no. 171 760, Roche Diagnostics GmbH, Mannheim, Germany) were used as control serum. The CV for the method was 0.33 percent.

f. High density lipoprotein cholesterol (HDL-C)

Low-density lipoproteins (LDL) was precipitated qualitatively by the addition of phosphotungstic acid in the presence of magnesium ions (Cat no.CH 2652 Randox, Crumlin, UK). HDL-C was isolated by centrifugation of the sample. The cholesterol content of the isolate was then determined, using exactly the same method as described for the measurement of the TC as described above. The intensity of the produced colour was measured photometrically at 365nm. A special control was used as standard (Boehringer Mannheim-Roche Diagnostic, Mannheim, Germany). Values are expressed in mmol/L. The CV for the method was 1.9%.

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g. %HDL-C

The %HDL-C was determined using the following calculation:

$$\%\text{HDL-C} = \frac{\text{HDL-C}}{\text{TC}} \times 100$$

h. Low density lipoprotein cholesterol (LDL-C)

LDL cholesterol (mmol/L) was determined using the following calculation (Randox, Crumlin, UK):

$$\text{LDL cholesterol} = \text{total cholesterol} - \frac{\text{triglycerides}}{2.2} - \text{HDL cholesterol}$$

Values were expressed in mmol/L. The CV for the method was 1.9%.

i. Serum-triglycerides

Serum triglyceride concentration was determined by using a colorimetric assay supplied by Roche, Diagnostics, Germany (cat. no. 1730711). The method is based on the work by Wahlefeld using a lipoprotein lipase from micro-organisms for the rapid and complete hydrolysis of triglycerides to glycerol, followed by oxidation to dihydroxyacetone phosphate and hydrogen peroxide. The hydrogen peroxide thus reacts with 4-aminophenazone and 4-chlorophenol under the catalytic action of peroxidase to form a red dye (Trinder endpoint reaction). The method

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was calibrated against the Calibrator for Automated Systems (cat. no. 759 350, Roche Diagnostics GmbH, Mannheim, Germany). Precinorm U /normal values (cat. no. 171 735, Roche Diagnostics GmbH, Mannheim, Germany) and Precipath U /abnormal values (cat. no. 171 760, Roche Diagnostics GmbH, Mannheim, Germany) were used as control serum. The CV for this method was 1.3%.

j. Non-esterified fatty acids (NEFA)

The optimised colorimetric assay for the enzymatic determination of free fatty acids (non-esterified fatty acids, NEFA) in duplicate was used to determine the free fatty acid concentration (cat. no. FA 115, Randox Laboratories Ltd, Crumlin, United Kingdom) in plasma samples. The method involves the formation of Acyl-CoA, AMP and PPi in the presence of Acyl-CoA Synthetase. The Acyl-CoA is converted to 2,3,-trans-Enoyl-CoA and H_2O_2 in the presence of Acyl-CoA Oxidase. The H_2O_2 is reacted with a chromogen in the presence of peroxidase, which can be measured photometrically. The intensity of the colour of the chromogen is directly proportional to the concentration of the NEFA in the sample.

k. Plasma fibrinogen

Plasma fibrinogen was determined using the Dade Behring Coagulation Analyser with Multifibrin®-U reagent (Cat. No. OWZG 15; Dade Behring,

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Marburg, Germany). Fibrinogen Standards 1-4 (Cat. No. OWCS 11; Dade Behring, Marburg, Germany) were used for calibration purposes. Control Plasma N for normal values (Cat. No. ORKL 17; Dade Behring, Marburg, Germany) and for Control Plasma P for pathological ranges (Cat. No. OUPZ 13; Dade Behring, Marburg, Germany) were included for control purposes.

I. Full blood counts determination

A full blood count was performed on the Coulter® MicroDiff 18, cell counter.

3.4.9 Anthropometric measurements

Anthropometry involves obtaining physical measurements of an individual, then relating these measurements to standards that reflect, among others, health and nutritional status (Lee and Nieman, 1996; Gibson 2000). All the anthropometric measurements were taken by the trained researcher throughout the study. For standardisation purposes the anthropometric measurements were measured by the same fieldworker throughout the study.

a. Weight

Body weight was measured according to a standard method described by Lee and Nieman (1996) and Charlton **et al**. (1996) using a calibrated

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Seca digital electronic scale which weighs to the nearest 0.1kg. 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.

b. Height

Standing height of subjects wearing light clothing and no shoes was measured to the nearest 0.5 cm using a statiometer as described by Charlton **et al**. (1996). 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 ("Frankfort plan"). The top of the statiometer was lowered to rest flat on the top of the head.

c. Body Mass Index (BMI)

BMI was calculated using the standard formula: (weight (kg) / height (m^2)) (Pressman and Adams, 1990, Hammond, 2004).

d. Waist and hip circumferences

Waist and hip circumferences were measured in duplicate, around the

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smallest and widest part of the waist and hips respectively, to the nearest 0.1cm. Waist circumference was measured at the lowest level of the umbilicus. Hip circumference was measured at the largest diameter below the umbilicus or the maximum circumference over the buttocks taken perpendicularly on the axial line of the trunk as described by Charlton **et al.** (1996).

e. Blood pressure

Blood pressure was recorded using a sphygmomanometer and a stethoscope before blood samples were collected, according to the methods used by DeBono and Boon (1991). The subjects were seated with the 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 branchial artery and the cuff was inflated to a level well above that which abolishes the Korotkov 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 dropped further, the sounds became louder and then usually suddenly became muffled and later disappeared, at which stage the diastolic pressure was measured. Three intermittent readings were taken at two-minute intervals, and the lowest value was recorded.

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3.5 QUESTIONNAIRES

Questionnaires used in this study included a screening questionnaire (**Appendix 2**), a food frequency questionnaire (**Appendix 3**) and a tolerance questionnaire (**Appendix 4**).

3.5.1 Screening questionnaire

The screening questionnaire was developed to select subjects with specific characteristics according to the inclusion and exclusion criteria of the study. Questions regarding age, activity level, smoking habits, alcohol intake and a medical history were recorded (**Appendix 2**).

3.5.2 Food Frequency Questionnaire (FFQ- Appendix 3)

The purpose of dietary assessment is to estimate food consumption of dietary intake in individuals or groups of people (Nelson, 2000). Usual dietary intake is valuable in assessing nutritional status when used in combination with biochemical, anthropometric and clinical data (Lee and Nieman, 1996; Dwyer, 1998). Data obtained from this questionnaire will not be discussed in this document.

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3.5.3 Tolerance questionnaire

The tolerance questionnaire (**Appendix 4**) was completed after the study to determine whether any of the subjects experienced any possible side-effects during the intervention that could be related to the contents of the capsules. Volunteers were asked if they had experienced any of the following symptoms: nausea, constipation, diarrhoea, decrease or increase in appetite, and whether the number of capsules consumed was acceptable.

3.5.4 Standardisation of the FFQ

The fieldworkers were trained to ask the same questions and to use the FFQ, the food models and other dietary survey aids in the same way, to obtain reliable results.

3.6 SUPPLEMENTS

3.6.1 Capsules

Capsules were filled by QuattroMed, in Bethlehem, Free State. The capsules were enterically coated, to ensure that the capsules would pass the stomach and dissolve in the large gut. The capsules were designed

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in such a way that they contained equal amounts of the sodium and calcium salts, in order to prevent excess intake of these ions by subjects. The molar concentrations of SCFAs were calculated using 15g of non-digested glucide, which produce 1.78 mol SCFA per mol hexose, as reference (Roberfroid **et al.,** 1993).

3.6.1 Supplement 1

Supplement 1 contained a mixture of:

- 70% acetate, consisting of 0.4618g sodium and 0.2989g calcium acetate (2 x 0.057685185 mol SCFA),
- 15% propionate, made up of 0.0698g sodium and 0.0677g calcium propionate (2 x 0.012361111 mol SCFA), and
- 15% butyrate, made up of 0.0801g sodium and 0.0779g calcium butyrate (2 x 0.012361111 mol SCFA).

The capsules each weighed 1.0562g. Each subject consumed 8 capsules per day.

3.6.2 Supplement 2

Supplement 2 contained a mixture of:

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- 50% acetate, consisting of 0.3299g sodium and 0.2135g calcium acetate (2 x 0.041203704 mol SCFA);
- 50% propionate, made up of 0.2328g sodium and 0.2257g calcium propionate (2 x 0.041203704 mol SCFA), and 0% butyrate.

The capsules weighed 1.0019g each. Each subject consumed 8 capsules per day.

