The *in vitro* faecal evaluation of prebiotic effects of rooibos phenolic compounds on the gut microbiota of vervet monkeys (*Chlorocebus pygerythrus*)

By

Noluxabiso Mangwana

213301784

Dissertation submitted in fulfilment of the requirements for the degree

Master of Environmental Health

in the Faculty of

Applied Sciences

at the

Cape Peninsula University of Technology

Supervisor: Dr Richard Mundembe (CPUT)

Prof Christo Muller (SAMRC)

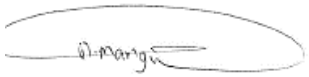
Co. Supervisor: Dr Ntevheleni Thovhogi (SAMRC)

Cape Town

March 2020

DECLARATION OF COPYRIGHT

I, Noluxabiso Mangwana, declare that the contents of this dissertation represent my own unaided work, and that the dissertation has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.



31 March 2020

Signature

Date

ABSTRACT

Background

The development of metabolic disease is accompanied by changes in gut microbiota phenotype, including a decrease of beneficial bacteria and increase of pernicious bacteria of the gastrointestinal tract. A Western (high-fat and high-sugar) diet, sedentary lifestyle and altered gut microbiota diversity have been associated with an increased risk of developing metabolic diseases such as type 2 diabetes and its associated risk factor, obesity. Many researchers have studied the link between the gut microbiota and diet. Hence our *in vitro* study is aimed at investigating the potential prebiotic effect of an aspalathin-rich unfermented rooibos extract, Afriplex GRT™ and aspalathin on the faecal bacterial diversity of vervet monkeys fed Western diet.

Methodology

A total of six vervet monkeys (Chlorocebus pygerythrus) were selected from monkeys fed either a maize based normal diet (standard diet group; n=3) or a high fat diet (Western diet group; n=3) for more than 5-years. Faecal samples were collected from the animals in both groups at the Primate Unit and Delft Animal Centre (PUDAC) between 7 – 9 AM. Faecal samples from the two groups were divided into culture-independent baseline samples (before culture) and culture-dependent samples (after anaerobic culture). The culture-dependent samples were cultured under anaerobic conditions at 37°C for 10 hours, with or without Afriplex GRT™ extract or aspalathin. Bacterial genomic DNA (gDNA) was extracted from all samples using the NucleoSpin® DNA Stool extraction kit. Purified gDNA was sent for metagenomic sequencing for 16S rRNA gene analysis of microbial diversity using an Ion Torrent Next-generation Sequencing platform.

Results

Results indicated that the Western diet affects the abundance of several bacterial species. Afriplex GRT™ and aspalathin significantly enhanced the relative abundance of health promoting butyrate-producing bacteria such as *Faecalibacterium prausnitzii* in both standard and Western diet groups ($p= 0.02$ and $p=0.04$, respectively). A similar trend was observed in other beneficial bacteria such as *Eubacterium spp*., *Sutterella spp*., and *Dorea longicatena*.

Conclusion

Based on the data observed, it can be suggested that Afriplex GRT™ has a beneficial prebiotic effect on gut microbiota diversity and gut health.

Keywords: Vervet monkey, Aspalathin, Rooibos, Afriplex GRT™, Gut microbiota and Western diet.

ACKNOWLEDMENTS

I would firstly like to thank God Almighty, '*Umveli nqanga ngokundigcina kwaze kwabalapha'.* Without Him, none of this would have been possible.

I would like to thank my family and friends for all the love, support, prayers and motivation. I would like to thank all my friends especially Farai Muzanenhamo for keeping me sane throughout this time. Miss Asive '*Marhadebe*' Myataza, thank you for being selfless and having a big heart, you always put other people ahead of yourself. Miss Tracey Jooste for all your technical assistance and always willing to listen to my boloney.

I would also like to thank the South African Medical Research Council (SAMRC), Biomedical Research and Innovation Platform (BRIP) and the Research Capacity Development for granting me the opportunity to pursue this project with them, and specifically to Prof Christo JF Muller for trusting me with this project.

A special thanks to my supervisors, Dr Ntevheleni Thovhogi, Prof Christo JF Muller and Dr Richard Mundembe, for sharing your knowledge and giving your expert advice to me, also for your support, motivation and encouragement.

Finally, I would like to thank myself for not giving up or giving in throughout the entire project.

The work herein was made possible through funding by the South African Medical Research Council through its Division of Research Capacity Development under the Internship Scholarship Programme.

DEDICATIONS

This dissertation is dedicated to my family both living and dead

To my gran ***Pinkie Mdidimba***

My mother ***Nonzame Mangwana*** *and my father* ***Robert Tshalisi***

THANK YOU FOR EVERYTHING

TABLE OF CONTENTS

DECLARATION OF COPYRIGHT ii

ABSTRACT iii

ACKNOWLEDMENTS iv

DEDICATIONS v

TABLE OF CONTENTS vi

LIST OF FIGURES xi

LIST OF TABLES xiii

GLOSSARY xiv

ABBREVIATIONS xvi

1. INTRODUCTION 1

1.1. RESEARCH BACKGROUND 2

1.2. RESEARCH PROBLEM 3

1.3. RESEARCH AIM AND OBJECTIVES 3

1.4. RESEARCH QUESTIONS 4

1.5. PURPOSE AND SIGNIFICANCE OF THE STUDY 4

1.6. DELINEATION OF THE STUDY 4

1.7. OVERVIEW OF DISSERTATION CHAPTERS 5

2. LITERATURE REVIEW 6

2.1. GUT MICROBIOTA 7

2.1.1. Gastrointestinal Track 8

2.1.2. Gut biogeography 10

2.1.3. Composition of gut microbiota 11

2.1.4. Enterotypes 13

2.1.5. Probiotic bacteria 15

2.1.6. Gut microbiota metabolites 15
2.1.7. Short chain fatty acids 16
2.1.8. The gut barrier function 19
2.1.9. Dysregulation of the gut microbiota 19
2.1.10. Effects of diet on gut microbiota regulation 20
2.1.11. Specific species related to gut dysbiosis 21
2.2. METABOLIC SYNDROME 25
2.2.1. Diabetes Mellitus 25
2.2.2. Obesity 26
2.2.3. Diabetes and Obesity association with gut microbiota alterations 27
2.3. ROOIBOS PHENOLIC COMPOUNDS 28
2.3.1. Rooibos plant 28
2.3.2. Bioactive properties of rooibos 29
2.3.3. Dietary polyphenols 30
2.3.4. Aspalathin 30
2.3.5. Afriplex Green Rooibos Extract® (GRT) 32
2.3.6. Bioavailability of aspalathin 32
2.4. USE OF NON-HUMAN PRIMATE IN RESEARCH 34
2.5. CULTURING SYSTEMS 36
2.5.1. Anaerobic chamber 36
2.5.2. Speedy Breedy system 37
2.6. METAGENOMICS 39
2.6.1. Types of metagenomic analysis 39
2.6.2. Ion torrent 40
2.7.3. Ion torrent principle 40
2.7.4. Ion torrent library preparation 40
3. METHODOLOGY 42

3.1. ANIMAL SELECTION 43
3.2. ANIMAL CARE AND ETHICS APPROVAL 43
3.3. SAMPLE COLLECTION AND HANDLING 45
3.4. SPEEDY BREEDY CULTURING 45
3.5. ANAEROBIC CHAMBER 45
3.6. TREATMENT CONCENTRATIONS 46
3.6.1. Aspalathin 46
3.6.2. Afriplex GRT™ 46
3.7. DEOXYRIBONUCLEIC ACID (DNA) EXTRACTION 47
3.8. POLYMERASE CHAIN REACTION (PCR) 47
3.8.1. Primer preparation 47
3.8.2. Conventional PCR Composition 48
3.9. THE 16S METAGENOMIC SEQUENCING (ION TORRENT NEXT GENERATION SEQUENCING) 50
3.9.1. Amplification and Library preparation 50
3.9.2. Template preparation, enrichment and sequencing 50
3.10. AMPLICON DATA PROCESSING AND ANALYSIS 51
3.11. STATISTICAL ANALYSIS 51
4. RESULTS 52
4.1. RESULTS STRUCTURE 53
4.2. OBSERVED CULTURABLE BACTERIA 54
4.3. DNA, PCR AND GEL ELECTROPHORESIS 56
4.4. ALPHA DIVERSITY 57
4.4.1. Preliminary optimization work 57
4.4.2. Results 59
4.5. BETA DIVERSITY 62
4.5.1. Effect of diet on faecal sample obtained from the standard diet fed monkeys 63

4.5.2. Effect of diet on faecal sample obtained from the Western diet fed monkeys. 64
4.6. EFFECT OF DIET ON NON-CULTURED SAMPLES 66
4.6.1. Gut microbiota phyla distribution 66
4.6.2. Gut microbiota genera distribution 68
4.6.3. Overall species distribution 71
4.6.4. Beneficial species on the gut microbiota 73
4.6.5. Harmful species on the gut microbiota 74
4.7. EFFECT OF ANAEROBIC CULTURING 76
4.7.1. Effect of culturing and favoured species during culturing 77
4.8. EFFECT OF ROOIBOS PHENOLIC COMPOUNDS ON CULTURED GUT MICROBIAL DIVERSITY 79
4.8.1. Effect of aspalathin and Afriplex GRT™ on phyla 79
4.8.2. Effect of Aspalathin and Afriplex GRT™ on the genera 80
4.8.3. Effect of aspalathin and Afriplex GRT™ on relative abundance of specific genera of standard diet 81
4.8.4. Effect of aspalathin and Afriplex GRT™ on relative abundance of specific genera of Western diet 82
4.8.5. Effect of aspalathin and Afriplex GRT™ on relative abundance of specific species of the standard diet 84
4.9. SUMMARY TABLE 91
5. DISCUSSION 92
5.1. ALPHA DIVERSITY RICHNESS OF THE VERVET MONKEYS 93
5.2. EFFECT OF DIET ON THE GUT MICROBIOTA 93
5.3. IMPACT OF CULTURING ON THE GUT MICROBIOTA DIVERSITY SHIFTS OR GROWTH 95
5.3.1. Choice of anaerobic culturing system 95
5.3.2. Comparing culture-independent and culture-independent metagenomics techniques 96
5.3.3. Species favoured due to culturing 96

5.4. SHIFTS IN THE MICROBIAL COMMUNITY INFLUENCED BY ROOIBOS PHENOLIC COMPOUNDS, i.e. ASPALATHIN AND AFRIPLEX GRT™ 97

6. CONCLUSION 99

6.1. CONCLUSION 100

6.2. STUDY LIMITATIONS 100

6.3. FUTURE WORK 101

7. REFERENCES 102

LIST OF FIGURES

Figure 1: Gastrointestinal tract illustration......9

Figure 2: Krona chart representing bacterial composition in the gut microbiota,. 12

Figure 3: List of all the essential metabolites produced by the gut microbiota......18

Figure 4: **Geographical distribution of rooibos (*Aspalathus linearis*) in South Africa**.....28

Figure 5: *Aspalathus linearis* flowering plant 29

Figure 6: **Chemical structure of Aspalathin**.....31

Figure 7:**Illustration of the anaerobic glove box (Pictured captured at CPUT)**.....36

Figure 8:Schematic representation of Speedy Breedy culturing system.....38

Figure 9: **The Workflow of the study** 44

Figure 10: Schematic representation of the results......53

Figure 11: **Bacterial images of Gram stained cultured faecal samples observed under 100X magnification using the light microscope**.....55

Figure 12: The 1.5% agarose gel electrophoresis.. 56

Figure 13: Comparison of two anaerobic culturing methods, anaerobic chamber and Speedy Breedy culturing systems. 57

Figure 14: **Comparison of aspalathin concentrations** 58

Figure 15: **The representation of the sequences obtained per sample**.....60

Figure 16: Representation of the principle coordinate analysis (PCoA) plot based on species, measured using Bray-Curtis distance 62

Figure 17: **Representation of the PCoA for baseline (culture-independent) and cultured-dependent samples obtained from the standard diet-fed monkeys**.. 63

Figure 18: Representation of the PCoA of the faecal samples obtained from the Western diet monkeys 64

Figure 19: Distribution of the gut microbiota phyla.....66

Figure 20: Representation of the three dominant gut microbiota phyla.....67

Figure 21: Representation of the overall genus distribution as classified by diet 68

Figure 22: Representation of the gut microbiota genera obtained from culture-independent faecal samples classified by diet 69

Figure 23: A-B) The Relative abundance of the overall distribution of the gut microbiota species at the species level 72

Figure 24: Beneficial gut bacterial species 73

Figure 25: **Harmful gut bacterial species** 74

Figure 26: Representation of the most dominant phyla 76

Figure 27: The effect of diet on cultured species. 77

Figure 28: The effect of aspalathin and GRT on cultured gut microbiota phyla 79

Figure 29: Representation of relative abundance of the cultured genera level 80

Figure 30: Relative abundance of standard diet cultured gut microbiota genera using aspalathin and GRT. 81

Figure 31: Relative abundance of Western diet cultured gut microbiota genera using aspalathin and GRT 82

Figure 32: **The effect of rooibos phenolic compounds (aspalathin or GRT) on standard diet samples**.. 84

Figure 33: **The effect of aspalathin or GRT on standard diet faecal samples**..... 85

Figure 34: **The effect of aspalathin or GRT on standard diet faecal samples**..... 86

Figure 35: The effect of Aspalathin and GRT on Western diet monkey faecal samples 87

Figure 36: **The effect of aspalathin or GRT on Western diet monkey faecal samples** 88

Figure 37: The effect of aspalathin or GRT on Western diet monkey faecal samples..... 89

LIST OF TABLES

Table 1: **Gut microbiota enterotypes classification on different phyla and effect of diet on gut microbiota in different phyla** 14

Table 2: Characteristics of the monkeys selected for the study 43

Table 3: The list of primers and annealing temperature used 47

Table 4: The PCR reaction mixture. 48

Table 5: PCR program. 49

Table 6: Effect of Western diet before and after culturing, and GRT™ treatment 91

Table S1: DNA concentrations and ratio. 127

GLOSSARY

Biofilm	It is a collection of microbial species that reside together in the matrix and are found in the surface
Commensals	It is a biological interaction where one species benefit, and the other species neither benefits nor is harmed. It is also a term used for resident gut bacteria
Digesta	A mass of dietary fibre substance that is digested as it passes through the gastrointestinal tract
Dysbiosis	A deviation from a normal microbial community, such as an imbalance in the abundance, membership or localization of microorganisms
Enterotype	A cluster of bacterial species that characterizes individuals gut microbiota
Gnotobiotic animals	Formerly germ-free animals that now carry a defined microbiota. The composition of the microbiota in these animals is usually determined experimentally
Indigenous organisms	Organisms that are native to a particular area
Insulin resistance	It is described as a physiological condition where beta-cells secrete normal amounts of insulin hormone, but unable to respond normally in the target tissues of the liver, skeletal muscle and adipocytes
Interleukin	Cytokine produced by the leucocytes
Microaerophilic	Microorganisms that can survive in environments with low oxygen concentrations. E.g. the epithelial cells in the gut

Microbiota	The assortment of microorganisms that inhabit a particular area on a host. The microorganisms may include bacteria, fungi, single celled eukaryotes, and viruses
Mutualists	Organisms that mutually benefit from each other
Prebiotic	It is a non-digestible food compound that is beneficial to the host by selectively stimulating growth activity of commensal gut bacteria
Probiotics	They are living microbial food supplements that beneficially affect the host by improving its intestinal microbial balance
Short chain fatty acids	Are produced in the caecum and the proximal colon by the anaerobic gut bacteria through fermentation of dietary fibres and non-digestible carbohydrates

ABBREVIATIONS

Afriplex GRT™	Aspalathin-rich Green Rooibos Extract
AHR	Aryl Hydrocarbon Receptor
BC	Before Christ
BHI	Brain Heart Infusion
BMI	Body Mass Index
CAF	Central Analytical Facilities
CMOS	Complementary metal-oxide-semiconductor
CPUT	Cape Peninsula University of Technology
DPB	Dulbecco's phosphate buffered saline
IDF	International Diabetes Federation
IgA	Immunoglobin A
ISFET	Ion sensitive field-effect transistor
ITS	Internal transcribed spacer region
MAC	Microbiota Accessible Carbohydrates
MRD	Maximum Recovery Diluent
mTORC1	mechanistic target of rapamycin complex 1
NGS	Next Generation Sequencing
NHP	Non-human Primates
OTU	Operational Taxonomic Units
PUDAC	Primate Unit and Delft Animal Centre
SCFA	Short Chain Fatty Acids
SD	Standard diet
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
WD	Western diet
WGS	Whole genome sequencing
WHO	World Health Organization

CHAPTER I

1. INTRODUCTION

1.1. RESEARCH BACKGROUND

"Bad digestion is at the root of all evil" and "death sits in the bowels" are famous quotes by Hippocrates, the father of medicine in the 4th century, *BC* (Gregoire, 2019). Indeed, this is true when it comes to metabolic diseases. The gut microbiota is an assortment of microorganisms in the gastrointestinal tract that work together to harvest nutrients from the non-fermented carbohydrates when they reach the colon (Hills *et al.*, 2019). The gut microbiota plays a crucial role in biosynthesis of vitamins, as it synthesizes vitamin B12 and vitamin K and steroid hormones. The gut microbiota also interacts with neurotransmitters and plays a role in the absorption and extraction of various nutrients and metabolites, including bile salts, branched-chain aromatic amino acids and drugs, and it produces short chain fatty acids (Gentile & Weir, 2018). The gut microbiota acts by modulating the immune and inflammatory response, as well as regulates the mobility and integrity of gut barrier, and controls neuronal signalling (Sircana *et al*., 2018).