3.6.3 Supplement 3

The placebo was filled with sodium chloride, calcium chloride and CMC sodium carmelel DV (S), a non-fermentable form of cellulose. The capsules weighed approximately 1.0288g each. Subjects had to take 8 placebo capsules per day.

3.7 FIELDWORKERS AND STANDARDISATION OF TECHNIQUES

3.7.1 Fieldworkers

The fieldworkers used in this study included:

- A qualified nurse and a group of trained medical personnel of the SANDF. These people were responsible for taking blood samples and measuring blood pressure.

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- Four qualified dieticians who were standardised to use the validated food frequency questionnaire.
- A trained primary health care worker of the SANDF who helped with the translation of the questionnaires where necessary.
- A post-graduate student trained to take anthropometric measurements.

3.7.2 Standardisation of blood sampling

The nurse and the operational medical personnel received special training in the measurement of blood pressure and drawing of the various blood samples. This was necessary, as some research techniques were different to those used for everyday medical purposes, especially the quantity of blood taken on each visit. Some samples used for the determination of specific metabolic parameters had special prerequisites that had to be adhered to, i.e. blood tubes that should be taken without stasis; as well as some samples that should be mixed well with the contents of the blood tube in order to prevent clotting of the blood sample within the tube (citrated blood tubes, etc.). The time-limits for blood sampling were also adhered to strictly (all samples were taken before 10 a.m.) in order to prevent daily variation on metabolic parameters from having an impact on the study results.

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3.8 PILOT STUDY

A pilot study was conducted on a group of 20 volunteers. However, all measurements were simulated and no blood samples were taken from the individuals. The adapted FFQ were also tested on five members by the qualified dieticians to apply earlier training in order to ensure that all the questions of the FFQ were clear, and to identify any other problems that could occur.

3.9 MANAGEMENT OF THE STUDY

Daily management is an essential component of quality assurance (Dennis and 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 and 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.

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- 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 outsider participating neither in the project nor in the execution of any aspects of the project. All the capsules looked similar. Packages were numbered by an outsider and kept in a sealed envelope in his office. 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.

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- The SANDF section head of the members ensured that the capsules were taken every day. Each subject's capsules were kept in his own bag, clearly marked with his number.

- It 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. Subjects were given supplements on a daily basis when they had their meals at the mesh. This was also a measure of compliance. Each subject had their own bag of capsules kept at the mesh and given to them under direct supervision of the mesh supervisor, dietician in charge of the study, or mesh attendant. 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 during their daily visit to the mesh. Compliance to the study was therefore very high and in most cases, only subjects that dropped out did not have a 100% compliance. Drop-outs included mainly those individuals relocated to other units within the defence force. One subject died during the study period, due

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to accidental death. This death was in no way related to the study.

- Informal social functions were arranged at regular intervals.

- The subjects were followed up weekly. 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.

3.10 STATISTICAL ANALYSIS

Results were summarised using means, standard deviations (SD), numerical variables and frequencies and percentages (categorical variables). Changes within groups from baseline one (day 8) to baseline two (day 36); and from baseline 2 (day 8) to the end of the intervention period (day 36), were compared using the paired-t-test, with a p-level of < 0.05 as significant. Differences in changes between groups were measured using the independent t-test method. The analyses were performed by the Department of Biostatistics, UFS.

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3.11 LIMITATIONS OF THE STUDY

A sample of 75 subjects who met the inclusion criteria during the screening phase was initially recruited for the study. Only 58 subjects (Group 1: 17; Group 2: 21; Group 3: 20) completed the study. However, it was estimated that a group of 15 volunteers per group would have been sufficient for statistical changes to be measured as clinically significant. Also, this study had a very short intervention period. Long-term effects of SCFAs on the lipid profiles may differ from the short-term effects. It is therefore suggested that the study be repeated with a longer intervention period.

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CHAPTER 4

RESULTS

4.1 INTRODUCTION

The results of the randomised clinical intervention trial will be presented in this chapter. The aim is to report the changes in lipid profiles and other measured metabolic parameters after supplementation with two different combinations of SCFAs and a placebo. Background characteristics of the group as a whole will be presented; after which the group will be broken down into the respective supplement groups. Comparisons between the metabolic effects of the supplements within the different study groups will then be presented. Baseline two (day 8) will be used as a reference baseline to compare possible changes in the lipid parameters of subjects. As explained in the Methods chapter, subjects were all given a placebo for one week for standardisation purposes.

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4.2 BASELINE CHARACTERISTICS OF THE STUDY GROUP AS A WHOLE

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 to the study. The subjects all lived on the premises of the SANDF, and followed exactly the same basic diet, supplied at the mess-hall of the SANDF. A registered dietician monitored the dietary intake of the study group in order to limit any major changes in intake during the study.

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 had used any chronic medication, had a history of previous illness (such as coronary heart disease, diabetes, etc.) or had had known or visible physical disabilities prior to the study.

A full-time medical team on the premises of the SANDF monitored any changes in the health of 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 subject files. Subjects were examined for any possible side-effects that may have been directly or indirectly caused by the respective supplements

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(allergic reactions, etc.). Subjects were asked to report suspected side-effects or observations or anything out of the ordinary. Records were kept of these reports.

4.2.2 Anthropometric measurements of the study group as a whole (see Table 4.1)

No overweight or obese subjects were included in the study group. The Body Mass Index (BMI) of the group as a whole fell within the estimated limits of the normal reference range for healthy adult males (18.5 to 24.9 kg/m^2). All other anthropometric measurements reflected the healthy physical dimensions of the study group.

The mean blood pressure of the study group at baseline 1 was 118 ± 12 over 83 ± 12 mmHg.

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Table 4.1 Anthropometric measurements of the study group as a whole

VARIABLE		Baseline 1	Baseline 2
Height	**X**	170.5	
(cm)	**SD**	6.50	
Weight	**X**	66.3	67.2
(cm)	**SD**	8.90	8.90
BMI	**X**	22.8	23.0
(kg/m^2)	**SD**	2.30	2.40
Waist	**X**	77.2	77.7
(cm)	**SD**	6.9	7.1
HIP	**X**	95.4	95.9
(cm)	**SD**	5.9	6.2
SBP	**X**	118	118
(mmHg)	**SD**	11	12
DBP	**X**	81	83
(mmHg)	**SD**	16	12

(X = mean; SD = standard deviation; BMI = Body Mass Index; SBP = Systolic Blood Pressure; DBP = Diastolic Blood Pressure)

4.2.3 Lipid profiles of the study group as a whole (see Table 4.2)

The serum-Total Cholesterol (TC) of the study group at day 8 (baseline 2) was 4.46 ± 0.84 mmol/L. The High-Density Lipoprotein Cholesterol (HDL-C) comprised 28.5 ± 7.3% of the circulating serum-total cholesterol. The fasting serum-triglyceride (TG) level for this group was 1.17 ± 0.61 mmol/L. Fasting non-esterified fatty acid (NEFA) levels were 0.368 ± 0.170 mmol/L.

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Table 4.2 Lipid profiles of the study group as a whole

VARIABLE		Baseline 1	Baseline 2
TC	X	4.36	4.46
(mmol/L)	SD	0.88	0.84
TG	X	1.17	1.17
(mmol/L)	SD	0.50	0.61
HDL-C	X	1.41	1.24
(mmol/L)	SD	0.47	0.28
LDL-C	X	2.35	2.67
(mmol/L)	SD	0.83	0.78
%HDL-C	X	33.7	28.5
(%)	SD	9.5	7.3
NEFA	X	0.48	0.37
(mmol/L)	SD	0.27	0.17

(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)

4.2.4 Glycometabolic indicators of the study group as a whole (see Table 4.3)

The fasting serum-glucose level of the group as a whole at day 8 (baseline 2) was 5.47 ± 0.88 mmol/L. The group had a fasting serum-insulin level of 13.7 ± 21.3 µIU/mL.

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Table 4.3 Glycometabolic indicators of the study group as a whole

VARIABLE		Baseline 1	Baseline 2
Glucose	**X**	5.32	5.47
(mmol/L)	**SD**	0.83	0.88
Insulin	**X**	10.7	13.7
(µIU/mL)	**SD**	16.6	21.3

(X = mean; SD = standard deviation)

4.2.5 Other metabolic variables of the study group as a whole (see Table 4.4)

Some more general metabolic indicators of the health status of subjects were included in the laboratory analyses. The serum Total Protein (TP) concentration at day 8 (baseline 2) was 79.9 ± 5.6 g/L, of which the albumin made up 44.8 ± 2.6 g/L.