The impact of diet on health and quality of life are associated with changes in the diversity of the gut microbiota in both humans and animals (Amato *et al*., 2015). Disturbance of the gut microbiota (dysbiosis) is a major cause of ill health (reviewed by Hills *et al.*, 2019). In the recent years, research shows that gut dysbiosis is associated with chronic low grade inflammation which underlies the development of metabolic diseases including diabetes (Brail *et al*., 2018; Koh *et al*., 2018). Growing evidence from clinical studies showed that individuals with insulin resistance are characterized by altered composition of the gut microbiota, i.e. a high *Firmicutes/Bacteroidetes* ratio compared with healthy individuals (Zhang, *et al*., 2012).

The human diet and gut microbiota have evolved from mainly a plant-based diet to more carnivorous diet with increased digestibility of food due to cooking and other food processing techniques (Amato *et al*., 2015). Non-human Primates (NHP) models are used to study the gut microbiome due to their close phylogenetic relationship with humans and similar dietary requirements. Microbial profile has been established, that confirms that metabolic diseases in vervet monkeys corresponds to metabolic diseases in humans, thereby confirming the relevance of this model to study gut microbial aberrations (Clayton *et al*., 2016).

Polyphenols have the ability to modify the gut microbiota, mediated by their antimicrobial activity of the polyphenols themselves and their breakdown products via enzymatic and bacterial biotransformation (Possemiers *et al*., 2011). Rooibos phenolic compounds have been shown to influence the gut microbiota diversity (Muller *et al.*, 2017). The major bioactive rooibos polyphenol, aspalathin, is a *C*-glucosyl dihydrochalcone that is poorly absorbed in the small intestine, mainly due to its hydrophilic nature and the resistance of aspalathin *C-C* bond to enzymatic hydrolysis (Zhang *et al*., 2007). Aspalathin is therefore able

to reach the large intestine unaltered in substantial quantities. Studies have reported faecal bacteria residing in the colon of the *Lactococcus* and *Enterococcus* genus can hydrolyse the stable *C*-*C* bond of various *C*-glycosides, including flavone *C*-glucosides (Braune and Blaut, 2011; Nakamura *et al*., 2011; Kim *et al*., 2014). Current findings have also demonstrated that the aspalathin-rich green rooibos extract Afriplex GRT™ profoundly affected the microbiota phenotype, suggesting that the beneficial effects of rooibos on the metabolism could be attributed to its beneficial pre-biotic effects on the microbiome. Whilst some attention has been given to flavone *C*-glucosides, to our knowledge dihydrochalcone *C*-glucosides, such as aspalathin, have not yet been studied in NHPs before.

1.2. RESEARCH PROBLEM

The incidence of major metabolic diseases such as obesity and diabetes continue to escalate exponentially world-wide. The impact of diet on health and quality of life has been associated with gut microbiota diversity. However, a paucity of information remains about the bacteria species involved, their substrate specificity, and mechanism of degradation of rooibos phenolic compounds. In view of the bioactivity of aspalathin, insight into its microbial biotransformation would advance our understanding of the relative importance of this unique rooibos flavonoid as a modulating factor of gut biodiversity. The project intends to provide answers to the role of rooibos polyphenols in the modulation of gut microbiota, specifically the enhancement of more beneficial microbiota populations, and suppressing harmful diet induced bacteria, which are the major causal factors for the development of metabolic diseases such as obesity and diabetes.

1.3. RESEARCH AIM AND OBJECTIVES

AIM

The aim of this study was to perform anaerobic culture on faecal samples, collected from vervet monkeys fed a standard or Western diet, to assess the potential prebiotic effect of rooibos bioactive phenolic compounds using aspalathin and aspalathin-rich green rooibos extract Afriplex GRT™ on the gut microbiota.

OBJECTIVES:

- To culture and isolate microbes from the gut microbiota of the vervet monkey faecal samples using anaerobic chamber and Speedy Breedy culturing systems, and

- To identify bacterial species using Bioinformatics tools, Ion Torrent Next-generation Sequencing and Ion Torrent Software Torrent Suite™ software.

1.4. RESEARCH QUESTIONS

Rooibos is known to exhibit preventive effects against diabetes and obesity. We propose that a major factor mediating these effects is through the regulation of gut microbiota by rooibos polyphenols. To this end we would like to address the following questions:

- What is the effect of diet on the gut microbiota?
- What are the effects of Afriplex GRT™ and aspalathin on the gut microbiota in vervet monkeys on normal and Western diets?
- Does culturing have an impact on the gut microbiota diversity shifts or growth?
- How will Afriplex GRT™ and aspalathin affect faecal microbial communities during culture?

1.5. PURPOSE AND SIGNIFICANCE OF THE STUDY

The project focuses on the effect of a Westernized diet (high fat and high sugar) on the gut microbiota. The impact of the diet on health and quality of life has been associated with gut microbiota diversity. Within the South African context, metabolic disease, particularly obesity, ranks amongst the highest in the world (Agyemang *et al*., 2016). Our research aims to establish a greater understanding of the role that flavonoids, such as aspalathin, a *C*-glycoside dihydrochalcone, uniquely found in Rooibos spp., play in the modulation of gut microbiota. Specifically, its enhancement of more beneficial microbiota populations and suppression of pathogenic bacteria, major causal factors for the development of metabolic disease, were studied.

1.6. DELINEATION OF THE STUDY

This study focused on the use vervet monkeys as a study model, i.e. three monkeys fed a standard diet and three monkeys fed a Western diet (high fat and high sugar) diet. The study also investigates the role that Afriplex GRT™ and aspalathin plays on the biotransformation of gut microbiota *in vitro*.

1.7. OVERVIEW OF DISSERTATION CHAPTERS

Chapter 2: *Literature* review

The literature chapter reviews existing work relating to the gut microbiota diversity, localization of the gut microbiota, metabolites produced by the gut microbiota and the dysregulation of the gut microbiota. It also focuses on the use of a NHP, the vervet monkey as a study model. This chapter further investigates the prebiotic effects of Afriplex GRT™, an aspalathin enriched rooibos extract and pure aspalathin on anaerobic faecal sample cultures. Two culturing systems used to culture the bacteria and the principles behind each technique used for identification of the gut microbiota are also described.

Chapter 3: Methodology

This chapter discusses the experimental design, and the methodology followed in the study. The objective of this chapter was to illustrate the scientific workflow and how results were obtained. Techniques employed in this study include basic polymerase chain reaction (PCR) and Ion Torrent Next-generation Sequencing.

Chapter 4& 5: Results and Discussion

This chapter reports on data gathered, the first section reports on the quality of the metagenomic sequences received which were discussed under the alpha and beta diversity sections. The gut microbiota diversity shifts were analysed and classified by diet of the monkeys.

Chapter 6: Conclusion

The final take home message of the study was discussed based on our findings in context with other related scientific information in chapter 4.

Chapter 7: References

Includes all references used in this dissertation.

CHAPTER II

2. LITERATURE REVIEW

2.1. GUT MICROBIOTA

Gut microbiota is an assembly of all the microorganism communities in the gut (Berg, 1996). The gut consists of all three domains of life; eukaryotes (plants, animals and fungi), archaea and bacteria. In 1719 Van Leeuwenhoek had made the first discovery of the gut microbiota, where he observed faeces of a child under a microscope and he discovered *Escherichia coli* and *Bacillus bifidus*. Thirty decades later, an enormous amount of research had been done to better understand the gut microbiome (Willing & Jansson, 2010). The gut microbiome contains over 3 million genes that encodes for thousands of metabolites, whereas the human microbiome encodes for only 23000 genes (Rinninella *et al*., 2019). Quantifying and culturing anaerobic gut microbiota have been difficult due to the nutritional and cultivation requirements which were unknown. Due to the advancement in technology 16S rRNA gene sequencing has allowed researchers to phylogenetically identify and quantify components of the gut microbiota using DNA or RNA extracted directly from faecal matter (Thursby & Juge, 2017, Rinninella *et al*., 2019).

The microbial community of the gut is determined by age, environment, diet and phylogeny of the host. The human colon contains about 200 g of living organisms at a concentration of 10^{12} microbes/gram gut content. The gut microbiota has shown to interact with the host in different ways that can be beneficial or harmful to the host, this includes controlling the host's inflammatory response to the gut, synthesizing proteins and small molecules that are utilized by the host and regulates the amount of energy available in the diet (Sweeney & Morton, 2013).

The function of a healthy gut microbiota to host is to extract energy from digestible and indigestible nutrients, synthesize vitamin K and B, and enhances pathogenic resistance. The gut microbiota also modulates the immune and inflammatory response, regulates the mobility and integrity of gut barrier, and regulates neuronal signalling (Sircana *et al*., 2018).

Certain communal microbial assemblages can contribute to causing diseases (Possemiers *et al.,* 2011). Therefore, the gut bacteria have a crucial immune function that protects against pathogenic bacteria colonization and growth by competitive processes such as modifying the pH, secreting antimicrobial peptides, metabolising nutrients, producing bacteriocins and affecting cell signalling pathways. Gut microbiota also prevent bacterial invasions of pathogens by maintaining intestinal epithelial integrity (Khosravi & Mazmanian, 2013).

2.1.1. Gastrointestinal Track

The gastrointestinal tract is an organ system that extracts and absorbs nutrients from ingested food, and expels the remaining waste as faeces. The food path starts when it enters the mouth, through the oesophagus, stomach, small and large intestines, and exits through the anus (Cheng *et al*., 2010). The first part of the GI tract is the oesophagus, which is a tube that connects the mouth and the stomach. The stomach holds food and liquids, contains acids and enzymes that break-down food for further enzymatic breakdown and absorption in the small intestines, and for microbial action in the large intestines. The small intestine consists of three parts namely; the duodenum, jejunum and ileum. The ileum is where most of the nutrients get absorbed into the bloodstream (Cheng *et al*., 2010).

The colon is part of the large intestine, which is the final part of the digestive system and hosts most bacteria responsible for fermentation of indigestible carbohydrates and other micronutrients. The colon consists of five parts: descending colon, ascending colon, transverse colon, sigmoid colon and caecum, as indicated in the figure (1C). Its function is to reabsorb fluids and process waste products from the body and prepare for its elimination (Cheng *et al*., 2010). The gastrointestinal (GI) tract illustrated in figure (1) represents one of the largest interfaces (250 – 400 m^2) between the host, environmental factors and antigens in the human body (Cheng *et al*., 2010).

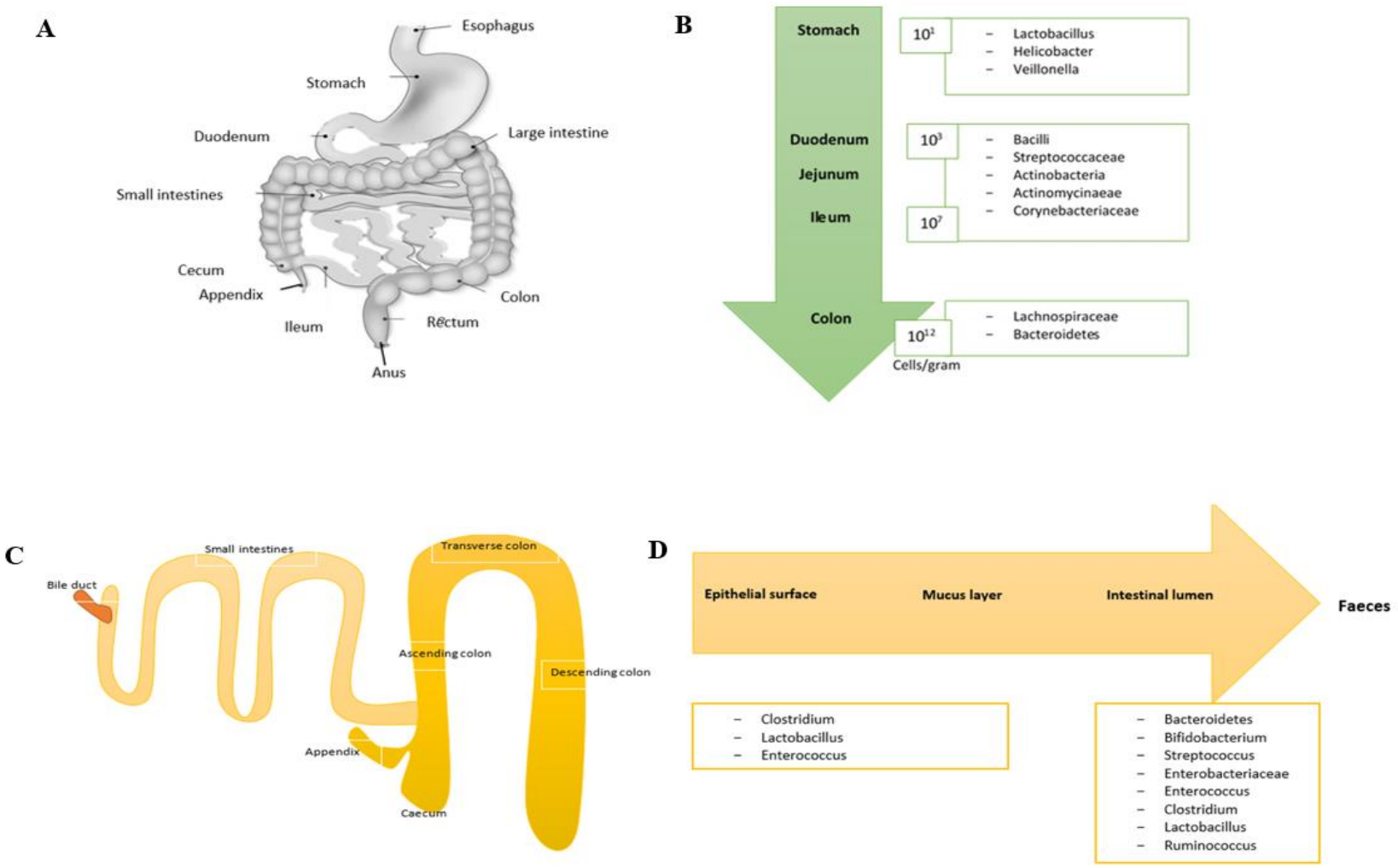


Figure 1: Gastrointestinal tract illustration. A) The complete gastrointestinal tract. **B**) Variety of microbial composition and number across the gastrointestinal tract. In the stomach there are less microbial diversity compared to the colon. **C** & **D**) The lower human gastrointestinal tract where most of the microbiota reside.

2.1.2. Gut biogeography

The mammalian digestive tract contains microbes that have adapted to the regional environment within the digestive tract, such as the stomach, small intestines, cecum and the colon figure (1A). The stomach and duodenum have low microbial density with 10^3 to 10^5 CFU per gram contents as illustrated in figure (1B). It is populated by facultative anaerobic species such as *Lactobacillus, Streptococcus* and *Enterococcus* genera. Populations are limited due to acidity, presence of bile secretion and because of the fast rate of digestion passage (Willing & Jansson, 2010).

The proximal small intestines indicated in figure (1C) is where microbes tend to attach to the intestinal lumen and mucus layer lining, figure (1D). The small intestine microbial community is dominated by rapidly growing facultative anaerobes that can endure the presence of bile acids and antimicrobials, while they compete for simple carbohydrates that are available in the area. Bile acids that are secreted via the bile duct at the proximal end of the small intestine are bactericidal to certain bacterial species due to its surfactant properties (Donaldson *et al.,* 2015). A study done by Islam *et al.* (2011), showed that when feeding mice excess bile acids stimulate the growth of *Firmicutes* and inhibits the growth of *Bacteroidetes*. The study also showed that using cholic acid simplifies the composition of the gut microbiota of rats (Islam *et al.,* 2011). The microbial density of the ileum increases from 10^8 to 10^9 CFU per gram of contents due to a slower rate of digesta passage, and increased in numbers of *Clostridiales* and *Bacteriodetes* (Willing & Jansson, 2010).

Microbial community complexity increases when it reaches the large intestine where the microbial density increases from 10^{10} to 10^{12} CFU per gram of contents accordingly, and the microbial community is dominated by gram-positive genera such as; *Clostridium*, *Bacillus*, *Ruminococcus*, and *Fusobacterium* (Willing & Jansson, 2010). The cecum is where the small and large intestine connects. The cecum and colon serve as a site for metabolism of resistant polysaccharides that were not digestion in the small intestines. The cecum and colon contain the densest and diverse bacterial communities (Donaldson *et al.,* 2015). The colon contains lower concentration level of anti-microbials, slower transit time and depleted simple carbons aids the growth of fermentative polysaccharide-degrading anaerobes (Islam *et al.,* 2011).