Blood cell counts were also measured as part of a full blood count, and the results are reported in Table 4.4. The haemoglobin levels at day 8 (baseline 2), measured as part of the full blood count, were 15.8 ± 1.5 g/dl. The concentration of plasma-fibrinogen, an acute phase protein, was 3.28 ± 1.26 g/L.

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Table 4.4 Other metabolic variables of the study group as a whole

VARIABLE		Baseline 1	Baseline 2
TP	X	80.50	79.90
(g/L)	SD	6.30	5.60
Albumin	X	45.20	44.80
(g/L)	SD	3.80	2.60
TP/Albumin	X	1.88	1.79
	SD	0.47	0.13
WBC	X	5.95	5.66
($x10^3/\mu L$)	SD	2.05	1.51
RBC	X	5.16	5.17
($x10^3/\mu L$)	SD	0.53	0.57
Haemoglobin	X	15.9	15.8
(g/dL)	SD	1.1	1.5
Fibrinogen	X	2.77	3.28
(g/L)	SD	0.91	1.26
Albumin/	X	17.7	16.1
Fibrinogen	SD	4.8	5.8

(X = mean; SD = standard deviation; TP = serum-Total Protein; WBC = White Blood Count; RBC = Red Blood Count)

4.3 RESULTS OF THE INTERVENTION STUDY

4.3.1 The Placebo Group

4.3.1.1 Changes in the anthropometrical measurements of the placebo group (see Table 4.5)

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No significant changes in any of the anthropometrical variables were measured in the placebo group during the intervention phase of the study.

Table 4.5 Changes in the anthropometrical measurements of the placebo group

VARIABLE		Day 0	Day 8	Day 22	Day 36	Day 43
Height	**X**	1.72				
(cm)	**SD**	0.07				
Weight	**X**	68.6	69.6	69.9	69.6	69.7
(kg)	**SD**	10.9	10.5	11.5	10.3	10.8
BMI	**X**	23.2	23.4	23.7	23.6	23.6
(kg/m^2)	**SD**	2.9	2.8	3.0	2.7	2.8
Waist	**X**	78.9	80.2	78.2	78.4	78.1
(cm)	**SD**	8.6	8.7	7.7	8.1	7.4
Hip	**X**	95.9	96.9	98.1	97.5	98.0
(cm)	**SD**	8.8	9.2	6.0	6.5	6.8
SBP	**X**	119	124	121	116	115
(mmHg)	**SD**	15	15	14	11	11
DBP	**X**	85	87	85	83	83
(mmHg)	**SD**	15	16	16	10	12

(X = mean; SD = standard deviation; BMI = Body Mass Index; SBP = Systolic Blood Pressure; DBP = Diastolic Blood Pressure; Means with the same symbol differ significantly from each other with a $p < 0.05$ – paired t-tests)

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4.3.1.2 Changes in lipid profiles of the placebo group (see Table 4.6)

Compared to baseline 2, no changes were measured in any of the lipid measurements.

Table 4.6 Changes in the lipid profiles of the placebo group:

VARIABLE		Day 0	Day 8	Day 22	Day 36	Day 43
TC (mmol/L)	X	4.23	4.36	4.29	4.30	4.42
	SD	0.98	0.71	0.97	0.49	0.88
TG (mmol/L)	X	1.29	1.24	1.12	1.27	1.18
	SD	0.39	0.69	0.47	0.41	0.75
HDL-C (mmol/L)	X	1.26	1.18	1.34	1.20	1.43
	SD	0.24	0.23	0.27	0.23	0.31
LDL-C (mmol/L)	X	2.22	2.47	2.42	2.45	2.54
	SD	0.74	0.80	0.76	0.50	0.78
%HDL-C (%)	X	32.2	28.3	32.3	30.50	32.70
	SD	6.30	8.00	7.10	6.10	9.10
NEFA (mmol/L)	X	3.33	3.54	3.68	4.92	3.65
	SD	1.99	1.47	1.99	1.90	2.43

(X = mean ; SD = standard deviation ; means with the same symbol differ significantly from each other with a $p < 0.05$ – paired t-tests ; 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)

4.3.1.3 Changes in the glycometabolic indicators of the placebo group (see Table 4.7)

No significant differences in any of the glycometabolic parameters of the placebo group were measured between any of the visits. The

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measured decrease in serum-insulin levels from day 8 (baseline 2) to day 22 was not significant.

Table 4.7 Changes in glycometabolic indicators of the placebo group

VARIABLE		Day 0	Day 8	Day 22	Day 36	Day 43
Glucose (mmol/L)	**X**	5.08	5.65	5.12	5.15	5.52
	SD	0.57	1.16	0.93	1.21	1.07
Insulin (µIU/mL)	**X**	8.30	10.10	21.60	9.10	14.5
	SD	8.90	14.90	20.70	15.30	9.70

(X = mean; SD = standard deviation; means with the same symbol differ significantly from each other with a $p < 0.05$ – paired t-tests)

4.3.1.4 Changes in other metabolic variables of the placebo group (see Table 4.8)

None of the other measured metabolic variables in the placebo group showed any significant change from the baseline to the end of the supplementation phase.

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Table 4.8 Changes in other metabolic variables of the placebo group

VARIABLE		Day 0	Day 8	Day 22	Day 36	Day 43
TP	X	76.9	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
TP/Albumin	X	1.8	1.8	1.8	1.8	1.7
	SD	0.1	0.1	0.1	0.1	0.1
WBC	X	6.31	5.78	5.05	5.29	5.73
($x10^3/\mu L$)	SD	2.11	1.51	1.32	1.31	1.70
RBC	X	5.25	5.05	4.97	5.01	5.21
($x10^3/\mu L$)	SD	0.46	0.46	0.53	0.43	0.63
Haemoglobin	X	16.1	15.2	14.9	15.8	15.9
(g/dL)	SD	1.1	0.9	1.2	1.1	1.1
Fibrinogen	X	2.82	3.53	2.88	2.68	2.92
(g/L)	SD	0.9	1.19	0.69	0.84	1.29
Albumin/	X	17.06	16.69	16.15	18.81	19.23
Fibrinogen	SD	4.87	5.27	4.36	7.6	7.22

(X = mean; SD = standard deviation; Means with the same symbol differ significantly from each other with a $p < 0.05$ – paired t-tests; TP = serum-Total Protein WBC = White Blood Count; RBC = Red Blood Count)

4.3.2 The acetate/propionate/butyrate Group

4.3.2.1 Changes in anthropometrical measurements of the acetate/propionate/butyrate-supplement group (See Table 4.9)

No significant differences in any of the anthropometrical variables were measured after supplementation with a combination of acetate, propionate and butyrate.

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Table 4.9 Changes in the anthropometrical measurements of the acetate/propionate/butyrate-supplement group

VARIABLE		Day 0	Day 8	Day 22	Day 36	Day 43
Height	**X**	1.69				
(cm)	**SD**	0.10				
Weight	**X**	65.1	66.1	65.6	65.7	66.6
(kg)	**SD**	7.4	8.2	8.1	7.8	7.4
BMI	**X**	22.5	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**	4.4	5.1	5.1	4.7	4.8
SBP	**X**	120	116	117	116	118
(mmHg)	**SD**	10	12	7	8	8
DBP	**X**	80	81	79	79	83
(mmHg)	**SD**	9	9	7	8	9

(X = mean; SD = standard deviation; means with the same symbol differ significantly from each other with a $p < 0.05$ – paired t-tests; BMI = Body Mass Index; SBP = Systolic Blood Pressure; DBP = Diastolic Blood Pressure)

4.3.2.2 Changes in the lipid profiles of the acetate/propionate butyrate-supplement group (see Table 4.10)

The acetate/propionate/butyrate-supplement caused a decrease in serum-TC from 4.95 ± 0.81 mmol/L to 4.57 ± 0.87 mmol/L (p=0.088) after 4 weeks of supplementation. However, this decrease was not significant. A significant decrease in serum LDL-C was measured from baseline to the end of the supplementation phase (from 3.10 ±

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0.78 mmol/L to 2.61 ± 0.94 mmol/L; p =0.001). There was also a significant increase in the %HDL-C after the four weeks of supplementation (p=0.017).

It is interesting to note the expected significant decrease in NEFA levels from day 8 (baseline 2) to the end of the supplementation phase (from 0.385 ± 0.185 mmol/L to 0.287 ± 0.146 mmol/L; p = 0.020). No other significant changes in any of the lipid variables in this group were observed.