2.1.3. Composition of gut microbiota

Gut microbiota is composed of a number of microorganisms, including bacteria, yeasts and viruses. Various bacterial phyla present in the gut include; *Actinobacteria, Bacteroidetes, Deferribacteres, Fibrobacteria, Firmicutes, Fusobacteria, Lentisphaerae, Proteobacteria, Spirochaetes, Synergistetes, Tenericutes, Verrucomicrobia* and others. There are three major phyla which are *Firmicutes, Bacteroidetes* and *Proteobacteria*. However, more than 90% of the gut microbiota are *Firmicutes and Bacteroidetes* (Possemiers *et al.,* 2011). The *Firmicutes* phylum consists of more than 200 different genera such as *Lactobacillus, Streptococcus, Clostridium, Sarcina, Mogibacterium, Eubacterium, Coprococcus* and many others. *Clostridium* genera consist of 95% of the *Firmicutes.* Bacteroidetes consists of many genera such as *Bifidobacterium, Collinsella, Olsenella, Bacteroides, Prevotella, Fibrobacter,* and others (Arumugam *et al*., 2011). An example of the gut microbiota taxonomy is illustrated in figure (2).

It is known that the gut microbiota differs from individual to individual, as suggested by a study conducted by Tap *et al.,* (2009), where 17 subjects were studied in order to define the phylogenetic core. The study found that there was no operational taxonomic unit (OTU) defined at 2% dissimilarity. Although the microbiota differs between individuals, its functions largely remain preserved between individuals. Each individual's microbiota is characterized by specific combinations of bacterial species due to inter-individual and intra-individual variations throughout human life. These assemblages are called enterotypes (Rinninella *et al.,* 2019). Other sequencing studies suggest that core microbiome exists at the genera level (Willing & Jansson, 2010). Additionally, a study conducted by Turnbaugh *et al.* (2008) suggests that core microbiota could be defined as a functional level than at the species level. Thursby & Juge, (2017) reported that gut microbiota can be characterized based on the community type, which is called enterotype.

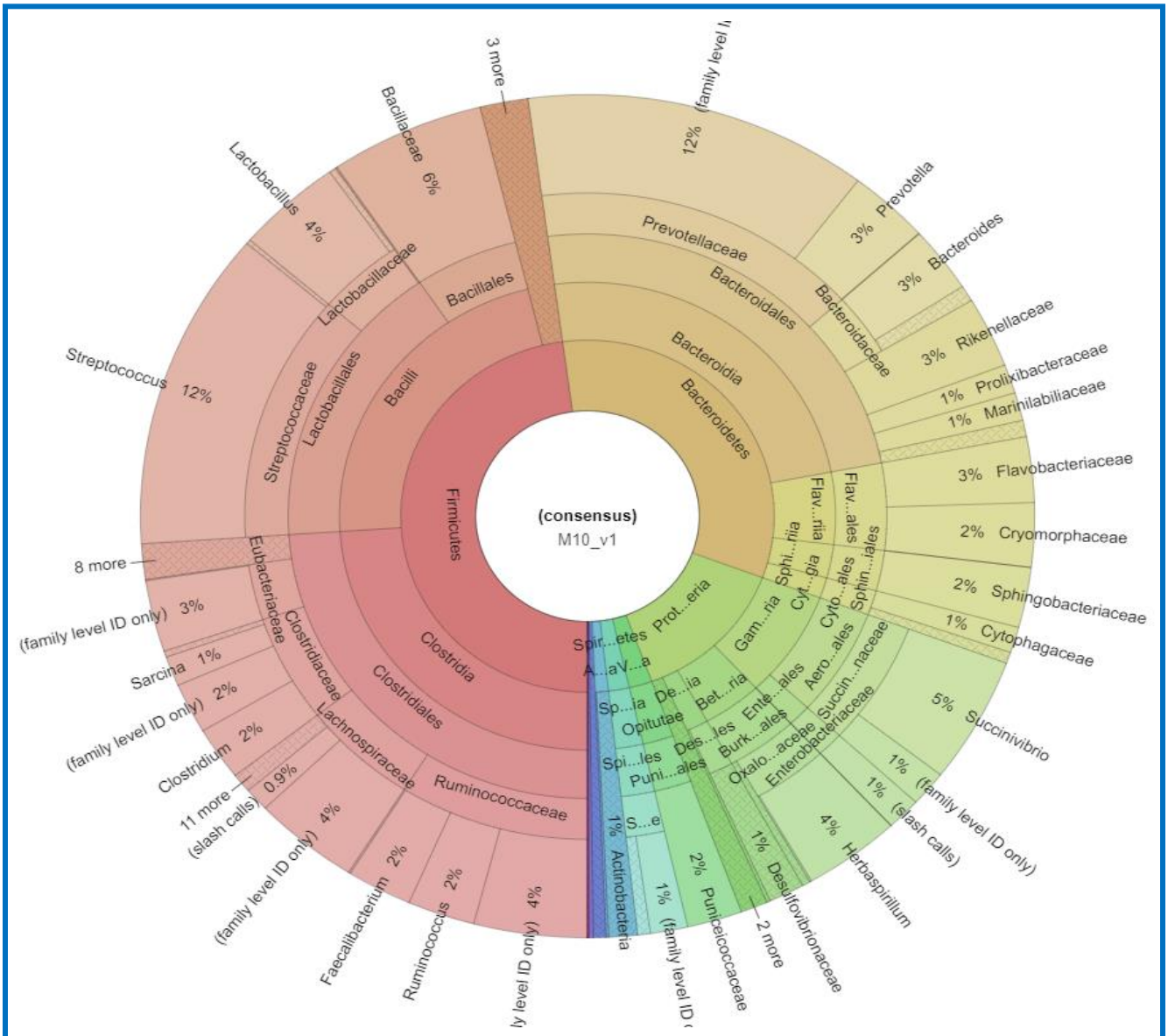


Figure 2: Krona chart representing bacterial composition in the gut microbiota, cited from Thermo-Fischer ion torrent software (https://ionreporter.thermofisher.com/ir/secure/analyses/ff8081816c09ddea016c48d9d3a860f1/metagenomics//shared/data/CAF-NT/nthovhogi@mrc.ac.za/SB-14_v1/SB-14_v1_20190731162529899/outputs//MetagenomicsActor-00/S021_SB-12_v1_consensus_krona.html?collapse=false&key=false).

2.1.4. Enterotypes

Enterotype bacteria is a cluster of bacteria that characterizes individuals gut microbiota. There are three enterotypes which are characterized by three dominant bacteria in each enterotype. Each enterotype class is identifiable by the variation in the levels of one of the three genera, enterotype I (*Bacteroidetes)*, enterotype II (*Prevotella)*, and enterotype III (*Ruminococcus)* (Arumugam *et al*., 2011). Enterotypes are grouped by function and enumeration of bacteria. Enterotypes characterize individuals and they remain stable throughout adulthood, they can be restored if they are modified (Rinninella *et al.,* 2019). Each enterotype with its distinctive cluster of bacteria and respective functional characteristics, each has a distinctive way of energy generation from fermentable substrates available in the colon. Enterotype I produce its energy from carbohydrates using glycolysis and pentose phosphate pathways. Enterotypes II and III degrade mucin glycoproteins of the gut mucosal layer (Rinninella *et al.,* 2019).

Enterotype I contain eight species, (Table 1) and is enriched in *Bacteroidetes* which co-occurs with *Parabacteroides*. Drivers of this enterotype derive its energy from carbohydrates and proteins through fermentation. The species in this enterotype have high saccharolytic potential due to the genes encoding enzymes (proteases, galactosidases, hexosaminidases), they use the glycolysis and pentose phosphate pathways to harvest its energy (Arumugam *et al*., 2011).

Enterotype II, contains six species, it is enriched in *Prevotella* and co-occurs with *Desulfovibrio* which act in synergy to degrade mucin glycoproteins present in the mucosal layer of the gut. *Prevotella* is a mucin degrader and *Desulfovibrio* removes sulphate by enhancing the rate-limiting mucin desulphation step. Enterotype III is more frequent and it is enriched in *Ruminococcus* and co-occurs with *Akkermansia*. Both these species are able to degrade mucin. These are enriched in membrane transporters, mostly of sugar. These species update simple sugars and hydrolyse mucin. Enterotypes I and II are enriched in the biosynthesis of vitamins; biotin, riboflavin, pantothenate, ascorbate, thiamine, and folate (Arumugam *et al*., 2011).

Table 1: **Gut microbiota enterotypes classification on different phyla and effect of diet on gut microbiota in different phyla**, (Adopted from Rinninella *et al*., 2019)

GUT MICROBIOTA ABUNDANCE									
		Actinobacteria	*Bacteroidetes*	*Firmicutes*	*Proteobacteria*	*Verrucomicrobiota*	*Euyarchaeota*	*Spirochaetes*	Ref
Enterotype	I	*Slackia*	*Bacteroides* *Parabacteroides*	*Clostridiales* *Alkaliphilus* *Lactobacillus* *Catenibacterium*	*Geobacter*		*Methanobrevibacter smithii*		(Arumugam *et al*., 2011, Rinninella *et al*., 2019)
	II	*Eggerthella*	*Prevotella*	*Veillonella* *Ruminococcaceae* *Holdemania* *Peptostreptococcaceae* *Staphylococcus* *Leuconostoc*	*Desulfovibrionaceae* *Rhodospirillum* *Helicobacter* *Escherichia* *Shigella*	*Akkermansia municiphila*			
	III	*Gordonibacter*	*Sphingobacteria*	*Ruminococcus* *Staphylococcus* *Marvinbryantia* *Symbiobacterium* *Ruminococcaceae* *Dialister*		*Akkermansia municiphila*			
Types of diet	High fibre diet	*Bifidobacterium*↓	*Prevotella*↑ *Bacteroides*↓	*Eubacterium*↑ *Oscillibacter*↑ *Butyricicoccus*↑ *Sporobacter*↑ *Blautia*↓ *Dorea*↓ *Lanchnospiraceae*↓ *Roseburia*↓ *Feacalibacterium*↓ *Ruminococcus*↓ *Erysipelotrichaceae*↓	*Succinivibrio*↑			Trepnema↑	
	High-fat diet	*Actinobacteria*↑	*Bacteroidetes*↑ *Alistipes*↑ *Barnesiella*↑	*Rosebaria*↓ *Eubacterium rectale*↓ *Ruminococcus bromi*↓	*Bilophila*↑				

2.1.5. Probiotic bacteria

Probiotics are live microbial food supplements that benefit the host by improving its intestinal microbial balance (Fuller, 1992). *Lactobacilli* and *Bifidobacterium* are the two genera that are usually associated with producing probiotics, they are; *i) Lactobacilli* genus that secretes SCFA by fermentation of carbohydrates which produces pyruvate via the glycolytic pathway and ii) *Bifidobacterium* genus bacteria that ferments carbohydrates to produce acetate and lactate. Probiotics are characterised by their ability to withstand conditions in the gastrointestinal tract such as; resisting salivary enzymes, stomach acid, small intestine bile secretion and enzyme secretion, pH changes and other food components encountered along the passage, and they also are able to compete with resident microbiota (Binns, 2013). These food supplements (probiotics) are usually fermented milk products and lyophilized bacteria (Collins & Gibson, 1999). Taking in probiotics have advantages such as; alleviating symptoms of lactose malabsorption, suppresses cancer, increases natural resistance to infectious diseases of the intestinal tract, and reduces serum cholesterol concentrations (Collins & Gibson, 1999).

Probiotics are reported to play a role in prevention of health problems such as, diarrhoea caused by infection (Plaza-Díaz *et al*., 2018), antibiotic-associated diarrhoea (Newberry, 2012), irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) (Plaza-Diaz *et al*., 2019), allergic disorders such as atopic dermatitis (eczema) (Rather *et al*., 2016) and allergic rhinitis (Berings *et al*., 2017). Probiotics are also used to treat gut dysbiosis, as it restores the microbial diversity (Plaza-Diaz *et al*., 2019). Probiotic VSL#3 is a multi-strain probiotic tested on overweight or obese patients. It has shown to reduce the concentrations of lipids and inflammatory markers such as high-sensitivity *C*-reactive protein, and it enhances insulin sensitivity and produces changes in the composition of gut microbiota (Plaza-Diaz *et al*., 2019). Probiotic strain L. plantarum TENSIA is shown to decrease BMI and blood pressure on obese patients (Sharafedtinov *et al.*, 2013). Metabolic activity and growth of probiotic bacteria are promoted by prebiotic compounds. Combination of probiotics and prebiotics are called symbiotics (LeBlanc *et al*., 2017).

2.1.6. Gut microbiota metabolites

The gut microbiota is the central mammalian regulator for energy intake, as it processes nutrients to produce compounds that can be utilized by the host. The gut microbiota synthesizes vital metabolites that are not produced by the host and produces vitamins. Changes in the microbiota composition are likely to influence the biochemical pathways and metabolic regulation of the host (Brial *et al.*, 2018). Microbial end-products are metabolites that the host uses in metabolic homeostasis, neurobiology, and immunological processes

(Brown & Hazen, 2018). Gut microbiota metabolites are produced from different sources such as proteins, fibres, choline, tyrosine and tryptophan which produces branched chain amino acids and short-chain fatty acids (SCFA) metabolites (Brial *et al.,* 2018) as illustrated in figure 3.

Carbohydrates and proteins are depolymerised by actions of gut microbiota to produce mono and oligomeric compounds, which are further broken down into SCFA, CO_2 and H_2. Proteins are broken down into peptides and amino acids, and then into SCFA, CO_2 and H_2, branched-chain fatty acids (BCAAs), ammonia (NH_3), hydrogen sulphide (H_2S), amines, phenols, indoles and mercaptans (Blaut & Clavel, 2007). SCFA are used as a source of energy by the host. Butyric acid, a short-chain fatty acid is a source of energy for the epithelial cells lining in the colon and it impacts cell growth and differentiation. The gases that are produced i.e. carbon dioxide, hydrogen, and methane may contribute to the equilibrium of the microbiota. These gases can cause flatulence and distension, which leads to intestinal discomfort when dietary intake of fermentable substrates is suddenly increased (Binns, 2013).

The gut microbiota breaks down flavonoids, which normally contains 2 phenyl-rings and an oxygen-containing heterocyclic ring with high metabolic potential. Gut microbiota catalyses reactions using the following catalytic reactions; *O*- and *C*- deglycosylation, demethylation, dehydroxylation, ester cleavage, reduction of carbon-carbon double bonds, isomerization, ring fission, extension and truncation of the aliphatic carbon chain and decarboxylation (Braune & Blaut, 2016).

2.1.7. Short chain fatty acids

Short-chain fatty acids (SCFA) are produced in the caecum and the proximal colon by the anaerobic gut bacteria through fermentation of dietary fibres and non-digestible carbohydrates. The gut microbiota ferments carbohydrates that are not used by the host to produce carbon dioxide (CO_2), hydrogen (H_2) methane (CH_4) and SCFA. The most abundant SCFA are butyrate, acetate and propionate. SCFA acts as an energy source for the mucosal cells of the gut microbiota. The gut microbiota can utilize plant-derived non-nutritional substance like flavonoids into nutritional metabolites (LeBlanc *et al.,* 2017, Brial *et al.,* 2018).

The SCFA are produced to have localized effects in the hosts gut. Propionate activates intestinal gluconeogenesis. The effects of SCFA are shown by the stimulation of liver gluconeogenesis mediated by propionate, which results in new lipid and protein synthesis. Butyrate acts as a source of energy to the gut mucosal cells, while acetate acts as a precursor for cholesterol synthesis (Byrne *et al.,* 2015).

Experiments done on animal models (rats) show that there are associations between SCFAs, host metabolism and disease. Rats supplemented with a diet enriched with butyrate and propionate, showed improved glucose tolerance ,while the rats on a high-fat diet supplemented with butyrate reduced glucose intolerance, insulin resistance, obesity, enhanced thermogenesis and mitochondrial function (De Vadder *et al.,* 2014). Experimental models and data conducted from human studies show that elevated SCFA can contribute to lowering blood pressure and improving vascular phenotypes (Brial *et al*., 2018).

The SCFA have been reported to have numerous physiological, biochemical and molecular effects in many tissues, including muscle, brain tissue, liver, intestines and adipose tissue. It is proposed that acetate produced by *Bifidobacterium* can protect the host from lethal infections by improving the intestinal defence mediated by epithelial cells (Plaza-Diaz *et al*., 2019).

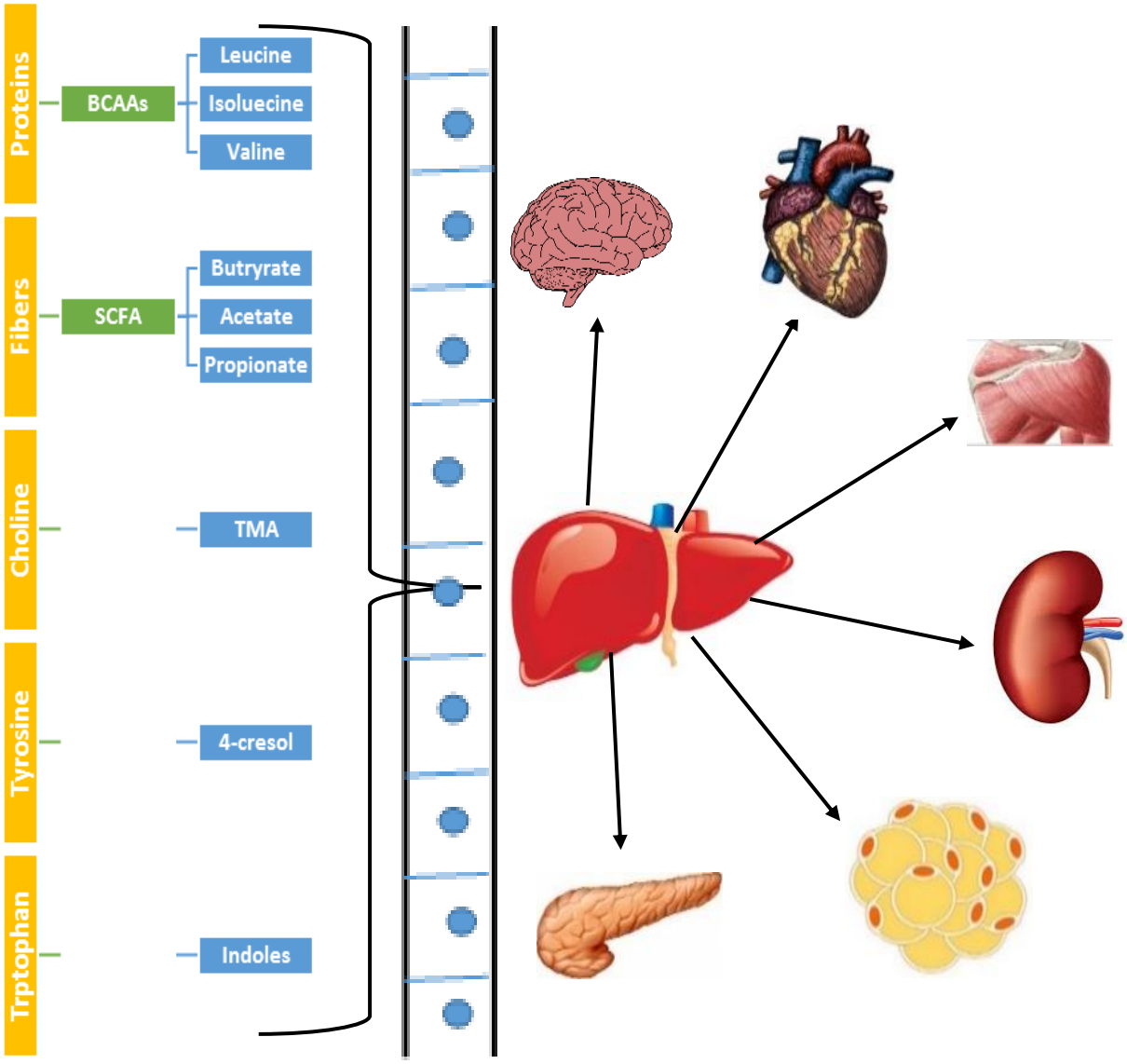


Figure 3: List of the essential metabolites produced by the gut microbiota. Once produced in the gut they are sent into the liver via the portal system to be modified before reaching the general circulation (Plaza-Diaz *et al*., 2019).

2.1.8. The gut barrier function

The gut barrier consists of a mucosal layer overlaying an epithelial cell layer which prevents the passage of harmful intraluminal substances including foreign antigens, microorganisms and their toxins into the body circulation (Assimakopoulos *et al*., 2018). The barrier comprises of several integrated components including physical, biochemical and immunological factors. Physical factors are the mucus layers and epithelia, biochemical factors that include enzymes, antimicrobial proteins, and immunological factors such as immunoglobin A (IgA) and epithelia-associated immune cells (Thursby & Juge, 2017). Mucus is present throughout the gastrointestinal tract. In the colon, the mucus lining is thicker because it is crucial in mediating the host-microbiota relationship. Colon mucus is divided into two layers consisting of a dense impenetrable inner layer and a loose outer coating which is penetrable by bacteria. The outer mucus layer consists of mucin proteins and diverse repertoire of *O*-glycans which provides energy and are preferred binding sites for commensal bacteria (Johansson *et al*., 2015).

The intestinal mucus layers are built around highly glycosylated gel-forming mucin MUC2, secreted by goblet cells. There are two groups of mucins, the normal gel forming and the transmembrane mucin. Type 1 is composed of MUC2, MUC_5AC that also forms extremely large polymers. The second group of mucins (MUC1, MUC3) are transmembrane mucins that cover the apical surfaces of the epithelial cells (Johansson *et al*., 2011). The glycan structures present in mucins are very complex and diverse and are based on 4 core mucin-type *O*-glycans containing *N*-acetylgalactosamine, galactose and *N*-acetylglucosamine. The *O*-glycans make up 80% of the MUC2 total molecular weight (Thursby & Juge, 2017). The gut barrier protects the host against infections and disturbance of the gut barrier layer causes to inflammation in the gut which leads to development of inflammation-based diseases including diabetes and obesity (Assimakopoulos *et al*., 2018).

2.1.9. Dysregulation of the gut microbiota

Dysbiosis is an imbalance of the gut commensal bacteria which leads to susceptibility to infection, decreased lymphocyte, and intestinal macrophage proliferation and low serum immunoglobulin levels (Clayton *et al*., 2018). Dysregulation of the gut microbiome can cause disease or conditions such as Crohn's disease, colon cancer, stress and anxiety, food allergies, asthma, autism, eczema, hepatic encephalopathy, diabetes, obesity and other metabolic related diseases (Holmes *et al.*, 2012, Clayton *et al.*, 2018).

Studies done in humans and animals show that the composition of gut microbiota in lean and obese species are different (Cani *et al.*, 2007). Increase in *Firmicutes: Bacteroidetes* (F:B) ratio is a key signal to obesity (Holmes *et al.*, 2012). It has been suggested that the F:B ratio correlates with body weight, with a ratio

being higher for obese people. It has been suggested that dietary modulations with polyphenols can reshape the gut microbial community and enhance host-microbial interactions to provide beneficial effects (Parkar *et al.*, 2013).

2.1.10. Effects of diet on gut microbiota regulation

Diet is a vital external factor that affects the gut microbiota, it can alter the microbial gut diversity and ecology. Diet-induced alteration can be detected within 24 – 48 hours after dietary manipulation (David *et al*., 2014). Diet alterations happen independently of the body weight and adiposity (Gentile & Weir, 2018).

Westernized diet composed of high-fat, high-sugar diet (HFD) has been associated with altered gut microbiota which decreases the gut microbiota's richness and diversity (Murphy *et al*., 2015). HFD has been reported to decrease *Bacteroidetes* phylum, while increasing *Firmicutes* and *Proteobacteria* phyla (Gentile & Weir, 2018, Schnorr *et al*., 2014, Yastunenko *et al*., 2012). Parks and colleagues (2013), reported that body fat percentage growth is positively associated with a relative abundance of the *Firmicutes* genera *Lactococcus* and with *Bacteroidetes* genera *Allobaculum*, and are negatively associated with the abundance of phylum *Verrucomicrobia* genera *Akkermansia* (Parks *et al.*, 2013). A human study conducted by Munukka and colleagues (2012), suggests that overweight/obese women with metabolic disorder had a higher proportion of bacteria belonging to the *Eubacterium rectale – Clostridium coccoides* than women without metabolic disorder and normal weight women (Munukka *et al*., 2012). High fat diet feeding is also reported to alter the gut barrier function by reducing tight junction proteins which lead to a leaky gut with increased circulating lipopolysaccharides (LPS) which is a cell wall component found on Gram negative bacteria (Gentile & Weir, 2018). Circulating LPS is known to have metabolic consequence to the host due to an increase of LPS circulating, which poses as a potent inflammatory response via Toll-like 4 receptor signalling which has been associated with development of cardiovascular and metabolic disease (Gentile & Weir, 2018).

Table (1) illustrates the enterotype classes present under each phylum, it also lists the changes in the gut microbiota under high fibre diet and high fat diet. Microbiota accessible carbohydrates (MAC) refers to a carbohydrate that can be degraded by gut microbiota. There are carbohydrates that cannot be degraded by microbes such as cellulose (Sonnenburg *et al*., 2016). MACs play a vital role in microbiota function and composition. A study conducted by Sonnenburg and colleagues (2016), where mice fed a diet low in MACs experienced a decrease of numerous taxa and loss of diversity is doubled over generations, and it was not recovered after reintroduction of MACs. MACs are responsible for SCFA production, therefore a reduction in MAC intake reduces SCFA production (Gentile & Weir, 2018).

A study conducted by O'Keefe and colleagues (2015) looked at the effect of fat and fibre in increasing risk of colon cancer on rural South Africans and African Americans. In the study, they compared the microbiota and metabolome changes of rural South Africans who were eating a standard diet which was high in fibre, low in fat, and African Americans who ate a Western diet which was high in fat, and low in fibre. They found that the African Americans had lower gut microbial diversity and higher colon cancer biomarkers compared to the Africans. When they swapped the diets of the two populations, they observed microbiota and metabolome shifts within two weeks. The rural South African population presented with increased colon cancer biomarkers while the African American population's colon cancer risk biomarkers were decreased (O'Keefe *et al*., 2015).

2.1.11. Specific species related to gut dysbiosis

2.1.11.1. Faecalibacterium prausnitzii

Faecalibacterium prausnitzii is the sole known species to the genus *Faecalibacterium* (Lopez-Siles *et al*., 2017). *Faecalibacterium prausnitzii* belongs to the *Firmicutes* phylum, it is a Gram negative, rod, anaerobic bacteria. It is non-motile and non-spore forming. It is one of the most abundant bacteria in the gut microbiota and it makes up about 5% of the gut microbiota. *Faecalibacterium prausnitzii* is reported to be one of the main butyrate producing organisms (Martín *et al*., 2018). This species is reported to contain anti-inflammatory properties through its ability to induce tolerogenic cytokine by secreting very low levels of pro-inflammatory cytokines like interleukin 12 (IL – 12) and promotes the secretion of anti-inflammatory cytokines like IL – 10 (Lopez-Siles *et al*., 2017).

Several studies show that *Faecalibacterium prausnitzii* cells or supernatant without the cells could reduce the severity of acute chronic and low-grade chemical induced inflammation in murine models (Sokol *et al*., 2008, Martín *et a*l., 2015). These anti-inflammatory effects were associated with secretion of metabolites responsible for blocking IL – 8 activation and upregulation of regulatory T- cells (Lopez-Siles *et al*., 2017). The *Faecalibacterium prausnitzii* abundance has been found to decrease in patients suffering from diseases such as irritable bowel syndrome, colorectal cancer, inflammatory bowel disease and obesity. Contrary to what is known, a study by Balamurugan and colleagues (2009) showed a higher abundance of *F. prausnitzii* species in obese children, and that the higher abundance of the bacterium contributed to increased energy salvage from non-absorbed carbohydrates (Balamuragan *et al*., 2009). *F. prausnitzii* consumes acetate while producing butyrate. In addition, it reduces the effect of acetate on mucus, and also prevents overproduction of mucus in the gut (Makki *et al*., 2018).

2.1.11.2. Lactobacillus murinus

Lactobacillus murinus is a Gram positive, rod-shaped non-spore forming bacterium. Research conducted by Pan and colleagues (2018) showed that mice that were put on a calorie restricted diet exhibited increased in abundance of *L. murinus* species after a period of 14 days, suggesting that the species is a key mediator of the anti-inflammatory and anti-aging effects of calorie restriction (Pan *et al.*, 2018). In another *in vitro* study by (Pan *et al*., 2018) the effect of *L. murinus* on inflammation were assessed using Caco-2 cells stimulated with TNF-a. When the cells were cultured with bacterial culture supernatant from the *L. murinus* cells, the researchers discovered that IL - 8 secretion was significantly reduced, implying that some soluble substances secreted by the *L. murinus* inhibited the production of IL - 8. Recent studies show that *L. murinus* protects against necrotizing enterocolitis by acting as an effective first colonizer in gastrointestinal tract of rat model (Isani *et al*., 2018).

2.1.11.3. Lactobacillus reuteri

Lactobacillus reuteri is a known and commonly used probiotic found in many sites in the body, such as skin, breast milk, urinary tract and the gastrointestinal tract. *L. reuteri* is known to be an effective prophylactic for diarrhoea (Urbańska *et al*., 2016). *L. rueteri* produces ethanol, organic acids and reuterin is a metabolite possessing antimicrobial properties. Reuterin can inhibit a wide range of microorganisms, mainly Gram-negative microorganisms. In human studies conducted to investigate the effect of *L. reuteri* in patients with diabetes, they administered *L. reuteri* DSM 17938 strain for 3 months and discovered that the supplementation did not significantly change the microbial structure but that the treatment was effective based on the baseline line values of each individual (Mu *et al*., 2018, Mobini *et al*., 2017).

L. reuteri has been shown to have anti-inflammatory effects by inducing anti-inflammatory T-regulatory (Treg) cells (Mu *et al*., 2018). In studies that correlate *L. reuteri* to obesity shows that high levels of *Lactobacillus* are found on obese individuals (both adults and children). An animal study conducted by Fåk and Bäckhed (2012) showed that the beneficial *L. reuteri* effect on obesity was strain dependent. Three different strains of *L. reuteri* were used to test the influence of diet induced obesity, where it was shown that *L. reuteri* PTA 4659 effectively reduced the body weight of mice fed high fat diet, whereas mice treated with *L. reuteri* L6798 gained more weight, liver and adipose weights were consistent with the body weight changes (Fåk & Bäckhed, 2012).

2.1.11.4. Lactobacillus apodemi

Lactobacillus apodemi is named after the species it was first isolated from, *Apodemus speciosus*. *L. apodemi* is a Gram positive, non-spore forming, non-motile rod-shaped bacteria. It mostly occurs in single cells or paired cells. When cultured on agar plate, it forms white, convex and smooth colonies with an opaque border. Grows best under microaerophilic environments (Osawa, 2006). *L. apodemi* is able to degrade tannin by producing tannase (Osawa, 2006).

2.1.11.5. Succinivibrio dextrinosolvens

Succinivibrio dextrinosolvens is a Gram negative, curved bacillus with a monotritrichous flagella. It was first isolated from bovine rumen. *S. dextrinosolvens* is founds on ruminating species. There were few reports of this species found in human, and those patients were reported to suffer from bacteremia and gastrointestinal tract disorders. It was assumed that this species is present in human microbiota but in small numbers. It is mostly present on high starch diet. *S. dextrinosolvens* produces acetate, succinate lactate and formate after fermentation (Hespell, 1992).

2.1.11.6. Prevotella copri

Prevotella copri is a Gram negative, rod-shaped, non-spore forming, non-motile bacteria, it is an obligate anaerobe. *Prevotella* genus abundance is associated with plant-rich diets, while the *Prevotella* genus is also associated with inflammation and certain diseases in the gut (Ley, 2016), *Prevotella copri* is known as a fibre degrader, it also produces SCFA and also helps improve glucose metabolism (De Filippo *et al*., 2010). There are other studies which associate *P. copri* to inflammatory conditions, insulin resistance and glucose intolerance (Kovatcheva-Datchary *et al.,* 2015). Some effects of *P. copri* are strain dependent (De Filippis *et al*., 2019). *P. copri* is known to be an important biomarker for diet (Precup & Vodnar, 2019).

2.1.11.7. Prevotella sterecorea

Prevotella stercorea is a Gram negative, non-motile rod-shaped and non-spore forming bacterium (Hayashi *et al*., 2007). *P. stercorea* is a bacterium associated with plant-rich diet, as it ferments fibre. A review compiled by Precup & Vodnar (2019) highlights that *P. copri* and *P. stercorea* growth were stimulated by β-carotene and vitamin A found in fruits like mangoes and bananas (Precup & Vodnar, 2019).

2.1.11.8. Herbaspirillum

Herbaspirillum is a Gram negative, non-fermentative, curved or sometimes helical bacilli bacterium. It is mostly found on plants as it is nitrogen fixing bacteria and helps the plant to grow. *Herbaspirillum* has been isolated from human infections sites (Marques *et al*., 2014) but are rarely associated with the cause of infection. *Herbaspirillum* has been isolated from blood and sputum of people with leukaemia, cystic fibrosis, bacteraemia and cellulitis (Tan & Oehler, 2005). There are few strains that are associated with being human pathogens, information is lacking on their pathogenicity, but it is speculated that environmental strains may cause pathogenicity to humans (Marques *et al*., 2014).