Table 4.10 Changes in the lipid profiles of the acetate/propionate/butyrate - supplement group

VARIABLE		Day 0	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*	34.90
(%)	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 ; Means with the same symbol differ significantly from each other with a $p < 0.05$ – paired t-tests ; 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)

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4.3.2.3 Changes in the glycometabolic indicators of the acetate/propionate/butyrate-supplement group (see Table 4.11)

The mean fasting serum-glucose levels decreased significantly during supplementation (from day 8 to day 36; p=0.028). The circulating serum-insulin levels also showed a concomitant decrease during the supplementation (p<0.001).

Table 4.11 Changes in the glycometabolic indicators of the acetate/propionate/butyrate-supplement group

VARIABLE		Day 0	Day 8	Day 22	Day 36	Day 43
Glucose	**X**	5.43	5.41*	5.46	4.96*	5.72
(mmol/L)	**SD**	1.37	0.52	0.96	0.85	0.73
Insulin	**X**	6.70	5.80*	10.90	1.70*	15.80
(µIU/mL)	**SD**	3.60	0.77	4.90	0.36	9.90

(X = mean; SD = standard deviation; Means with the same symbol differ significantly from each other with a $p < 0.05$ – paired t-tests

4.3.2.4 Changes in the other metabolic variables of the acetate/propionate/butyrate-supplement group (See Table 4.12)

Both the serum-TP (from 81.1 ± 6.2 g/L at day 8 to 75.9 ±8.4 g/L at day 36; p=0.001) and the serum-albumin levels (from 45.3 ± 2.5 g/L to 43.3 ± 2.9 g/L; p=0.020) of this subject group decreased

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significantly from both baselines to the end of the supplementation period. However, these values remained within the normal range.

There were no significant changes in any of the blood cell counts during the study.

Table 4.12 Changes in other metabolic variables of the acetate/propionate/butyrate-supplement group

VARIABLE		Day 0	Day 8	Day 22	Day 36	Day 43
TP	X	81.90*	81.10#	79.70	75.90*#	78.20
(g/L)	SD	6.50	6.20	6.40	8.40	5.20
Albumin	X	46.80*	45.30#	44.70	43.80*#	45.40
(g/L)	SD	4.70	4.40	3.90	3.10	2.40
TP/Albumin	X	1.87*	1.79#	1.79	1.73*#	1.72
	SD	0.49	0.11	0.09	0.14	0.12
WBC	X	5.96	5.77	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	5.22	5.17	5.32	5.23
($x10^3/\mu L$)	SD	0.65	0.63	0.49	0.54	0.55
Haemoglobin	X	16.2*	16.1	15.5	15.7*	15.6
(g/dL)	SD	1.2	1.9	1.3	1.2	1.5
Fibrinogen	X	2.73	2.98	2.58	2.94	2.60
(g/L)	SD	0.68	0.95	0.61	0.97	0.79
Albumin/	X	17.8	17.4	17.9	16.8	20.3
Fibrinogen	SD	5.7	5.9	3.8	6.5	8.9

(X = mean; SD = standard deviation; means with the same symbol differ significantly from each other with a $p < 0.05$ – paired t-tests; TP = serum-Total Protein; WBC = White Blood Count RBC = Red Blood Count)

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4.3.3 The acetate/propionate Group

4.3.3.1 Changes in the anthropometrical measurements of the acetate/propionate-supplement group (See Table 4.13)

No significant changes were observed in any of the anthropometrical measurements of the acetate/propionate-supplement group over the duration of the study.

Table 4.13 Changes in anthropometrical measurements of the acetate/propionate supplement group

VARIABLE		Day 0	Day 8	Day 22	Day 36	Day 43
Height (cm)	**X**	170.00				
	SD	0.10				
Weight (kg)	**X**	65.70	66.40	67.20	66.50	67.60
	SD	8.40	8.50	8.50	8.80	7.70
BMI (kg/m^2)	**X**	22.70	22.90	23.10	22.90	23.10
	SD	2.30	2.30	2.50	2.30	2.10
Waist (cm)	**X**	77.50	77.00	76.70	76.40	78.50
	SD	6.60	7.10	9.00	7.20	7.20
Hip (cm)	**X**	95.00	94.40	94.10	94.40	95.70
	SD	4.30	4.00	8.10	4.30	4.80
SBP (mmHg)	**X**	115	115	118	116	116
	SD	7	9	9	8	6
DBP (mmHg)	**X**	81	80	80	82	81
	SD	8	8	10	6	6

(X = mean; SD = standard deviation; means with the same symbol differ significantly from each other with a $p < 0.05$ – paired t-tests; BMI = Body Mass Index; SBP = Systolic Blood Pressure; DBP = Diastolic Blood Pressure

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4.3.3 The acetate/propionate Group

4.3.3.1 Changes in the anthropometrical measurements of the acetate/propionate-supplement group (See Table 4.13)

No significant changes were observed in any of the anthropometrical measurements of the acetate/propionate-supplement group over the duration of the study.

Table 4.13 Changes in anthropometrical measurements of the acetate/propionate supplement group

VARIABLE		Day 0	Day 8	Day 22	Day 36	Day 43
Height (cm)	X SD	170.00 0.10				
Weight (kg)	X SD	65.70 8.40	66.40 8.50	67.20 8.50	66.50 8.80	67.60 7.70
BMI (kg/m^2)	X SD	22.70 2.30	22.90 2.30	23.10 2.50	22.90 2.30	23.10 2.10
Waist (cm)	X SD	77.50 6.60	77.00 7.10	76.70 9.00	76.40 7.20	78.50 7.20
Hip (cm)	X SD	95.00 4.30	94.40 4.00	94.10 8.10	94.40 4.30	95.70 4.80
SBP (mmHg)	X SD	115 7	115 9	118 9	116 8	116 6
DBP (mmHg)	X SD	81 8	80 8	80 10	82 6	81 6

(X = mean; SD = standard deviation; means with the same symbol differ significantly from each other with a $p < 0.05$ – paired t-tests; BMI = Body Mass Index; SBP = Systolic Blood Pressure; DBP = Diastolic Blood Pressure

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4.3.3.2 Changes in the lipid profiles of the acetate/propionate-supplement group (See Table 4.14)

There was no measured change in the serum-TC levels of this group from day 8 (baseline) to the end of supplementation. It is interesting to note the significant (p=0.044) increase in HDL-C levels from 1.21 ± 0.24 mmol/L ± to 1.35 ± 0.34 mmol/L from day 8 (baseline 2) to the end of the supplementation. Furthermore, there was an expected significant decrease in the NEFA concentration of this group after supplementation with the acetate/propionate mixture (p=0.023).

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Table 4.14 Changes in the lipid profiles of the acetate/propionate supplement group

VARIABLE		Day 0	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 ; means with the same symbol differ significantly from each other with a $p < 0.05$ – paired t-tests ; 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)

4.3.3.3 Changes in glycometabolic indicators of the acetate/propionate-supplement group (See Table 4.15)

The fasting serum-glucose levels of this group decreased significantly from baseline 2 to the end of supplementation (from 6.65 ± 0.98 mmol/L at baseline 2 to 5.65 ± 0.24 mmol/L, p=0.001).

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Table 4.15 Changes in glycometabolic indicators of the acetate/propionate-supplement group

VARIABLE		Day 0	Day 8	Day 22	Day 36	Day 43
Glucose (mmol/L)	**X**	5.87	6.65*	5.77	5.65*	5.03
	SD	0.60	0.98	0.94	0.42	0.53
Insulin (µIU/mL)	**X**	7.90	12.62	7.10	14.01	13.80
	SD	4.60	22.61	5.50	24.83	9.70

(X = mean; SD = standard deviation; means with the same symbol differ significantly from each other with a $p < 0.05$ – paired t-tests)

4.3.3.4 Changes of other metabolic variables of the acetate/propionate-supplement group (See Table 4.16)

Serum-albumin levels increased significantly from day 8 to the end of supplementation ($p=0.005$). A significant decrease from baseline (both baseline 1 and 2) to the end of the experimental phase in the ratio of TP to Albumin was therefore also observed ($p=0.006$). The mean Red Blood Cell Count of this group of subjects also increased significantly from 5.05 ± 0.57 x 10^3/µL at day 8, to 5.24 ± 0.6 x 10^3/µL at the end of supplementation ($p=0.050$).

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Table 4.16 Other metabolic variables of the acetate/propionate supplement group

VARIABLE		Day 0	Day 8	Day 22	Day 36	Day 43
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
TP/Albumin	X	1.84*	1.80#	1.79	1.74*#	1.78
	SD	0.20	0.16	0.14	0.18	0.19
WBC	X	5.65	5.43	6.17	5.11	5.41
($x10^3/\mu L$)	SD	1.57	1.6	2.14	1.58	1.69
RBC	X	4.91*	5.05#	5.11	5.24#	5.16
($x10^3/\mu L$)	SD	0.38	0.57	0.50	0.60	0.77
Haemoglobin	X	15.5	15.9	16.1	16.2	15.8
(g/dL)	SD	0.9	1.3	1.6	1.4	1.7
Fibrinogen	X	2.58	3.36	3.05	2.85	2.72
(g/L)	SD	0.55	1.55	1.18	0.84	0.81
Albumin/	X	18.0	15.6	17.0	16.5	16.2
Fibrinogen	SD	3.7	6.2	4.9	4.7	5.0

(X = mean; SD = standard deviation; means with the same symbol differ significantly from each other with a $p < 0.05$ – paired t-tests; TP = serum-Total Protein; WBC = White Blood Count; RBC = Red Blood Count)

4.4 A COMPARISON BETWEEN THE EFFECTS ON THE DIFFERENT SUPPLEMENT GROUPS (See table 4.16)

The comparisons between the changes from baseline to the end of supplementation within the different variables between the three different supplement groups are presented in Table 4.6. Only the

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most important differences between changes within the different supplementation groups relevant to this study will be highlighted in this section.

There was a small increase in BMI of the placebo group from baseline to the end of supplementation, that differed significantly from the change in BMI in both the acetate/propionate/butyrate and the acetate/ propionate supplemented groups.

The increase in serum-TC levels within the acetate/propionate supplemented group from the baseline to the end of supplementation was significantly greater when compared to the decrease in serum-TC levels of the acetate/propionate/butyrate supplemented group for the same time period.

The increase in HDL-C levels and %HDL-C from baseline to end of supplementation of both the acetate/propionate/butyrate and the acetate/propionate supplemented groups differed significantly from the change in HDL-C levels within the placebo group for the same period of time. However, the increase in the %HDL-C in the acetate/propionate/butyrate supplemented group was significantly greater when compared to the increase in %HDL-C in the acetate/propionate supplemented group.

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The decrease in LDL-C levels of the acetate/propionate/butyrate supplemented group during the experimental phase was significantly greater when compared to the change in LDL-C levels in both the placebo and the acetate/propionate supplemented groups.

The measured decrease in NEFA levels of both the experimental groups differed significantly from the increase in NEFA levels of the placebo group during the experimental phase of the study.

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Table 4.17 Comparisons between mean changes in measured variables from day 8 to the end of supplementation (day 36) within the three different supplement groups

VARIABLE	Placebo Supplement	Acetate/Propionate/ Butyrate Supplement	Acetate/ Propionate Supplement
Weight (kg)	0.0	-0.4	0.1
BMI (kg/m^2)	0.2	-0.1*	0.0#
Waist (cm)	-1.80	0.10	-0.60
Hip (cm)	0.60	0.10*	0.00
SBP (mmHg)	-8	0	1
DBP (mmHg)	-4	-2	2
TC (mmol/L)	-0.01	-0.38	0.11#
TG (mmol/L)	0.00	0.10	0.08*
HDL-C (mmol/L)	0.00	0.19*	0.14*
LDL-C (mmol/L)	-0.00	-0.49*	-0.06#
%HDL-C	2.2	6.0*	3.0*#
NEFA (mmol/L)	1.38	-0.71*	-0.88*
Glucose (mmol/L)	-0.50	-0.45	-1.00
Insulin (µlU/mL)	-1.00	-4.10	1.39
TP (g/L)	-2.8	-5.2*	-1.1*#
Albumin (g/L)	-1.2	-1.5	2.6*#
TP/Albumin	0.00	-0.06	-0.06
WBC (x10^3/µL)	-0.49	0.11*	-0.32
RBC (x10^3/µL)	-0.04	0.10*	0.19*
Haemoglobin.(g/dL)	0.6	-0.4	0.3
Fibrinogen.(g/dL)	-0.85	-0.04	-0.51
Albumin/Fibrinogen	2.1	-0.6	0.9#

* mean changes differ significantly from the mean changes within the placebo group ($p<0.05$); #mean changes differ significantly from the mean changes within the acetate/propionate/butyrate supplement group ($p<0.05$). A negative (-) prefix denotes a decrease in measurement

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4.5 SUMMARY

The subject group used in this study was of a very homogenous nature due to the strict inclusion and exclusion criteria that were adhered to. All the subjects were male, and between the ages of 18 and 45 years. The study group followed exactly the same diet that was compiled by a registered dietician. No overweight or obese subjects were included in this study. All measured anthropometric characteristics and blood pressure mirrored a healthy physical status. The serum-TC of the group as a whole was 4.46 ±0.84 mmol/L with the serum-HDL-C and -LDL-C, 1.24±0.28 mmol/L and 2.67±0.78 mmol/L, respectively. None of the other measured metabolic variables were abnormal or indicative of any present abnormalities.

Blood was analysed after two and four weeks of supplementation with two different combinations of short-chain fatty acids and a placebo, in order to assess whether these fatty acids had had any metabolic effects that could be to the benefit of the subjects. The effect of the different supplements will mainly focus on changes in any of the measured lipid profiles.

No significant changes were measured in any of the lipid fractions, including the total cholesterol of the placebo group. The acetate/propionate/butyrate supplement caused a decrease, albeit

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not significant, in serum-TC from 4.95 ± 0.81 to 4.57 mmol/L ± 0.87 and HDL-C from 1.27 ± 0.28 to 1.46 ± 0.66 mmol/L. These two effects are mentioned because they are directly linked to the %HDL-C which increased significantly from 26.3 ± 6.5% to 32.3 ± 12.4%. The serum-LDL also decreased significantly from 3.10 ± 0.78 to 2.61 ± 0.94 mmol/L.

The combination of acetate and propionate caused no changes in the serum-TC levels of the subjects. However, a small significant increase in the serum-HDL (from 1.21 ± 0.24 to 1.35 ± 0.34 mmol/L) was measured. The supplement had no effect on the serum-LDL-C levels.

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CHAPTER 5
DISCUSSION

5.1 INTRODUCTION

The main aim of this study was to determine the possible effect of different combinations of SCFAs on the lipid profiles of westernised black men. By doing so, it is believed that the attributable risk contributed by high serum-cholesterol levels in this population group could be decreased. Two different combinations of SCFAs and a placebo supplement were used in order to determine whether the specific combinations of SCFAs would have any effect on the lipid profiles of the respective study groups. Other more general metabolic indicators, as well as the anthropometric status and the blood pressure of the subjects were also monitored.

In this chapter, the main changes from the reference baseline (Day 8) to the end of the supplementation phase (Day 36) in the three different supplementation groups will be discussed. The results of this study will also be compared with the data of other similar studies.

5.2 BASELINE CHARACTERISTICS OF THE GROUP AS A WHOLE

5.2.1 Demographic characteristics of the study group as a whole

The study group was very homogenous. This could mainly be ascribed to the strict inclusion criteria adhered to during the recruitment of

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volunteers. Recruited subjects had healthy physical dimensions (body weight, etc.). All subjects were living on the same military base, where they received the same controlled westernised diet at a central mess hall, and also participated in exactly the same physical activities. In addition, subjects with a history of previous or current cardiovascular events, diabetes, hyper- or hypotension were excluded from the study. Subjects with a history of familial diseases were also excluded from the study. It is therefore evident that the group was perfect to use for intervention purposes, due to the strict control that could be assured throughout the study.

5.2.2 Anthropometric measurements of the study group as a whole

The Body Mass Index (BMI) of the group as a whole fell within the estimated limits of the normal reference range for healthy adult males (18.5 to 24.9 kg/m^2, Tietz 1998). All other anthropometric dimensions of the group as a whole supported this outcome.

5.2.3 Lipid profiles of the study group as a whole

Serum total-cholesterol (TC) levels are directly, and high-density lipoprotein cholesterol (HDL-C) levels inversely, related to CHD (Kannel and Wilson, 1995). Subjects with healthy lipid profiles were recruited for this study. However, individuals with cholesterol levels in the higher range of the normal were recruited in order to ensure that it

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would still be possible to detect the hypothesised beneficial cholesterol-lowering effect of the SCFA supplements. Serum lipid fractions were measured to quantify the possible effects of the different combinations of supplements.