2.1.11.9. Bilophila wadsworthia

Bilophila wadsworthia is associated with high fat diet and is known to increase inflammation and glucose intolerance. *B. wadsworthia* causes bile acid dysmetabolism and leads to intestinal barrier dysfunction (Natividad *et al*., 2018). It causes acute inflammation as it generates hydrogen sulphide via taurine respiration (O'Keefe *et al*., 2015).

2.1.11.10. Roseburia spp.

Roseburia spp. are Gram-positive anaerobic bacteria There is a link between *Roseburia spp*. and gut health. They form part of the commensal gut bacteria that produce SCFAs and butyrate. *Roseburia spp*. have anti-inflammatory properties and maintains immunity. Disturbance in the *Roseburia spp*. abundance in the gut microbiota may affect various metabolic processes that are associated with several diseases such as obesity, T2D, irritable bowel syndrome and others (Tamanai-Shacoori *et al*., 2017). Depletion of *Roseburia spp.* decreases butyrate oxidation and decreases butyrate levels in unhealthy humans (Tamanai-Shacoori *et al*., 2017). A study conducted by Neyrinck and colleagues (2012) shows that *Roseburia spp*. abundance decreases with high-fat diet feeding and that chitin-glucan increases the abundance of *Roseburia spp.* in the gut microbiota (Neyrinck *et al.*, 2012).

2.2. METABOLIC SYNDROME

Metabolic syndrome (MetS) is an assortment of related conditions such as, glucose intolerance, insulin resistance, increased blood pressure, excess body fat around the waist and abnormal triglyceride levels. These occur together increasing the risk of stroke, diabetes and heart disease (Al-Qawasmeh & Tayyem, 2018). There are four metabolic risk factors associated with MetS; elevated post-prandial plasma glucose, elevated blood pressure, a prothrombotic state, and a pro-inflammatory state (Grundy, 2018). Obesity is a risk factor related to metabolic disease. Other parameters such as age and sex can influence MetS. It is reported that MetS are more prevalent in older people, than it is in children and adolescents, with greater risk in women than with men (Al-Qawasmeh & Tayyem, 2018). Metabolic syndrome complications can lead to atherosclerosis, heart disease, kidney disease, stroke and type 2 diabetes mellitus (T2DM) (Diamant, Blaak and de Vos, 2010; Sanderson *et al*., 2014).

The MetS risk factors cut-off points are dependent on country and population such as the elevated waist circumference measurements. It is known in the literature that the normal triglyceride levels should range at ≥150 mg/dL (1.7 mmol/L), high density lipoprotein cholesterol at around <40 mg/dl (1.0 mmol/L) in males, and <50 mg/dL (1.3 mmol/L) in females. The systolic blood pressure should be ≥130mm Hg with diastolic blood pressure of ≥85 mm Hg, and the elevated blood glucose levels of ≥100 mg/dL (Alberti *et al*., 2010). MetS are also linked to insulin resistance (IR) which is described as a physiological condition where beta-cells secrete increased amounts of insulin hormone, but target tissues such as the liver, skeletal muscle and adipocytes are unable to respond normally to the insulin. Insulin resistance is reported to be a precursor of diabetes mellitus (Al-Qawasmeh & Tayyem, 2018).

2.2.1. Diabetes Mellitus

Diabetes Mellitus is referred to as a group of diseases characterised by high blood glucose. There are three common types of diabetes mellitus; namely, type 1 diabetes, type 2 diabetes and gestational diabetes. Type 2 diabetes (T2D) is the most reported type of diabetes, which is a chronic condition that affects the way the body processes blood glucose, while type 1 diabetes (T1D) is a chronic condition in which the pancreas produces little or no insulin. Gestational diabetes is a form of diabetes with high blood sugar affecting pregnant women mainly in their third trimester of pregnancy (McCracken *et al*., 2018).

Type 1 diabetes (T1D) is due to an autoimmune process where the body's immune cells attack the pancreatic beta-cells and kill them, resulting in physical loss of beta-cells and therefore no insulin is produced. Type 2 diabetes (T2D) is a progressive disease characterised by loss of insulin sensitivity by insulin responsive

cells (insulin resistance), whereby the insulin produced is not efficiently utilized by the body to maintain normal blood glucose levels (Tuomi, 2005, Kim *et al*., 2018).

The prevalence of T2D amongst South Africans is high due to an increased genetic predisposition, sedentary lifestyle and unhealthy diet which contains high sugar and high saturated fats. In South Africa, there's an increasing number of people affected by T2D, as per the International Diabetes Federation (IDF), which estimates that 5.5% of South Africans between the ages of 20 and 79 years have diabetes, thus 1.83 million South Africans in this age group may be afflicted by T2D (IDF, 2017). There are a number of studies that have demonstrated gut microbiota playing a main role in the initiation and perpetuation of insulin resistance and type 2 diabetes by triggering low-grade inflammation, however, contribution of the gut microbiota in development of T2D still needs to be studied further (Backhed et al., 2004, Cani *et al.,* 2007, Everard & Cani, 2013).

2.2.2. Obesity

Obesity is a chronic disease defined by excessive adipose mass. Obesity is a result of an imbalance between food intake and energy expenditure resulting in accumulation of fat and adipose mass tissue. Adipose tissue can be broadly divided into two main anatomical depots namely visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT). Adipose tissue consists of adipocytes, connective tissue, nerve tissue, immune cells and blood vessels (Oikonomou & Antoniades, 2018). Clinically obesity is characterized by BMI greater than 30 kg/m^2 (Lee *et al*., 2018). World Health Organisation (2018) stats show how obesity has nearly tripled since 1975 worldwide, and in 2016 more than 1.9 billion 18-year-olds and older adults were overweight and 13% were obese (Anon, 2018B). A study conducted by Puoane *et al*., emphasized that obesity is a major health problem in developing countries, particularly in women. Obesity is associated with increased risk of developing metabolic associated diseases, such as coronary heart disease, diabetes, stroke and cancer (Puoane *et al*., 2002). Obesity treatment is targeted on therapeutic, nutritional and behavioural management. Several reports suggest that gut microbiota plays a crucial role in the development of fat mass and altered energy homeostasis. It is proposed that the gut microbiota has the capacity to increase energy harvested from diet and can modulate host signalling pathways that could influence the host metabolism and energy balance (Backhed *et al*., 2004).

Gut microbiota, Gram-negative bacteria produce lipopolysaccharides (LPS) that is a key molecule causing inflammation and other metabolic diseases. There are a number of studies conducted that show LPS as a major contributor to metabolic endotoxaemia. There's a relationship between fat feeding, obesity, type 2 diabetes and LPS (Everard & Cani, 2013).

2.2.3. Diabetes and Obesity association with gut microbiota alterations

Gut microbiota, obesity, and type 2 diabetes are all affected by the hosts genotype, lifestyle, diet, physical activity and antibiotics (Allin *et al*., 2015). There are a number of studies that support that obesity and diabetes can alter the gut microbiota. The first study that showed gut microbiota alterations was associated with obesity was performed on genetically obese *ob/ob* mice (Cani *et al*., 2007). The study revealed an increase in *Firmicutes* and a decrease in *Bacteroidetes*. A study conducted by Backhed *et al*., (2004) showed that gut microbiota plays a crucial role in development of fat mass and altered energy homeostasis. The study revealed that germ-free mice were leaner compared to mice that harboured microbiota since birth, and after inoculation of the germ-free mice with gut microbiota it induced an increase in fat mass and insulin resistance (Backhed *et al*., 2004).

Recently it has been discovered that the metabolites produced by the gut microbiota contribute to the onset of disease (Cani, 2019). A study by Arah Koh and colleagues (2018) describes how gut microbiota-derived metabolite imidazole propionate produced from histidine contributes to the development of insulin resistance and eventually causes T2D (Koh *et al*., 2018). They compared the portal vein plasma of participants with obesity and T2D with participants with the same BMI but without T2D, they found that levels of four metabolites derived from amino acids were higher in T2D than in the controls. They further showed that imidazole propionate contributes to insulin disorder, as the imidazole propionate is an inhibitor of the intracellular insulin receptor signalling cascade. Imidazole propionate activates p38ϒ- p62-mTORC1 pathway, which is the activation of p38ϒ MAPK which promotes p62 phosphorylation which activates mechanistic target of rapamycin complex 1 (mTORC1) (Koh *et al*., 2018).

Recently El Hadji Seck and colleagues (2018) showed a correlation between obesity, gut microbiota and salt intake. By using more than 1300 individuals from different geographical areas, they found that faecal salinity was increased amongst obese individuals and that a dose-dependent relationship exists between faecal salinity and body weight. They also observed that faecal salinity was a predictor of obesity and it was independent of geographical area, age and sex. Proving that salt levels in the gut and obesity are inter-linked (Seck *et al*., 2018). A human study conducted by Wu and colleagues (2010) showed an abundance of *Bifidobacterium* abundance to be associated with a lower incidence of overweight, obese and type 2 diabetes (Wu *et al.*, 2010). Seck also found that stool salinity was the key factor associated with depletion of *Bifidobacterium* and *Akkermansia muciniphila* (Seck *et al*., 2018).

2.3. ROOIBOS PHENOLIC COMPOUNDS

2.3.1. Rooibos plant

Aspalathus linearis family *Fabaceae*; tribe *Crotalarieae*, commonly known as rooibos, is found in the mountainous area of Cederberg mountains, Western Cape, South Africa (Joubert *et al.*, 2008). Cultivation of rooibos is mainly concentrated in the Clanwilliam area of the Cederberg and Bokkeveld mountains. Rooibos geographical distribution is limited, as it naturally grows in the Western Cape and southwestern areas of the Northern Cape (Morton, 1983). Its geographical distribution is illustrated in figure (4).

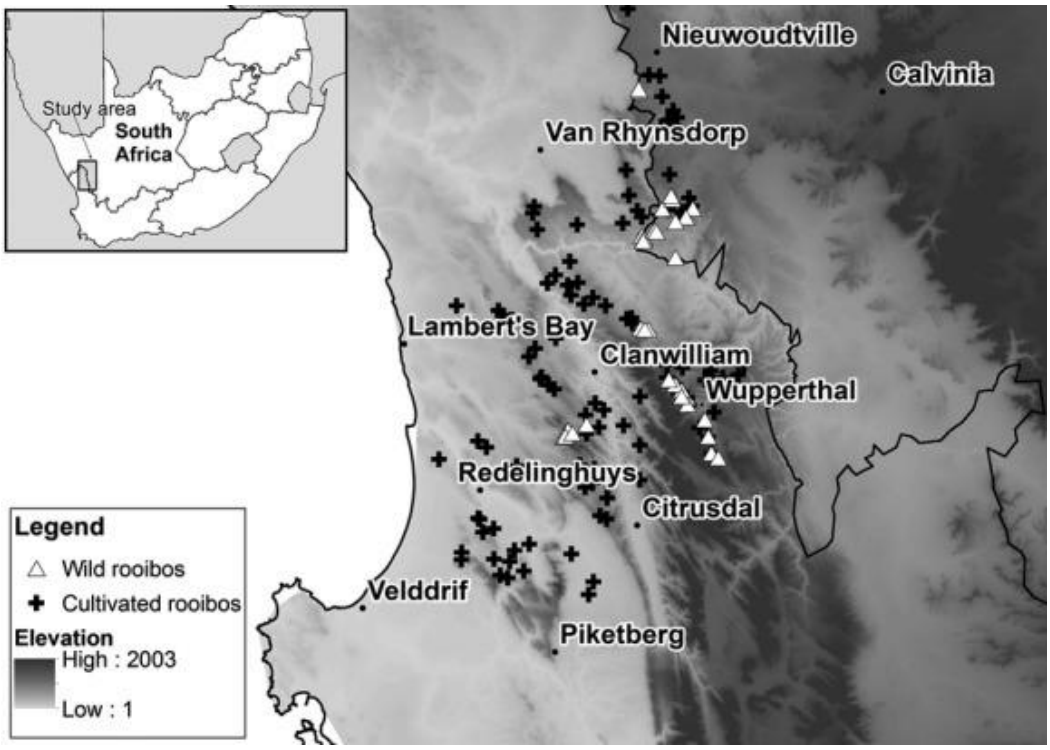


Figure 4: **Geographical distribution of rooibos (*Aspalathus linearis*) in South Africa** (Lötter & le Maitre, 2014). Rooibos is cultivated along the Lambert's Bay region up to Nieuwoudtville. The areas marked with a cross represents the cultivated rooibos area, and the triangle represents the area where rooibos grows widely. The wild type rooibos grows in elevated areas.

The rooibos plant, *Aspalathus linearis* is a shrub-like bush, with needle-like leaves with branches that are 60 cm in length which is green/brown in colour, the leaves are 2-6 cm in length, and they produce yellow flowers that occur in short clusters as indicated in figure (5) (Dahlgren *et al*., 1968). There are two types of rooibos which are used in tea production, namely the Nortier which can be cultivated and Cederberg which is the type that grows wildly (Morton, 1983).

2.3.2. Bioactive properties of rooibos

Rooibos is low in tannin content, alkaloids and caffeine free. It is known as herbal beverage amongst the locals; because it alleviates nervous tension, ingestion, heartburn and nausea. It also improves appetite, promotes sound sleep and alleviates infantile colic on infants (Morton, 1983; Schmid *et al*., 1998). Rooibos tea is available in two forms, as oxidized (fermented) rooibos or green (unfermented) rooibos. Green (unfermented) rooibos, contains much higher levels of polyphenols than the oxidized (fermented) rooibos, the product that is commonly prepared as herbal tea (Joubert *et al*., 2008).

Figure 5: *Aspalathus linearis* flowering plant, (https://www.superfoodly.com/red-rooibos-tea-benefits/)

2.3.3. Dietary polyphenols

Polyphenols are a group of phytochemicals which are found in plant-based foods that are associated with maintaining health. Dietary polyphenols possess anti-inflammatory and anti-oxidative properties that influence glucose and lipid metabolism (Hanhineva *et al*., 2010). Polyphenol intake is associated with lowering risks of major chronic diseases including diabetes, cardiovascular diseases and cancer and their recommended dietary intake is 1 g/day (Parkar *et al*., 2013). There are two major classes of polyphenols; hydroxycinnamic acids and flavonoids (Parkar *et al*., 2013). Flavonoids contain various structural classes such as flavanols, flavanones, anthocyanidins and isoflavones (Hanhineva *et al*., 2010). The hydroxycinnamic acids are a class of aromatic acids having a C6–C3 skeleton and a natural phenylpropenoic acid compounds, which occur as esters or glycosides.

Unfermented rooibos contains about 4% to 12% aspalathin, at the concentration is 49.9 mg/g and other less plentiful polyphenols such as nothofagin 10.8 mg/g, isoorientin 3.6mg/g, orientin 2.3 mg/g, rutin 1.3-1.7 mg/g, isovitexin 0.7 mg/g, vitexin 0.5mg/g, and other (Kreuz *et al*., 2008). Isoorientin has been associated with anti-hyperlipidemic and anti-mutagenic activity (Sezik *et al.*, 2005; Snijman *et al.*, 2007), anti-inflammatory, anti-nociceptive activity and gastroprotective activity (Küpeli *et al.*, 2004; Zucolotto *et al*., 2009) and hepatoprotective activity (Deliorman Orhan *et al.*, 2003). Orientin has been associated with anti-adipogenesis activity properties and anti-mutagenicity (Snijman *et al*., 2009).

2.3.4. Aspalathin

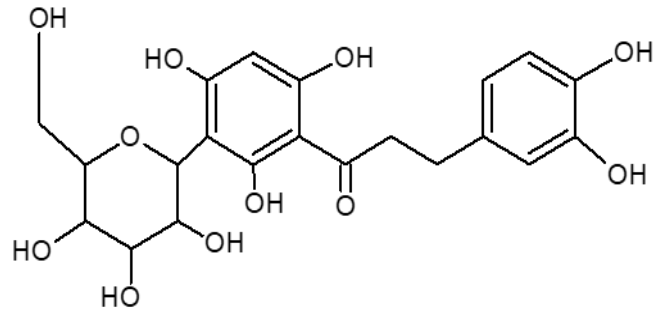
Aspalathin is a *C*-glucosyl dihydrochalcone flavonoid found in the rooibos plant species. Highest quantities of aspalathin are found in the unprocessed rooibos plant, and it is a chemical marker for *Aspalathus spp.* Aspalathin is found only in rooibos spp. and it is believed that it protects the plant against oxidative stress caused by environmental factors (Diamant, Blaak & de Vos, 2010). It is reported to have glucose-lowering effects, ameliorates insulin resistance *in vitro*, protects heart cells, lowers cardiovascular risk factors and protects beta cells against oxidative stress (Muller *et al*., 2017).

Aspalathin is found in the leaves of *Aspalathus linearis* along with its flavone derivatives isoorientin and orientin, with isoorientin being more abundant than orientin. The flavone equivalents of another *C*-glucosyl dihydrochalcone, nothofagin, are vitexin, and isovitexin which are present at significantly lower levels than orientin and isoorientin (Joubert *et al.*, 2013). There are other major compounds which are, quercetin-3-O-

robinobioside, rutin, hyperoside, and isoquercitrin, of which quercetin-3-O-robinobioside and rutin are present in the highest quantities (Muller *et al*., 2017).

Aspalathin chemical structure, indicated in figure (6) has been described as *3'-C, -D-glucopyranosyl-2',3,4,4',6'-pentahydroxydihydrochalcone* (Koeppen & Roux, 1966). Aspalathin has a *C*-glycoside (sugar) which is directly linked to the flavonoid nucleus via an acid resistant and enzyme resistant *C-C* bond. It has molecular formula, $C_{12}H_{24}O_{11}$, a molecular weight of 452.412 g/mol (Johnson *et al.*, 2018), and a chemical structure as indicated in figure (6a).

(A)



(B)

Molecular Formula:	$C_{21}H_{24}O_{11}$
Formula Weight:	452.40866
Composition:	C(55.75%) H(5.35%) O(38.90%)
Molar Refractivity:	108.66 ± 0.3 cm^3
Molar Volume:	272.1 ± 3.0 cm^3
Parachor:	851.5 ± 4.0 cm^3
Index of Refraction:	1.730 ± 0.02
Surface Tension:	95.9 ± 3.0 dyne/cm
Density:	1.662 ± 0.06 g/cm^3
Dielectric Constant:	Not available
Polarizability:	43.07 ± 0.5 $10^{-24}cm^3$
RDBE:	10
Monoisotopic Mass:	452.131862 Da
Nominal Mass:	452 Da
Average Mass:	452.4087 Da
M+:	452.131313 Da
M-:	452.13241 Da
[M+H]+:	453.139138 Da
[M+H]-:	453.140235 Da
[M-H]+:	451.123488 Da
[M-H]-:	451.124585 Da

Figure 6: **Chemical structure of Aspalathin** (**a**) Aspalathin consists of the glycosyl group and dihydrocholane structure, and it is *3'-C-,-D-glucopyranosyl-2',3,4,4',6'-pentahydroxydihydrochalcone,* (**b**) Molecular properties of aspalathin (ChemSketch freeware, ACD labs).

During fermentation of rooibos, a colour change is observed from green to red/brown colour, which is caused by the conversion of its reactive polyphenols such as aspalathin to its flavanone analogues isoorientin and orientin (Krafczyk & Glomb, 2008). Isoorientin is formed via *(R)/*(S)-eriodictyol-6-*C*-β-D-glucopyranoside convention of aspalathin, while orientin doesn't form via this mechanism. Orientin is formed as a result of isoorientin undergoing opening of its vinyl ester structure, bond rotation and closing of the ring structure (Krafczyk & Glomb, 2008).

2.3.5. Afriplex Green Rooibos Extract® (GRT)

Afriplex green rooibos extract is an aspalathin-rich extract formulated from green rooibos tea. Afriplex GRT™ is prepared as a standardized pharmaceutical grade extract by Afriplex (Pty) Ltd (Paarl, South Africa). Afriplex GRT™ contains ca. 12% aspalathin amongst the other polyphenolic green rooibos plant components. To-date, Afriplex GRT™ has been used in a number of studies conducted by the SAMRC (Muller *et al.,* 2017). Using a high fat and high sugar diet-induced vervet monkey model the effects of rooibos on the gut microbiota was compared to monkeys on maize diet. It was observed that due to the Afriplex GRT™ treatment *Firmicutes* to *Bacteroidetes* (F/B) ratio increased in diabetic vervet monkeys, (Muller, in *11th World Congress on Endocrinology and Metabolic Disorders*, 2017), this correlated with improved blood glucose and lipid parameters (Orlando *et al*., 2019). Bacterial species which are relevant to contributing positively to human metabolic disease also increased (Muller, in *11th World Congress on Endocrinology and Metabolic Disorders*, 2018). Based on previous and current studies, it was shown that Afriplex GRT™ has several health benefits such as having a positive influence on regulating blood glucose levels (Muller *et al.*, 2012) and regulating LDL-cholesterol levels in vervet monkeys (Orlando *et al.,* 2019).

2.3.6. Bioavailability of aspalathin

Bioavailability of bioactive food compounds is dependent on a number of factors such as; absorption, distribution, metabolism and excretion. Factors such as interaction with other food components limit or enhance the bioavailability of polyphenols in the digestive system (Rein *et al*., 2013). Aspalathin is ingested by drinking rooibos or consuming food supplemented with rooibos extract. Aspalathin is hydrophilic which results in poor bioavailability (Johnson *et al*., 2018).

A study conducted by Huang and colleagues (2008) using Caco-2 monolayer cells demonstrated that the absorption of aspalathin was better in green rooibos extracts than in pure compound aspalathin. This indicates that the action of aspalathin is aided by other plant components present in the extract. An *in vivo* study conducted by Kreuz and colleagues (2008), they found a number of metabolites in the urine of pigs fed with an aspalathin enriched, green rooibos extract equalling 157-167mg aspalathin/kg body weight/day. No aspalathin or metabolites were detected in the plasma of the pig, but aspalathin and several metabolites were detected in the urine. This showed that glycosylation of aspalathin was not prerequisite for absorption of aspalathin (Kreuz *et al*., 2008).

Human studies have been done on to establish the bioavailability of rooibos phenolic compounds. A study by Courts & Williamson (2009), served 300 mL of green rooibos infusion which contained 91.2 mg aspalathin/subject. Urine samples were collected and it was found that 0.74% of the total aspalathin

consumed was excreted over 12 hour period in the form of 3-*O*-methylaspalathin (162 mg) and 3-*O*-methylaspalathin glucuronide (87 mg), with the first metabolite predominant (Courts & Williamson, 2009). In another human study conducted by Stalmach and colleagues (2009), 500 mL of fermented rooibos and green rooibos beverage were consumed on different occasion by the same subject, and 8 metabolites were found in the human urine. The main metabolite found after ingestion of green rooibos was an *O*-methyl-aspalathin-*O*-glucuronide, while eriodictyl-*O*-sulfate was the main metabolite after ingestion of fermented rooibos (Stalmach *et al*., 2009).

We believe that use of plant-based prebiotic phenolic compounds can modulate changes in the gut microbiota, in particular the rooibos phenolic compound aspalathin is of interest. Prebiotics are defined as substrates that are selectively utilized by host microorganisms for the health benefit of the host (Gibson *et al*., 2017). For food to be classified as prebiotic, it must not be hydrolyzed or absorbed in the upper gastrointestinal tract, it must be a selective substrate for a limited number of beneficial commensal bacteria in the gut stimulating growth of the beneficial bacteria, and it must be able to alter the guts composition for a healthier gut microbiota composition (Gibson *et al.*, 2010). Aspalathin meets these criteria as it resists deglycosylation by enzymes of the small intestine and reaches the lower intestines in large amounts.

2.4. USE OF NON-HUMAN PRIMATE IN RESEARCH

Research involving non-human primates (NHPs) has played a vital role in many of the medical and scientific advances of the past century. NHPs are used because of their similarity to human physiology, neuroanatomy, reproduction, development, cognition, and social complexity. Yet, it is these very similarities that make the use of NHPs in biomedical research a considered decision (Phillips & Everling, 2014). NHPs used for biomedical research include; *Cebus albifron* (new world monkeys), *Lemur catta* (ring-tailed lemurs), *Saimiri sciureus* (squirrel monkeys), *Aotus trivirgatus* (owl monkeys), *Papio spp* (baboons), *Macaca mulatta* (rhesus macaques), *Saguinus spp* (marmosets) and *Chlorocebus aethiops* (African green monkey, vervet). The total number of non-human primates used in research was 71 317 in 2010 according to the United States Department of Agriculture (USDA) (Animal Welfare Institute, 2018).

Non-human primates are increasingly used in pharmaceutical and bioterrorism experiments. Researchers continue to promote the development of NHP models for a variety of human diseases and conditions. NHPs are natural hosts of infectious agents and they are also susceptible to many human infections such as measles and tuberculosis. Newly acquired NHPs are usually quarantined for 3 months before introducing them into a colony (Parrott, 2019). NHP models are being used to study the gut microbiome due to their close phylogenetic relationship and similar phylogeny with humans. However, the human gut microbiota has evolved from a mainly plant-based diet to a humanised diet in which the digestibility of food is increased by cooking and other food processing techniques. Fortunately, for researchers most NHPs readily accept a humanised diet, making NHPs ideal models to study diseases related to diet.

Vervet monkeys (*Chlorocebus pygerythrus*) also known as the African green monkey, are long-tailed monkeys. Vervet monkeys belong to the *Chlorocebus* genus, there are six species under the genus recognized in Africa namely; *Ch. aethiops, Ch. cynosures, Ch. djamdjamensis, Ch. pygerythrus, Ch. sabaeus,* and *Ch. tantalus,* of which *Ch. Aethiops* was recorded as occurring in Southern Africa (Groves, 2001).

Vervet monkeys are medium sized primates which live in highly social groups of up to 50 individuals, they have long canines which protrude 3.2cm from the gum line. Males are bigger than females. Males range from 420mm to 600mm in height and from 3.9kg to 8.0kg in weight, while the females range from 300mm to 495mm in height and 3.4kg to 5.3kg in weight. Vervet monkeys have approximately equal length of arms and legs, and they use all four limbs to move around on both land and trees, they can also swim (Ramulondi, 2014).

The vervet monkey is one of two African non-human primate species, endemic to Southern Africa, and which is most commonly utilized in biomedical research (Kavanagh *et al*., 2007; St Clair *et al*., 1981). Vervet monkeys are used in many fields including virology, bacteriology, parasitology, neurology, toxicology, reproduction and cell biology and they have proven to be particularly useful in the areas of cardiovascular and metabolic disease (Fairbanks *et al*., 2010; Fincham *et al*., 1998; Kavanagh *et al*., 2007; Louw *et al*., 1997; Martin *et al*., 1990; Rudel *et al*., 1981; Smuts *et al*., 1992; Suckling & Jackson, 1993; Wallace *et al*., 2005).

Non-human primates are used as models to study human disease as they are more biologically relevant research models and are unmatched in terms of their relevance than other animals used to study many human conditions (Clayton *et al*., 2018). Recently, Amato *et al*, conducted the study showing differences in response to diet between humans and vervet monkeys (Amato *et al*., 2015). Humans show relative abundances of *Firmicutes* and reduced relative abundances of *Bacteroidetes* while vervet monkeys had an opposite pattern of a reduction in *Firmicutes* relative abundances and an increase in *Bacteroidetes* relative abundances in response to a Western diet (Amato *et al*., 2015).

2.5. CULTURING SYSTEMS

There are various techniques of culturing anaerobic bacteria such as using the special anaerobic culture media (pre-reduced media), anaerobic jar (GasPak anaerobic system), anaerobic bags or pouches, anaerobic chamber (anaerobic glove box), and the Speed Breedy culturing system. Pre-reduced media and anaerobic jars can be used to collect and transport samples under aerobic conditions. In this study two methods used for anaerobic culture were the anaerobic (glovebox) chamber and the Speedy Breedy® precision respirometer.

2.5.1. Anaerobic chamber

Anaerobic chambers, also known as an anaerobic glove box, are atmosphere control units designed to be used when working with oxygen sensitive specimens, products with containment needs, and/or general isolation control. These units allow researchers to easily process, culture and examine samples without exposure to atmospheric oxygen.

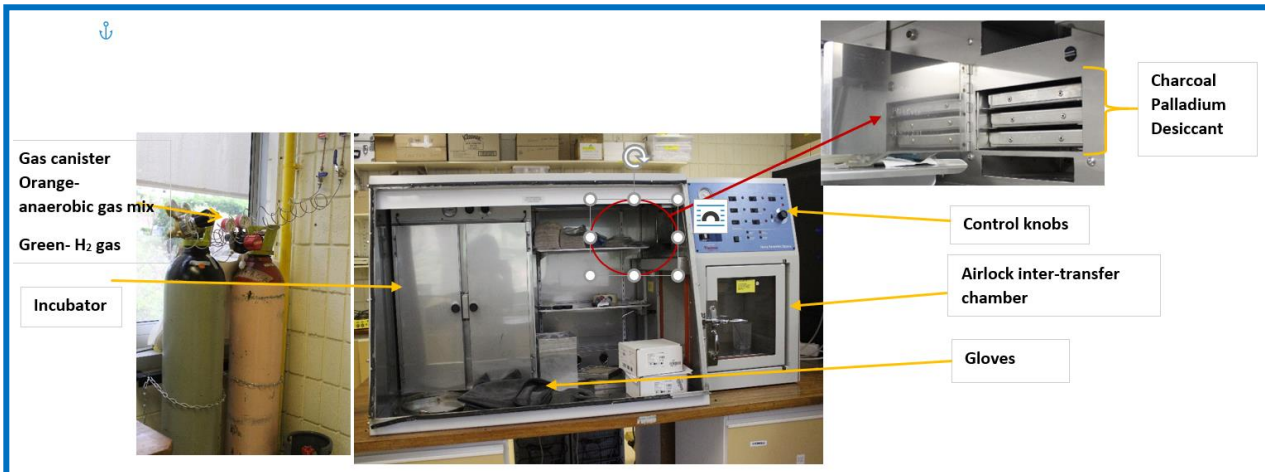


Figure 7:**Illustration of the anaerobic glove box (Image captured at Cape Peninsula University of Technology)**

Anaerobic chamber is an ideal anaerobic incubation system as it provides an oxygen-free environment at a stable pressure for culture of a large number of species. Anaerobic chamber's atmosphere is made up of 85% N_2, 10% CO_2 and 5% H_2. The chamber has glove port where rubber or neoprene gloves are used by the operator to perform manipulations within the chamber. There is an exchange chamber which is airlocked with the inner and outer chamber. The anaerobic chamber is fitted with a palladium catalyst and activated

charcoal. The palladium converts the O_2 present in the chamber into water using H_2 and the activated charcoal keeps CO_2 present in the chamber is for optimal growth of anaerobic bacteria species.

Media that was used for anaerobic culturing was maximum recovery diluent (MRD), Scheadler agar and Brain heart infusion (BHI) broth. MRD is a peptone saline diluent which is used for serial dilution. MRD contains low peptone digest concentration of animal tissue which helps maintain the organisms for 1-2 hours of dilution without multiplying (www.oxoid.com, 2018). Schaedler blood base agar is used for recovery of bacteria encountered in the gastrointestinal tract and found it to be successful in supporting growth of fastidious anaerobic microorganisms (Schaedler, 1965). Schaedler contains casein peptone, soy peptone, dextrose, yeast extracts, Tris (hydroxymethyl)-aminomethane, meat peptone, sodium chloride, L-cysteine, hemin, vitamin K, defibrinated sheep blood, agar and demineralized water at pH 7.6 (Schaedler, 1965). BHI medium is used to cultivate a wide range of microorganisms. It is a nutritive media which contains; calf brain infusion, beef/pig heart infusion, proteose peptone, dextrose peptone, sodium chloride and disodium phosphate (Sandven *et al*., 2009). These supply nutrients and proteins needed to supplement the growth of fastidious and non-fastidious growth of microbes.

2.5.2. Speedy Breedy system

The Speedy Breedy culturing system is a portable, sensitive, precision respirometer which detects and monitors microbial activity. These detections are determined as a consequence of pressure transients relating to gaseous exchanges within a closed culture vessel of a specific working volume, as a result of microbial respiration (*Speedybreedy.com*, 2018)**.** This device measures pressure consistently in the vessel. A rise in pressure occurs when fermentative organisms multiply inside the vessels. An increase in cell number is reflected by the increase in pressure measured as mbar/min as well as overall pressure in the vessel in millibars. Speedy Breedy detects the pressure in the mini-fermenter vessel by measuring the deformation of a membrane in response to the pressure generated by CO_2 produced by microorganisms. The deformation of the membrane is directly proportional to the pressure inside the vessel.

Speedy Breedy is a two-chamber vessel instrument (figure 8) that maintains culture conditions within purpose-designed disposable culture vessels. Samples are introduced via a rubber injection port into the 50 mL vessel. The vessel has a propeller that homogenizes the culture conditions for rapid microbial growth conditions. The mini-fermenter culture vessels are supplied with various dry media capsules designed for the specific purpose i.e. anaerobic microbial growth. Speedy Breedy is used in a number of applications such as testing for levels of contamination, or sterility. The Speedy Breedy culturing media used in this

study was specially prepared for anaerobic microbial culturing however the content is undisclosed (proprietary information).

Figure 8: Speedy Breedy culturing system, indicating the incubation chamber (a, b) and the culturing vessels (a, d). Speedy Breedy system consists of two chambers that can operate independently, controlled by a computer system (c) that monitors temperature and pressure during the microbial growth. The Speedy Breedy vessels (d) are supplied with the specific culturing media contained in a capsule which dissolves as the rotor rotates during microbial culture (Speedy Breedy, Bactest Limited, United Kingdom).