Mollentze **et al**. (1995) reported mean serum-TC levels of between 4.7 ± 1.2 mmol/L for black men in the age category 25-34 years living in Mangaung. Most of our subjects were from Mangaung. The authors also reported that the number of subjects in the moderate-risk hypercholesterolaemic category in this population is disturbing. An outstanding feature was that 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, who may be adopting a Western diet to a greater extent than their elders (Mollentze **et al**., 1995). However, serum-TC 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 Triglycerides (TG). The measured concentrations of these combined fractions predicted that the individuals used for this study were not at immediate high risk of cardiovascular events (Steyn **et al.,** 1991; Seedat **et al.,** 1992; Mollentze **et al.**, 1995; Slabber **et al.,** 1997). However, the lipid profiles do suggest that a current improvement in cardiovascular risk profiles is

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possible, and that it would be to the benefit of the subjects' health to sustain such an improvement when they grow older.

5.2.4 Glycometabolic indicators of the study group as a whole

The fasting serum-glucose and insulin levels of the subject group as a whole did not reflect the presence of any apparent glycometabolic disorders. Glucose levels of healthy individuals can fluctuate between 4.6 - 6.4 mmol/L (Painter et al., 1999). High glucose levels were reported for urban black populations in Durban, where the prevalence towards higher blood glucose levels (>7.9 mmol/L) is high (Seedat **et al.,** 1992). Fasting serum-glucose levels of between 4.7 ± 0.3 mmol/L were reported in a previous study conducted in our own laboratory using the same population source (De Wet, 1999). Insulin levels of a healthy individual should vary between 0-17 µIU/mL depending on the time of day and the state of fasting (Painter **et al**., 1999). Epidemiological studies report that insulin is also a risk factor for CHD (Kannel and Wilson, 1995). Furthermore, insulin is essential for the physiological regulation of HDL-C metabolism (Wolever **et al**., 2002).

It is suggested that the high level of physical activity, age and absence of obesity may benefit the subjects used in this study in terms of glycometabolic control.

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5.2.5 Other metabolic variables of the study group as a whole

A comprehensive profile of metabolic markers, all of which reflect the general health status of the individual, was analysed. Not all of these markers will be discussed, and only those of interest highlighted.

Albumin is acknowledged to be an important indicator of nutritional status and longevity (Carlson, 2004). The serum-albumin levels of this group fell within the normal reference range of between 35-52 g/L for healthy adult males (Painter **et al**., 1999), and it could therefore be said that the study population was apparently healthy. This claim is also supported by the other measured variables. The blood counts of both the Red and White Blood Cells fell within the normal range of 4.5-6.5 x 10^3/µlU and 4-11 x 10^3/µlU, respectively (Davidson 1991).

Plasma fibrinogen used as an acute phase marker showed that no infection or disease states which trigger an acute phase response were present throughout the study (Davidson 1991). The concentration of this protein fell within the normal reference range given for the specific population group (34-52 g/L; Painter **et al**., 1999). Plasma fibrinogen is also an accepted risk marker for cardiovascular events (Painter **et al**., 1999), however, it will not be discussed as such.

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5.3 CHANGES MEASURED DURING THE INTERVENTION PERIOD

5.3.1 Introduction

Other studies showed that the clinical effects of SCFA administration may be influenced by the method of administration (oral, rectal or intravenous). The capsules used for this study were coated with a thin layer of polymer. This prevented the acidity of the stomach from dissolving the capsules, and allowed enzymes in the small gut to digest them. This ensured that maximum distribution of the supplements took place within the colon, resembling the SCFA distribution when dietary fibre is fermented. Also, it seems that the frequency and concentration of the SCFA administered plays a significant role in the clinical manifestations of effects. Intravenous SCFA injections may cause acute acidosis at high concentrations, while rectal infusions of SCFAs may be influenced by the depth of administration. Capsulated SCFA supplements may therefore be a much safer and more trustworthy method of administration when compared to any other.

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5.3.2 The placebo group

5.3.2.1 Changes in the anthropometrical measurements of the placebo group

No significant changes in any of the anthropometric variables within the placebo group were measured during the study. It was not expected that the placebo supplement should cause any changes to the anthropometrical measurements of this group of individuals.

5.3.2.2 Changes in the lipid profiles of the placebo group

Serum-cholesterol levels of the placebo group did not show any changes from baseline to the end of the experimental phase. No other changes were measured in any of the cholesterol fractions.

5.3.2.3 Changes in glycometabolic indicators of the placebo group.

No significant differences in any of the glycometabolic parameters of the placebo group were measured between the visits. It was not expected that the placebo should introduce any glycometabolic changes in the study group.

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5.3.2.3 Changes in other metabolic variables of the placebo group

None of the metabolic variables within the placebo group showed any significant changes from baseline to the end of the study.

5.3.3 The acetate/ propionate/ butyrate group

5.3.3.1 Changes in the anthropometrical measurements of the acetate/propionate/butyrate-supplement group

No significant differences in any of the anthropometrical variables were measured. The tendency towards a decrease in group two of the BMI was not statistically significant.

5.3.3.2 Changes in the lipid profiles of the acetate/propionate/butyrate-supplement group

The acetate/propionate/butyrate-supplement caused a non-significant decrease in the serum TC of approximately 8% after four weeks of supplementation. This difference, however, is non-significant in statistical terms. In contrast, the serum-LDL-C levels significantly decreased (-16%) during the same period. It is evident that the small decrease in TC may be due to the decrease in LDL-C levels. Expressed as a fraction (percentage) of the TC, LDL-C decreased from 64% (day8) to 57% (day 36). The combination of fatty acids also

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caused a significant increase in %HDL-C levels. This increase is beneficial and may be exploited in future studies.

Numerous 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 **et al.,** 1984; Nishina and Freeland, 1990; Wright **et al.,** 1990).

Consumption of propionate reduces serum cholesterol in both animal and human models (Thacher **et al.,** 1981; Illman **et al.,** 1988; Stephan 1994). However, very little information is available where both the short-chain fatty acids acetate and propionate were given in combination.

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. NEFA concentration in this group decreased significantly during supplementation. A decrease in NEFA levels is associated with an increase in short-chain fatty acid concentrations and therefore serves as an indication that the SCFAs within the supplements were absorbed, and reached the peripheral circulation (Scheppach **et al.,** 1988; Akanji **et al.**, 1991; Veldman, 1996).

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Acetate derived from colonic fermentation could also be a substrate for hepatic lipid synthesis. The first step in acetate metabolism is its activation to acetyl-CoA by the enzyme acetyl-CoA synthetase, which is variably distributed in the cytosol and mitochondria of many tissues (Brockman, 1982; Scheppach **et al**., 1988). Acetyl-CoA, in turn, is the primary substrate for *de novo* cholesterol and triglyceride synthesis (McBurney and Thompson 1989; McBurney **et al**., 1988). However, addition of propionate to acetate supplements is reported to inhibit the incorporation of acetate into plasma triglycerides (Wolever **et al.,** 1995).

Very little information is available on the effect of butyrate on cholesterol metabolism in human subjects (Topping and Pant, 1995).

5.3.3.3 Changes in the glycometabolic indicators of the acetate/propionate/butyrate-supplement group

The combination of acetate, propionate and butyrate caused a statistically significant decrease in serum-glucose levels. A concomitant decrease in circulating insulin levels was also measured. This concomitant change is beneficial, and implies that the body requires less insulin to keep the glucose levels in circulation at a constant concentration. The results suggest that the supplement improves insulin resistance. A possible pathway to explore would be to investigate the possibility that the supplement increases the

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number of insulin receptors on the surface of the cell membrane. These receptors, once activated by insulin, enhance the uptake of glucose by the cells for energy production (Meisenberg en Simmons, 1998). In the study by De Wet (1999) in our own laboratory, a combination of three SCFAs (acetate, propionate and butyrate) also significantly decreased serum-glucose levels. Alamowitch **et al.** (1996) on the other hand, found that the acute ileal perfusion of the combination of acetate, propionate and butyrate did not significantly reduce serum-glucose levels in human subjects.