2.6. METAGENOMICS

Metagenomics is an analysis tool that can be used to study complex microbial communities collected directly from their environments, without culturing or isolation. It is an useful tool to elucidate various characteristics of a sample and to identify microorganisms species present and can provide insight into the metabolic activities and functional roles that the microbes play in the environmental samples (Ghosh *et al*., 2019). Various metagenomics analysis techniques provide information on taxonomic diversity (which species are present) and functional metagenomics (role each species play in the diversity). Taxonomic diversity analysis is achievable by amplification of targeted specific genes using PCR primers such as the variable regions V1 – V9 of 16S rRNA, 18S rRNA and ribosomal ITS before gene sequencing. There are two most commonly used methods of metagenomic analysis, which are the amplicon-based method and whole metagenomic shotgun sequencing (Ghosh *et al*., 2019).

2.6.1. Types of metagenomic analysis

Whole-genome shotgun (WGS) sequencing is a more precise method of sequencing as it randomly selects fragments from the entire genome. With this method of analysis, samples can be classified up to species level, but it is more expensive to run as it requires extensive analysis (Ranjan *et al*., 2016). WGS can be further be divided into two sequencing methods, namely sequence-based screens and functional screens. Sequence-based screens focus on describing microbial diversity and genome sequences of a sample, while the functional screens identify the functional gene product, but it does not identify which species it originates from (Madhavan *et al*., 2017). WGS allows higher sequencing depth for detection of rare taxa (Ghosh *et al*., 2019).

Another type of metagenomic analysis is the amplicon-based 16S rRNA sequencing method, this contains two amplicon-based methods focused on, which are the 16S rRNA and 18S rRNA/ribosomal ITS. The 16S rRNA sequencing is a widely used technique for microbial diversity analysis for environmental samples such as the soil and gut microbiota, amongst others. This method solely relies on investigating the hypervariable regions (V1-V9) of the bacterial 16S ribosomal RNA to make community-wide taxonomic assignments. 16S rRNA sequencing works by grouping almost identical sequences in the same operational taxonomic unit (OTU). Some degree of divergence is allowed, but sequence has to be >97% to be grouped under the same OTU. 16S rRNA has its disadvantages as it cannot distinguish between two species that have the same OTU. It is generally sensitive until the genus level (Morgan & Huttenhower, 2012). The 18S rRNA is normally used for fungal identification. The 18S rRNA region contains both hypervariable and conserved regions. The internal transcribed spacer region (ITS) is positioned between the 18S and the 5.85S

regions. ITS is used for analysing fungal diversity on samples (Ghosh *et al*., 2019). For our current study, we have used the amplicon-based method focusing on the 16S rRNA region.

2.6.2. Ion torrent

Ion torrent is a semiconductor sequencing method of our choice. Ion torrent sequences DNA by detecting hydrogen ions produced by the DNA sample through the release of hydrogen ion during polymerization of DNA on a semiconductor chip. Ion torrent is different from other sequencing technologies because it uses a pH-based sequencing strategy and there are no modified nucleotides or optics used.

2.7.3. Ion torrent principle

A semiconductor chip contains an array of microwells that each contains many copies of single stranded DNA template embedded on a microbead with a DNA polymerase. Each well gets flooded with a single unmodified deoxyribonucleoside triphosphate (dNTP) at a time. The incorporation of a nucleotide onto the single strands forms a covalent bond which releases pyrophosphate and a hydrogen ion. If the nucleotide in the well corresponds to the next available nucleotide, it incorporates into a growing complementary strand by DNA polymerase. If the nucleotide is not complimentary, then there's no assimilation. The hydrogen ion that gets released during polymerization changes the pH of the solution and that gets detected by ion-sensitive field-effect transistor (ISFET) which seats on a layer underneath the wells of the complementary metal-oxide- semiconductor (CMOS) chip. The unattached dNTP which remains uncharged are washed before the next cycle when a different dNTP is introduced. The ISFET transmits the chemical information into the computer to be digitalized, this is all recorded as sequencing, process happens every 15 seconds (Rusk, 2010, Pennisi, 2010).

2.7.4. Ion torrent library preparation

Next generation library is a collection of similar sized DNA fragments with known adapter sequences added to the 5'-end and the 3'-end. Library preparation contains four steps i.e., DNA fragmentation or target selection, adapter sequences, size selection, and final library quantification and quality control. DNA fragmentation takes place by a physical method or enzymatic methods of fragmentation. Mostly, when the quantification size is known, PCR amplification is used as the primers focus and amplify the region of interest within the desired size range. Adapter sequences are added to the DNA fragment, specific adapter sequences are added and the adapter sequences, usually 20 – 30 base pairs long, and their fragments are known. One adapter attaches to the 5'-end while the other attaches to the 3'-end. One adapter sequence contains the primer annealing site while the other adapter contains the DNA binding site where sequencing

will start. The third step of library preparation is selecting the library fragment size (Chen *et al*., 2018). There are two methods that can be used for this, it is either electrophoresis separation-based method or the bead-based size method. The bead-based separation employs the use of magnetic beads with variant buffer concentrations to isolate the DNA fragment size of interest. Size selection is critical as it affects the downstream process of DNA sequencing. The last step of library preparation is library quantification and quality control. Accurate analysis is vital as this affects sequencing, for analysis of these DNA fragments either bioanalyzer system is used or the qPCR is used. Bioanalyzer provides fragment size and library concentrations, while qPCR measures the amplifiable library information but lacks library size information. qPCR is the most accurate form of analysis to use (Chen *et al*., 2018).

CHAPTER III

3. METHODOLOGY

3.1. ANIMAL SELECTION

A total of six vervet monkeys (*Chlorocebus pygerythrus*) were randomly selected from two main groups: 1) the standard diet group (n=3) which received a maize based normal diet, and 2) the Western diet group (n=3) which was supplied with a high fat diet (Table 2). The normal diet comprised of 11% fat, 15% protein, 74% carbohydrates, while the Western diet consisted of 40% fat, 15% protein, and 44% carbohydrates. The animals were maintained on their respective diets for a minimum of 5 years prior to their selection for participation in this study. The monkeys were fed twice daily (morning and noon), receiving food at each time point, and they were supplemented with fresh fruits and vegetables. Physiological parameters such as body weight and fasting blood glucose concentrations were measured for the selected animals.

Table 2: Characteristics of the monkeys selected for the study

Monkey number	Diet	Sex	Body Weight (kg)	Age (years)	Blood glucose level (mmol/L)
M249	Standard diet	Male	5.34 kg	20	4.2
M268	Standard diet	Male	4.82 kg	19	4.0
M238	Standard diet	Male	4.95	20	6.3
M403	Western diet	Female	3.98 kg	12	6.5
M281	Western diet	Male	3.19 kg	18	10.4
M343	Western diet	Male	5.79 kg	16	12.4

3.2. ANIMAL CARE AND ETHICS APPROVAL

The vervet monkeys participating in this study were in-house bred and maintained at the Primate Unit and Delft Animal Centre (PUDAC) of the South African Medical Research Council (SAMRC), in accordance to all the relevant guidelines and standard operating procedures. All animals were housed in a sterile, airconditioned area with temperature ranging from 23 – 26^{o}C; humidity of 45-50% and 12-hour light cycle. The animals were contained individually in a single cage (900cm x 700cm x 1200cm) with foraging pans and grooming panels that allowed social interactions. Furthermore, animals were allowed to leave their home cages to interact with other animals with the aid of additional exercise cages. Ethical approval on the

use of animals was obtained from the Ethics Committee for Research on Animals (ECRA) of the SAMRC (Ref 08/18).

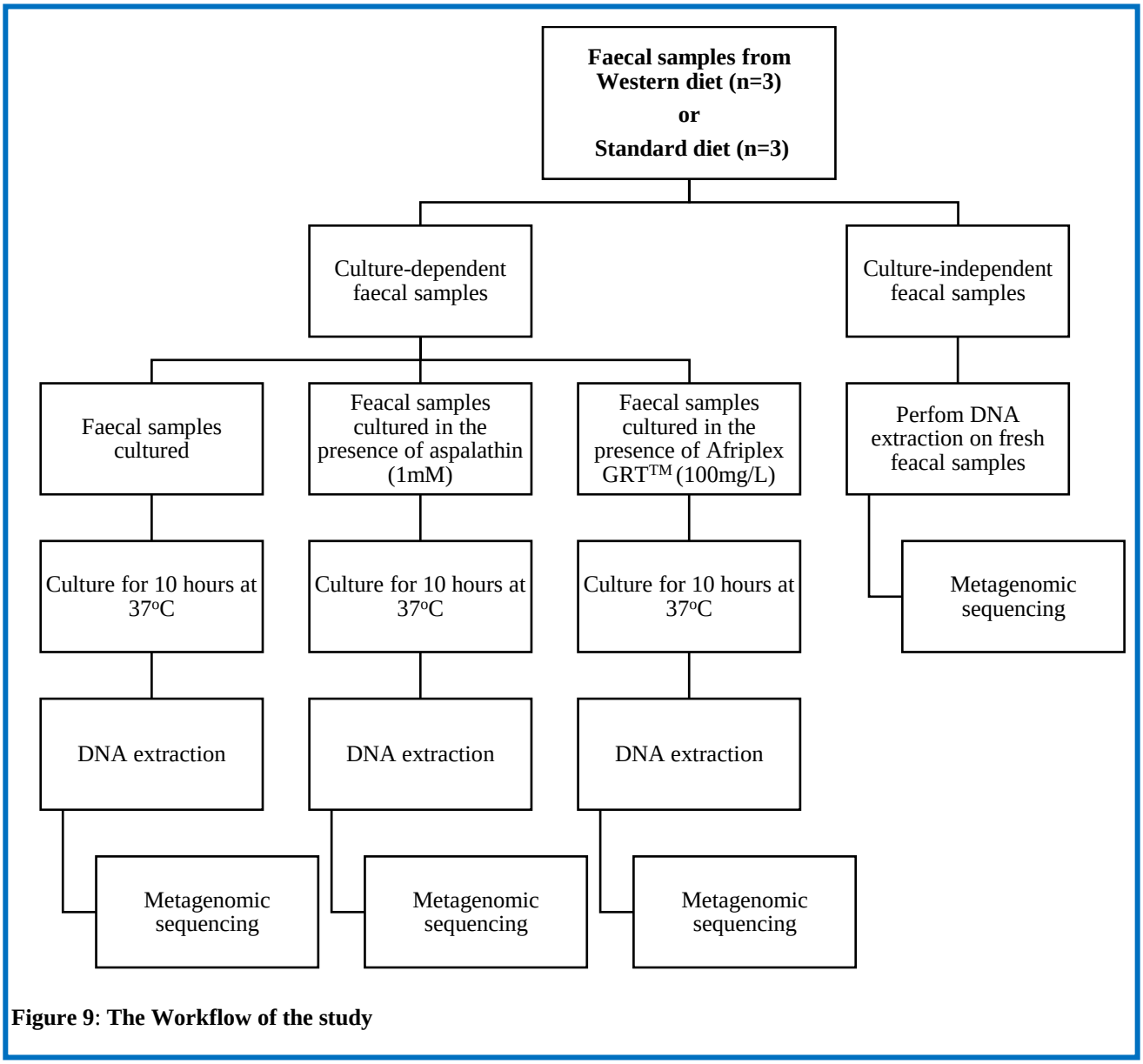


Figure 9: **The Workflow of the study**

3.3. SAMPLE COLLECTION AND HANDLING

The faecal samples were collected from the animals in both groups at PUDAC between 7- 9 AM. Faecal samples were collected from clean stainless-steel cage pans and transferred into sterile stool collection containers. The containers were immediately place into an anaerobic jar and kept under anaerobic conditions at room temperature using AnaeroGen (Thermo scientific, USA) until culture at the SAMRC and Cape Peninsula University of Technology, District six campus, respectively.

3.4. SPEEDY BREEDY CULTURING

A faecal slurry was prepared by dissolving one gram of faecal sample in 9mL of distilled water. The faecal samples were vortexed at the high speed for three minutes, followed by centrifugation at 13000xg for five minutes. The supernatant, composing of the gut microbiota, was retained as the object of interest. After the solution was centrifuged, 500µL of the solution was transferred into 50mL Greiner tube and it was topped up with distilled water for a final volume of 50mL without addition of any rooibos phenolic compounds. Samples cultured with Afriplex GRT™ and aspalathin were added to the faecal slurry solution, respectively. Afriplex GRT™ and aspalathin were dissolved in water and the final volume was made up to 50mL.

The sample solution was added aseptically to the Speedy Breedy fermentative vessels, using a 50mL sterile syringe. The samples were then cultured at 37°C for 10-hour period using Speedy Breedy culturing system. The bacterial growth curves were monitored using the Speedy Breedy software throughout culturing. The Speedy Breedy anaerobic culture media is an all culture broth which composed of proteose peptone, beef extract, yeast extract, malt extract, dextrose and ascorbic acid (pH= 7.2). The media was provided lab-ready inside the sterile anaerobic fermentative vessel provided by Speedy Breedy (Bactest Limited, UK). After culturing the samples were then centrifuged and the pellet used for DNA extraction.

3.5. ANAEROBIC CHAMBER

Maximum Recovery diluent (MRD) media was prepared and conditioned under an 85% N_2, 10% CO_2 and 5% H_2 anaerobic chamber for 24 hrs. Anaerobically, faecal slurry was prepared in a 50mL Greiner tube using the Maximum Recovery Diluent (MRD) media. One gram of faecal sample was dissolved in 10 mL of MRD. The mixture was vortexed and allowed to settle for 15 minutes. One millilitre of sample was aliquoted in 9mL of MRD media and serial dilutions were prepared up-to 10^{-8} dilution. One hundred

microliters of each sample were pipetted onto a 5% sheep's blood agar plate, and then incubated for 24 to 48 hours at 37^{o}C, to assess the growth of bacterial colonies.

After the 48 hours incubation period, colony forming units (CFU) were counted. Single colonies were picked and inoculated in 10mL Brain Heart Infusion broth (BHI) and incubated for 72 hrs at 37^{o}C. After cultivation, ten millilitres of cultured samples were removed, and used for subsequent DNA extractions and Gram staining.

Gram staining was done from the samples cultured on broth media and also on the plated single colonies according to the standard operational procedure. The slides were examined with x100 oil-immersion objective lens on a light microscope (Olympus, Life Bioscience).

3.6. TREATMENT CONCENTRATIONS

3.6.1. Aspalathin

In this study two concentrations of aspalathin tested to see which concentrations would invoke change in the gut microbiota. The two concentrations that were used were 100µM and 1mM. After repeated experiments that were conducted it was concluded that 1mM concentration was more effective than 100µM. Therefore, the 1mM concentration was used for the rest of the study. Aspalathin has a molar weight of 452g/mol that was adjusted to get the desired concentrations. The final volume for each sample was 50mL.

3.6.2. Afriplex GRT™

The Afriplex GRT™ concentration used, 100mg/L, was selected based on previously conducted cells culture studies, as this concentration was not toxic to the cells used, therefore, it was assumed it was safe to be used for anaerobic bacterial culture (Muller *et al*., 2012). The final volume was 50mL.

3.7. DEOXYRIBONUCLEIC ACID (DNA) EXTRACTION

Deoxyribonucleic acid (DNA) was extracted using the NucleoSpin® DNA Stool kit (Macherey-Nagel, Germany), according to the manufacturer's instructions. Briefly, 250mg weighed pellet or 500µL dissolved pellet was used for the extraction. Pellets were obtained from 50mL Speedy Breedy vessel or 10 mL from anaerobic chamber. Extracted DNA was eluted in 50µL of elution buffer and assessed in terms of concentration and purity using the NanoDrop ND-8000 (Thermo Scientific, USA). Samples with a DNA concentration less than 20ng/µL or a ratio of absorbance of A260/280 less than 1.8, and A260/230 less than 1.9 were repeated for further purification and to meet the quality standards of DNA. A supplementary table containing the quantity and quality of the DNA used for PCR and metagenomic sequencing is presented under Appendix A (Table S1).

3.8. POLYMERASE CHAIN REACTION (PCR)

3.8.1. Primer preparation

Quantification of different taxa by PCR was performed using primers targeting *Firmicutes, Bacteroidetes,* and *All Bacteria*. All primer sets were purchased from the Integrated DNA Technologies (Integrated DNA Technologies Inc., Coralville, United States) at a 100µM stock solution. Working solutions of each primer set were prepared at 10µM. Primers used are illustrated in table (3).