5.3.3.4 Other metabolic variables of the acetate/propionate/butyrate-supplement group

Both the serum-TP and albumin of this group showed a significant decrease from baseline to the end of the supplementation phase. Generally, a decrease in circulating albumin levels is not favourable. However, the decrease in the serum-TP levels were much greater compared to that of the albumin. The ratio of TP to albumin therefore decreased, which, in turn, is favourable. This change indicates that the fraction of the total protein comprised of albumin was greater at the end of the study, compared to the measured fraction at the reference baseline. As already mentioned, serum albumin is a marker for both longevity and nutritional status (Lindsey, 1996). It is important to note that even after a significant decrease, the concentration of both total protein and serum-albumin still fell within

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the normal reference range for healthy male adults (Painter **et al.**, 1999). No published data could be found regarding any changes in the albumin and/or total protein levels recorded after short-chain fatty acid supplementation.

A decrease in the haemoglobin levels in this group was observed. Veldman **et al.** (1997) also reported a decrease in haemoglobin levels after 4 weeks of supplementation with pectin (a soluble dietary fibre) using hypercholesterolaemic males as subjects. Similar results were also published by the same author after 4 weeks of supplementation with an enteric coated acetate supplement (Veldman **et al.**, 1999).

No other significant changes were measured in any of the other metabolic variables.

5.3.4 The acetate/propionate group

5.3.4.1 Changes in the anthropometrical measurements of the acetate/propionate-supplement group

There was no significant change in any of the anthropometrical measurements of the acetate/ propionate supplemented group over the duration of the study.

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5.3.4.2 Changes in the lipid profiles of the acetate/propionate-supplement group

Previous rectal infusion studies performed by Wolever **et al.,** (1991 and 1995) showed that colonic acetate was incorporated into serum cholesterol and triglycerides, and acutely raised the concentration of serum lipids, and that these effects are blocked by propionate. In this study group (receiving a combination of acetate and propionate salts) a beneficial statistically significant increase of the serum HDL-C was observed, without any changes in the other lipid fractions. A sodium propionate supplement also increased HDL-C and serum-TG levels in a study performed by Venter **et al.** (1989). The authors hypothesise that this effect may, in part, be mediated through the effects of propionate on hepatic carbohydrate metabolism.

Wolever **et al.** (1996) reported that the ratio of acetate to propionate in the peripheral serum of fasting normolidaemic males (n=62) were significantly related to the serum-TC (r=0.466; p= 0.0002) and LDL-C (r=0.384; p=0.0023). The authors also suggest that either increased acetate production relative to propionate concentration, or reduced propionate production relative to acetate concentration, increases cholesterol production.

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

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associated with an increase in short-chain fatty acid concentrations and therefore serves as an indication that the SCFAs within the supplements were absorbed and had reached the peripheral circulation (Crouse **et al.,** 1978; Scheppach **et al.,** 1988; Akanji **et al.**, 1991; Veldman, 1996).

5.3.4.3 Changes in the glycometabolic indicators of the acetate/propionate-supplement group

A significant decrease in the fasting serum-glucose levels was measured from baseline to end of supplementation with a mixture of acetate/propionate. In contrast with the acetate/propionate and butyrate supplement, no change in the serum-insulin concentrations was measured. Akanji **et al.** (1991) demonstrated a rise in acetate production and a decrease in fasting serum-glucose levels associated with a high fibre diet given to diabetic subjects. It is suggested that the blood glucose lowering effect of soluble dietary fibres may be related, in part, to short-chain fatty acids (SCFAs) generated during their fermentation (Thacker **et al.**, 1981). However, the physical characteristics of the fibre itself, plays a role as well. The expected effects of SCFAs on glucose metabolism are not clear-cut. Acetate could reduce blood glucose levels in the long-term by reducing NEFA concentrations (Crouse **et al.**, 1968), but propionate may have the opposite effect, being a gluconeogenic substrate in ruminants and

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horses (Judson **et al.**, 1968). In contradiction, Venter **et al.** (1989) found that a propionate supplement significantly decreased the fasting glucose levels as well as the maximum insulin increments during glucose tolerance tests. The authors suggest that the known beneficial effects of dietary fibre may in part be mediated through the effects of propionate on hepatic carbohydrate metabolism.

Wolever **et al.** (1991) found that a rectal infusion of acetate had no effect on blood glucose levels. However, a rectal infusion of propionate caused a significant increase in the measured serum-glucose levels in human subjects. A combination of these acids in the ratio of 3 parts acetate to 1 part propionate also had no effect on the serum-glucose levels. However, it caused a significant decrease in serum-insulin levels. It is important to remember that these effects were measured within 30 minutes after infusion of the fatty acids, thus excluding the effect of long-term use. A direct comparison between the results of this study, and those reported by Wolever **et al.** (1991), would therefore be impossible.

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5.3.4.4 Changes in the other metabolic variables of the acetate/propionate-supplement group

A significant increase in the serum-albumin concentration was observed from baseline to the end of the experimental supplementation. Albumin is a health-promoting protein and exerts beneficial effects within the framework of human pathology (Johnson **et al.**, 1999).

The observed statistically significant increase in the RBC count is clinically of no significance (Painter **et al.**, 1999). This increase will not be discussed, but needs further investigation. No other changes in any of the measured metabolic variables were observed in this group.

5.4 A COMPARISON BETWEEN THE EFFECTS OF THE DIFFERENT SUPPLEMENT GROUPS

Results from this study show that there was a definite and clear difference in response to the different SCFA mixtures. This section will be used to identify these differences. In order to explain these differences, it will also be necessary to focus on the different contents

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of the two experimental supplements, in order to differentiate between possible mechanisms involved during their metabolic actions. Only those differences that are of clinical significance will be discussed. The discussion will, furthermore, be limited to those markers relevant to the topic of this study. It should also be noted that very little literature is available on the long-term use of short-chain fatty acids, and most comparisons would make use of available data obtained by means of short-term human and *in vitro* studies. Also, most studies applied either rectal infusions or intravenous injections of SCFAs, which differs to 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). Another characteristic that could provoke different physiological responses to supplementation could also be ascribed to the exact consistency of the SCFA salts used during the study, whether it is the sodium, calcium or potassium salt, or even the free base of the acid. Also, the specific characteristics of each salt could influence the bioavailability of the supplement. All these aspects are important to consider when comparisons are made to other published data, and should be kept in mind when reading the following paragraphs.

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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 a mixture of acetate and propionate only, in a ratio of 50% acetate and 50% propionate. This implies that differences in metabolic response to the two different supplements could be ascribed to the following mechanism:

- Both the ratio of acetate to propionate, as well as the absence or presence of butyrate, which may each evoke a different physiological response.

The results from this study show that the oral consumption of acetate, propionate and butyrate has a definite influence on the cholesterol metabolism of apparently healthy male volunteers. These results are supported by other authors (Cummings and Macfairlane, 1991). It has long been hypothesised that SCFAs produced during colonic fermentation of dietary fibre may mediate, at least in part, the serum cholesterol-lowering effect associated with the intake of soluble fibre (Wolever **et al.,** 1996). The precise metabolic mechanism of the action of SCFAs in reducing plasma cholesterol concentrations, specifically LDL-C, is unknown (Wolever **et al.,** 1995).

In previous studies, interest has focused largely on the potential inhibition of cholesterol synthesis by acetate and propionate (Beynen

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et al., 1982; Illman **et al.**, 1988; Wright **et al.**, 1990; Venter **et al.**, 1990).

It was proposed by Wolever **et al.,** (1995) that acetate was used as a substrate for cholesterol synthesis and that it was inhibited by the presence of propionate. After an infusion of a ratio of acetate to propionate of 2:1 they reported that acetate was incorporated in cholesterol and triglycerides, and that propionate inhibited the incorporation of acetate into fatty acids due to the inhibition of acetyl –CoA. The outcome of the Wolever **et al.** (1995) study suggests that a higher intake of propionate may then have a more beneficial effect on the lipid profiles of human subjects. In our study the supplement containing acetate and propionate in a ratio of 1:1 had a small, albeit favourable, effect on the lipid profiles of the subject group. The acetate/propionate/butyrate (in a ratio of 7:1.5:1.5) supplement had an even more favourable effect on lipid metabolism when compared to the acetate and propionate supplement. A small decrease in the TC, with a significantly greater increase in the HDL-C fraction and a significantly greater decrease in the LDL-C fraction, was measured when compared to the acetate and propionate supplement. This suggests that, at least in males, either increased acetate production relative to propionate production, or reduced propionate production relative to acetate production, increases serum cholesterol concentrations (Wolever **et al.,** 1996). SCFAs produced during fermentation do not exist in isolation, and the proportion of acetate produced is always greater when compared to that of propionate

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(Mortensen **et al.**, 1988). The production of butyrate during the fermentation of dietary fibre may also have additional beneficial metabolic effects. However, Wolever **et al.** (1996) reported that there was no definite relationship between butyrate and lipid fractions in the fasting state.

Furthermore, the clinical non-significant differences between the changes within the different groups could possibly be attributed to the short duration of the study. Wolever **et al.** (2002) proposed a three to six month adaptation period for SCFAs to show a positive relation to the lipid profile of Type II diabetes patients, and they concluded that it may take many months for the changes in serum SCFAs to produce significant metabolic changes; and that serum acetate was significantly related to the long term adaptive changes in blood lipids. Also, in support of this, our study show there is a tendency towards body weight changes, and that these changes may, in part, be related to the changes in the different lipid fractions. However, 4 weeks of supplementation may not be adequate to show this relationship, and therefore requires further investigation.

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CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 INTRODUCTION

Few relationships are as well established as that between total serum cholesterol levels (TC) and the risk of coronary heart disease (CHD) (Willet, 1998). Serum-TC as an entity is not a stand-alone prediction of CHD. The total serum-cholesterol level is represented by several subcomponents, including the deleterious low-density lipoprotein (LDL) and very low density lipoprotein (VLDL) fractions, as well as the beneficial high-density lipoprotein (HDL) component. Furthermore, it is widely acknowledged that there exists a direct or indirect association between serum cholesterol and some lifestyle factors, especially dietary intake. Abundant data since World War II indicates that the dietary risk related to CHD is preventable. A variety of research studies has shown that dietary intervention could promote a healthy cholesterol profile. The use of soluble dietary to lower the LDL-C component of serum-TC in individuals with secondary hypercholesterolaemia has previously been published. The effect of a specific dietary component might therefore increase, decrease, or have no effect on the risk of CHD, depending on the cholesterol-specific component influenced (Willet, 1998). This may hold important therapeutic implications. Hopefully, results from this study will provide a foundation to investigate further possible ways in which the rising incidence of

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cardiovascular disease in the Black South African population could be decreased or prevented.

6.2 CONCLUSION

This study was used to investigate the possible effect of 28 days' exposure to two different combinations of short-chain fatty acids (SCFAs) orally administered in a group of local westernised Black men. The concentration of SCFAs used was equivalent to that generated by the fermentation of 15g of mixed fibre. The specific combinations of SCFAs gives further insight into the metabolic changes associated with the production and absorption of the respective fermentation products of dietary fibre.

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. It is believed that the increase of colonic acetate will have a positive effect on the lipid profile. Differences in metabolic response to the two different supplements could be ascribed to the following mechanism:

- The ratio of acetate to propionate, as well as the absence or presence of butyrate which may each evoke a collaborative physiological response.

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From the results it is evident that the inclusion of butyrate to the supplement was more beneficial in terms of lipid-lowering effects. A significant decrease in LDL-C concentration, as well as a significant increase in the ratio of HDL-C to the total cholesterol concentration, compared to only a small statistically significant increase in HDL-C concentration itself in the acetate and propionate supplemented group, is indeed promising. These profile changes associated with the additional intake of butyrate reflects a decrease in risk for the future development of CHD.

The prudent low fat, high-fibre diet is regarded as one of the few controllable risk factors in the prevention of degenerative diseases and is, therefore, also effective in controlling known coronary risk factors (hyperinsulinaemia, hyperlipidaemia, hypertension, obesity, etc.) as well as raised clotting factors (Vorster **et al.**, 1988; Hubbard **et al.**, 1994; Vorster **et al.**, 1997). It is evident from this study that the production of short-chain fatty acids as a by-product from microbial fermentation, does contribute, in part, to the beneficial metabolic effects associated with the intake of dietary fibre. The results also show that the metabolic effects depend on different combinations of SCFAs produced, and is therefore also influenced by the intake of specific types of dietary fibre. However, it is very important to acknowledge that the use of SCFA supplements should not replace the intake of dietary fibre. The physical characteristics of dietary fibre itself also contribute to its metabolic effects. It can therefore be concluded

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that short-chain fatty acid supplements could be used to the benefit of those individuals suffering from lifestyle diseases; but that the most appropriate ratio of acetate, propionate and butyrate supplementation needs further research.

6.3 RECOMMENDATIONS

6.3.1 Further research

This study clearly underlines the need for further investigation. More clinical intervention trials are required to investigate the most beneficial combination of SCFAs on lipid profiles in human subjects. Also, it will be required that different target groups suffering from lifestyle associated diseases with abnormal lipid profiles be investigated, such as individuals with insulin resistance, obese individuals, etc.

The findings of the present study also indicate that further research is needed to evaluate the effect of long-term use of SCFA supplements. It is advisable that a period of at least 6 months of supplementation be used to evaluate the metabolic changes that take place, if any.

6.3.2 Dietary recommendations and possible clinical use

The results of this study show that the intake of SCFA supplements which include butyrate as ingredient have more beneficial effects on

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the lipid profiles in westernised Black men, when compared to supplements that only include acetate and propionate as ingredients. However, effects on the total and LDL-cholesterol were not large enough to market it as a lipid-lowering drug. This suggests that short-chain fatty acids may be used as a food supplement. However, the appropriate combination of fatty acids, and the most appropriate method of application, needs further investigation.

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APPENDIX 1

CONSENT FORM

THE EFFECTS OF SHORT-CHAIN FATTY ACIDS ON GLYCOMETABOLIC CONTROL IN 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.

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

Signed/confirmed at ..... on 2001

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

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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 ……...2001

…….. (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 ……...2000

…….. (signature) ……
Translator Witness

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Appendix 2

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: _____kg _

LENGTH: _____cm

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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 AND HOW MANY DAYS IN A WEEK OR WEEKEND DO YOU

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USE ALCOHOL:

DAILY

WEEKLY

MONTHLY

OTHER: _____

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

	Per day
Beer (how many bottles/cans	
Brandy/whisky (how many tots)	
Vodka/gin (how many tots)	
Sjerrie/sweetwine (how many glasses)	
Table wine (how many glases)	
Other, specify:	

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

	Per day
Beer (how many bottles/cans	
Brandy/whisky (how many tots)	
Vodka/gin (how many tots)	
Sjerrie/sweetwine (how many glasses)	
Table wine (how many glases)	
Other, specify:	

BLOOD TESTS

BLOOD CHOLESTEROL: _____

FASTING GLUCOSE: _____

PLASMA FIBRINOGEN: _____

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FOR OFFICE USE ONLY:

SUBJECT: APPROVED REJECTED

ASSIGNED SUBJECT NO:

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APPENDIX 3

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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

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- Do you use any dietary supplem 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?

- 1 Regularly (> 4 times a week) [ ] 25
- 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?

- 1. Yes [ ] 31
- 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

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Do you drink tea (except Rooibos)

- 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?

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

41

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

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On a weekend day when you do y drinks do you usually have ?

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	per day			
Beer (how many bottles/cans)				59-6
Brandy/whisky (how many tots)				61-6
Vodka/gin (how many tots)				63-6
Sjerrie/ sweetwine (how many glasses)				65-6
Table wine (how many glases)				67-6
Other, specify:				69-7

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 cigarettes did you smoke a day? _____ 76

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

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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

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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							

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FOOD	DESCI		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:							

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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	Fried: in batter/crumbs						3018	
chicken with the	Fried but not coated						2925	
skin?	Roasted/grilled - with skin						2925	
Yes ☐ No ☐	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	

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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:	"Vetderm" 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/	Soups						3157	
peas/lentils	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	

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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	

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FOOD	DESCRIPTION	AMOUNT USUALLY EATEN	TIMES EATEN				CODE	AMOUNT/DAY
			Per day	Per week	Per month	Seldom/never		
	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						3876	
	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	

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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/ Salad dressing	Mayonnaise: Bought						3488	
	home-made						3506	
	Cooked said 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	

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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							

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FOOD	DESCRIPTION	AMOUNT USUALLY EATEN	TIMES EATEN				CODE	AMOUNT/DAY
			Per day	Per week	Per month	Seldom/n ever		
	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	Sweetened						3981	
Coke, Fanta	Diet						3990	
Mageu/Motogo	Sorghum beer						4056	
Alcoholic beverages such as sorghum beer	Specify:						4039	
Other, specify:	Beer average						4031	
	Wine						4033	
	Cider						4057	

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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	

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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		

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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 !

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APPENDIX: 4

QUESTIONNAIRE FOR SUBJECTS WHO COMPLETED THE STUDY

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

Respondent number: _____

Surname and Initials: _____

Employee Number: _____

Date of Birth: _____

---

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

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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:

_____

_____

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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

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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.

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