Table 3: The list of primers and annealing temperature used

Category	Primers		Base pairs (bp)		Annealing temperature (º C)
	Forward primer	Reverse primer			
Firmicutes	5’ ATG TGG TTT ATT TCG AAG CA3’	5’ AGC TGA CGA CAA CCA TGC AC 3’	20	20	49.15
Bacteroidetes	5’ CAT GTG GTT TAA TTC GAT GAT 3’	5’ AGC TGA CGA CAA CCA TGC AG 3’	21	20	48.35
All bacteria	5’ ACT CCT ACG GGA GGC AGC AG 3’	5’ ATT ACC GCG GCT GCT GG 3’	20	17	55

Melting temperature was calculated using the Marmur and Doty equation illustrated in equation (1) and annealing temperature was calculated using annealing temperature formula in equation (2).

$$Tm = 64.9 + \frac{41(G+C)-16.4}{G+C+A+T} \quad \textbf{Eq. (1)}$$

The above equation was chosen as the most appropriate equation as the primers used have base number of more than 13 bases. The annealing temperature was calculated based on the melting temperature obtained, using equation (2).

$$Ta = \frac{Tmf+Tmr}{2} - 5°C \quad \textbf{Eq. (2)}$$

The annealing temperature for each primer set were represented in table (3).

3.8.2. Conventional PCR Composition

The conventional PCR was performed using the Veriti 96 well Thermal Cycler (Applied Biosystems, California, United States) using Hot Star Taq polymerase kit (Qiagen, Germany). Given in the table below (4) are the Master Mix reagents making a 20µL total volume for each DNA product.

Table 4: The PCR reaction mixture. This table contains the reagents and volume needed for a successful PCR reaction

Order	Component	Volume (uL)	Final concentration	Company
1.	Nuclease free water	8.375	x1	Ambion (USA)
2.	Q solution	5	x1	Qiagen (Germany)
3.	10x PCR Buffer	2.5	x1	Qiagen (Germany)
4.	MgCl	2	x1	Qiagen (Germany)
5.	dNTPs	0.5	x1	Qiagen (Germany)
6.	Primer A (Forward)	1.25	10µM	Integrated DNA Technologies (Coralville, IA USA)
7.	Primer B (Reverse)	1.25	10µM	Integrated DNA Technologies (Coralville, IA USA)
8.	Taq DNA polymerase	0.125	x1	Qiagen (Germany)
9.	Template DNA	1	x1	-

The final reaction mixture was prepared for 32 samples in duplicate. Three primer sets were used as illustrated in table (3).

Table 5: PCR program. The PCR reaction is set for 35 cycles with different annealing times for each gene used.

Step	PCR temperature (ºC)	Time (minutes)	Cycle
Initial denature	95	15	1x
1. Denaturation	94	0.5 - 1	35x
2. Annealing	Table 2	0.5 - 1	
3. Extension	72	1	
Final extension	72	10	1x
Storage	4	∞	-

PCR program was set according to the parameters set on table (5). The integrity of extracted DNA was assessed using a 1.5% agarose gel.

3.9. THE 16S METAGENOMIC SEQUENCING (ION TORRENT NEXT GENERATION SEQUENCING)

3.9.1. Amplification and Library preparation

In order to unravel the effect of diet and/or phenolic compounds on gut bacteria, targeted 16S ribosomal RNA (rRNA) metagenomic sequencing was performed on thirty-two DNA samples using the Ion Torrent platform at the Central Analytical Facility (CAF), Stellenbosch University. To this end, hypervariable regions from polybacterial samples were amplified using the Ion 16S™ Metagenomics Kit, according to the manufacturer's guidelines. Target regions were amplified from 2µL DNA, across 25 cycles with two primers pools on the SimpliAmp Thermal Cycler (ThermoFisher Scientific, USA). The presence of amplified products was verified on the PerkinElmer LabChip® GXII Touch (PerkinElmer, Waltham, MA, USA), using the X-mark chip and HT DNA NGS 3K reagent kit according to the manufacturer's protocol. Following verification, PCR products from the two primer pools were combined for each sample, purified with Agencourt™ AMPure™ XP reagent and eluted in 15µL nuclease free water. Purified amplicons were quantified on the Qubit 4.0 Fluorometer using the Qubit 1x dsDNA HS assay kit according to the protocol.

Library preparation was performed from 100ng pooled amplicons for each sample using the NEXTflex DNA Sequencing Kit. Briefly, 40µL of each purified pooled PCR product was end-repaired at 22°C for 30 minutes, using 3µL Endrepair enzyme mix and 7µL End-repair buffer in a final volume of 50µL. The end-repaired products were purified with Agencourt™ AMPure™ XP reagent. Nineteen microlitres end repaired product was ligated to 4µL IonCode™ Barcode Adapter with the addition of 31.5µL Ligation mix at 22°C for 15 minutes. The adapter-ligated, barcoded libraries were purified with Agencourt™ AMPure™ XP reagent and quantified using the Ion Universal Library Quantitation Kit. Quantitative PCR (qPCR) amplification was performed using the StepOnePlus™ Real-time PCR system (ThermoFisher Scientific) and library fragment size distributions were assessed on the LabChip® GXII Touch (PerkinElmer, Waltham, MA, USA), using the X-mark chip and HT DNA NGS 3K reagent kit according to the manufacturer's protocol.

3.9.2. Template preparation, enrichment and sequencing

Libraries were diluted to a target concentration of 10pM. The diluted, barcoded, 16S libraries were combined in equimolar amounts for template preparation using the Ion 510™, Ion 520™ & Ion 530 Chef Kit. In brief, 25µL of the pooled library was loaded on the Ion Chef liquid handler using Reagents, Solutions and Supplies according to the protocol. Enriched, template positive ion sphere particles were loaded onto an Ion 530™ Chip. Massively parallel sequencing was performed on the Ion S5™ Gene Studio using the

Ion S5™ Sequencing Solutions and Sequencing Reagents Kits according to the manufacturer's protocol. Flow space calibration and BaseCaller analyses were performed using default analysis parameters in the Torrent Suite Version 5.12.0 Software. Raw run data was uploaded to the relevant IonReporter cloud account.

3.10. AMPLICON DATA PROCESSING AND ANALYSIS

Generated raw sequence data files (.fastq) were pre-processed to exclude reads shorter than 10 base pairs (bp) and subjected to taxonomic and diversity analyses using the Quantitative Insights In Microbial Ecology pipeline (QIIME 2) using standard scripts and parameters. The latter is an open source bioinformatics software package that aids with the preparation, analysis and visualisation of large datasets (Caporaso *et al*., 2010). The OTUs were taxonomically classified using the BLAST method, as implemented in QIIME. Curated MicroSEQ® 16S reference library v2013.1 and Curated greengenes V13.5 were used as the refencing databases. A maximum E-value of 1E-10 was used to record an OTU assignment. To measure the alpha diversity, multiple rarefaction analysis, i.e. iterative (10x) subsampling at different depth intervals, was performed and the Chao1 richness estimator, observed OTUs and Shannon diversity index computed for each rarefied OTU table (Chao, 1984; Shannon, 1948). To test for significant differences between the sample groups, non-parametric two-sample t-tests were performed for each alpha diversity metric. The OTUs were further grouped into phylotypes according to their taxonomic identities, ranging from the phylum to species level, and the relative sequence abundance of the identified taxa evaluated per sample. For genus cut-off, percentage identity value is 97%, while species cut-off value is 99%. The difference in percentage match between the top hit and the next hit is 0.2%, if a genus or a species is found within this range of percentage difference, then it will be reported as slash call.

3.11. STATISTICAL ANALYSIS

All the results were analysed using GraphPad Prism® 8. Statistical analysis was done using unpaired parametric t-test with Welch's correction and non-parametric Mann-Whitney test analysis. The results were expressed as the mean ± standard error of mean (SEM). A probability of $p < 0.05$ was considered significant.

CHAPTER IV

4. RESULTS

4.1. RESULTS STRUCTURE

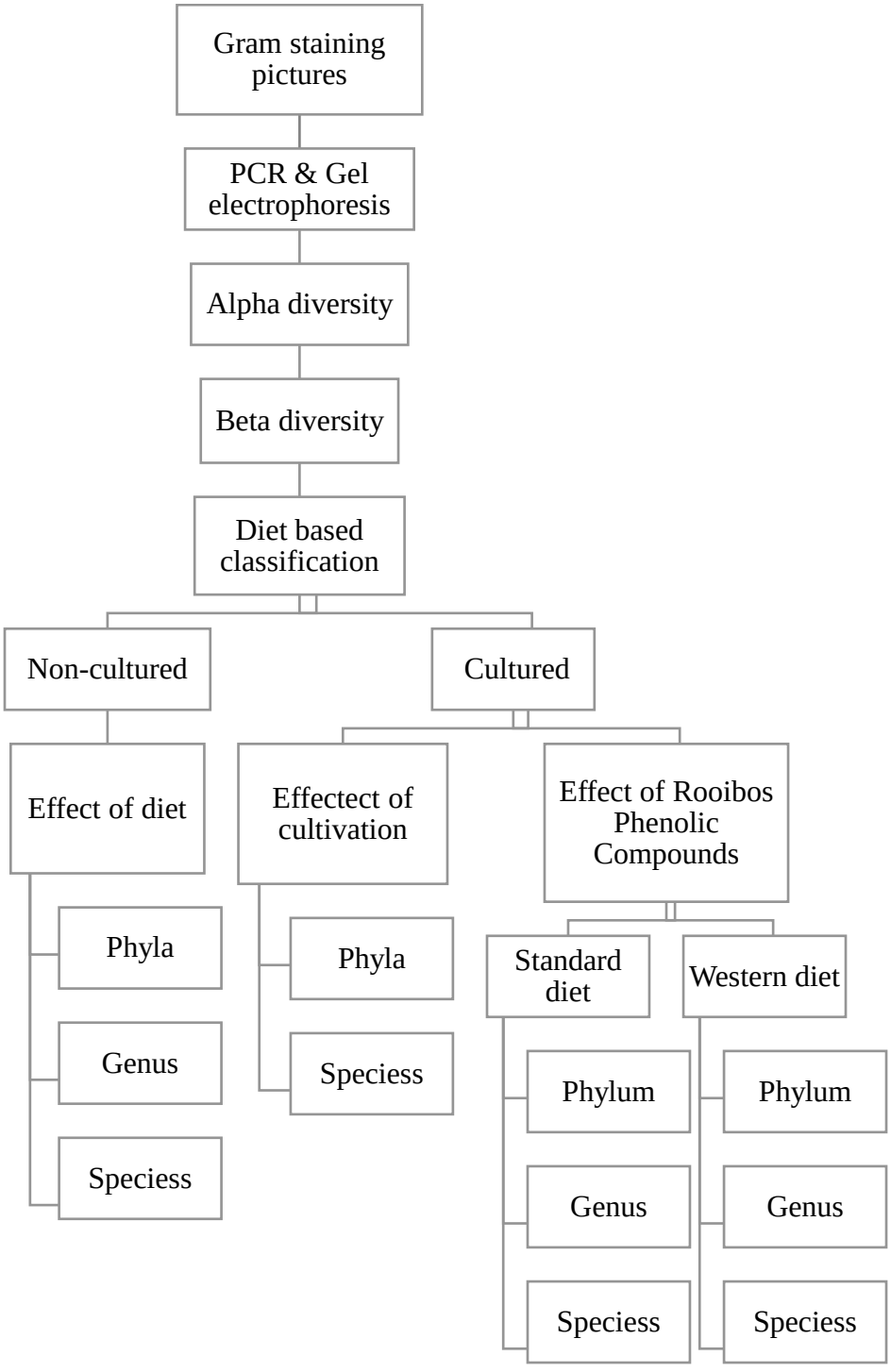


Figure 10:Schematic representation of the results.

4.2. OBSERVED CULTURABLE BACTERIA

The faecal samples were collected from vervet monkeys fed a normal maize diet and Western diet. Fresh stools were collected and immediately placed into an anaerobic jar to preserve the anaerobic environment until culture. In the laboratory baseline samples were taken before culture for analysis (culture-independent) and the rest of the samples were prepared and cultured under anaerobic conditions (culture-dependent) using anaerobic chamber and a Speedy Breedy culturing systems for 48 hours and 10 hours respectively.

For Gram staining after anaerobic culturing light microscopic images (figure 11) were captured under oil-immersion with a light microscope. Mixed cultures of Gram-negative and Gram-positive bacteria were observed in all samples, with Gram-negative being dominant. Gram-negative rods and Gram-positive cocci were also observed in all the images. In both experimental groups Gram-negative, short-chained rods were most dominant. Figure 11(A – B) demonstrates a cultured faecal sample obtained from the standard diet-fed monkeys and figure 11(C – D) were samples obtained from the Western diet-fed monkeys.

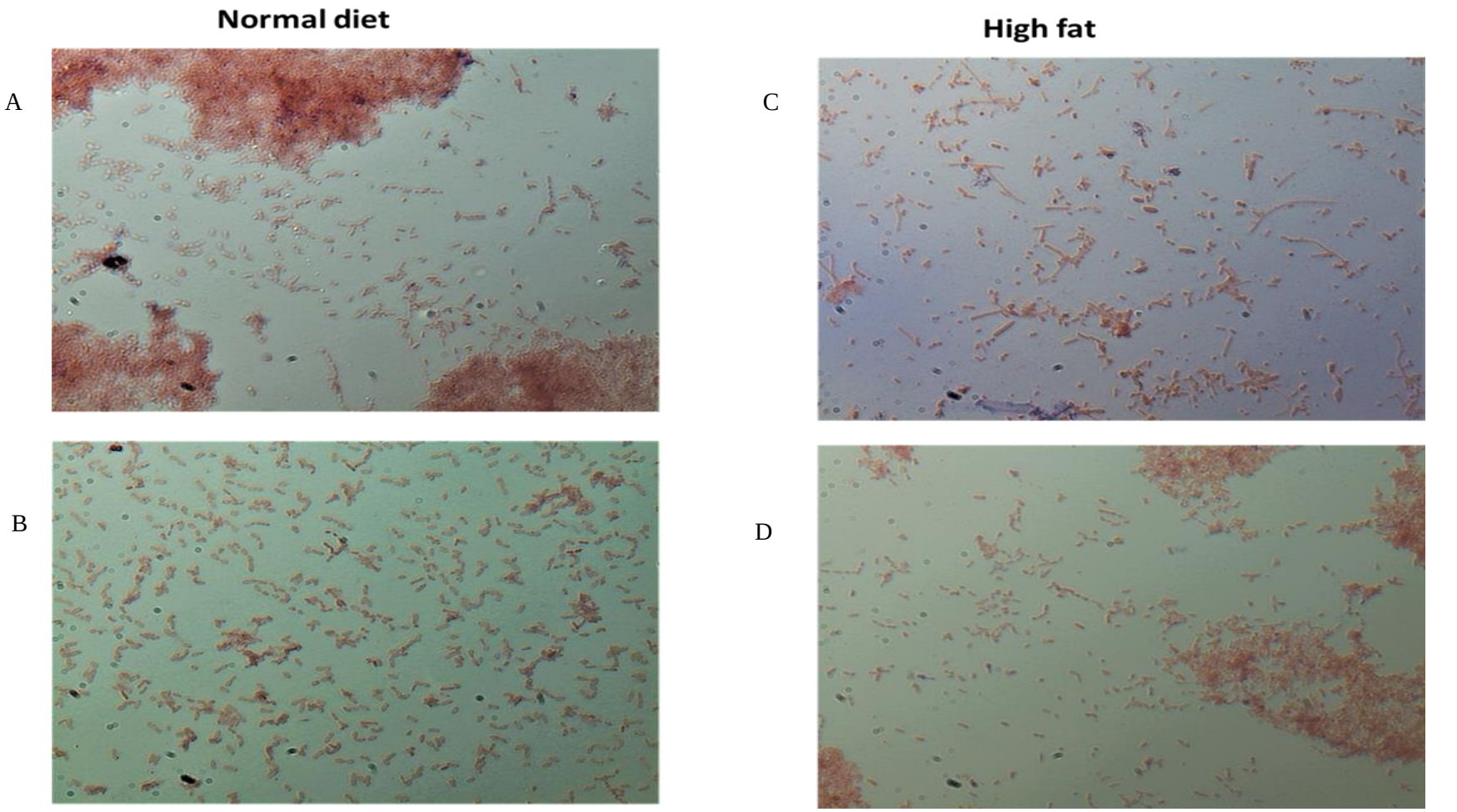


Figure 11: **Bacterial images of Gram stained cultured faecal samples observed under 1000X magnification using a light microscope**. Faecal samples obtained from standard diet-fed monkeys (A - B) demonstrated mixed cultures that were dominated by gram-negative short rods and few gram-positive cocci. Western diet images (C - D) was dominated with gram-negative short and long rods, with a few Gram-positive cocci.

4.3. DNA, PCR AND GEL ELECTROPHORESIS

The DNA from culture-independent and culture-dependent faecal samples were extracted using the NucleoSpin® DNA Stool (Macherey-Nagel) kit. Gel electrophoresis was run on 1.5% agarose Figure (12), with PCR products targeting the two most abundant phyla *Bacteroidetes* and Firmicutes. All bacteria targeted all other gut microbiota phyla present. The size of the Firmicutes and *Bacteroidetes* PCR products were found to be 100 bp, while all bacteria PCR products were about 150 bp. This indicates that all PCR reactions were a success, and all the targeted phyla were present, with all bacteria primer amplification products being bigger than those of *Firmicutes* and *Bacteroidetes*.

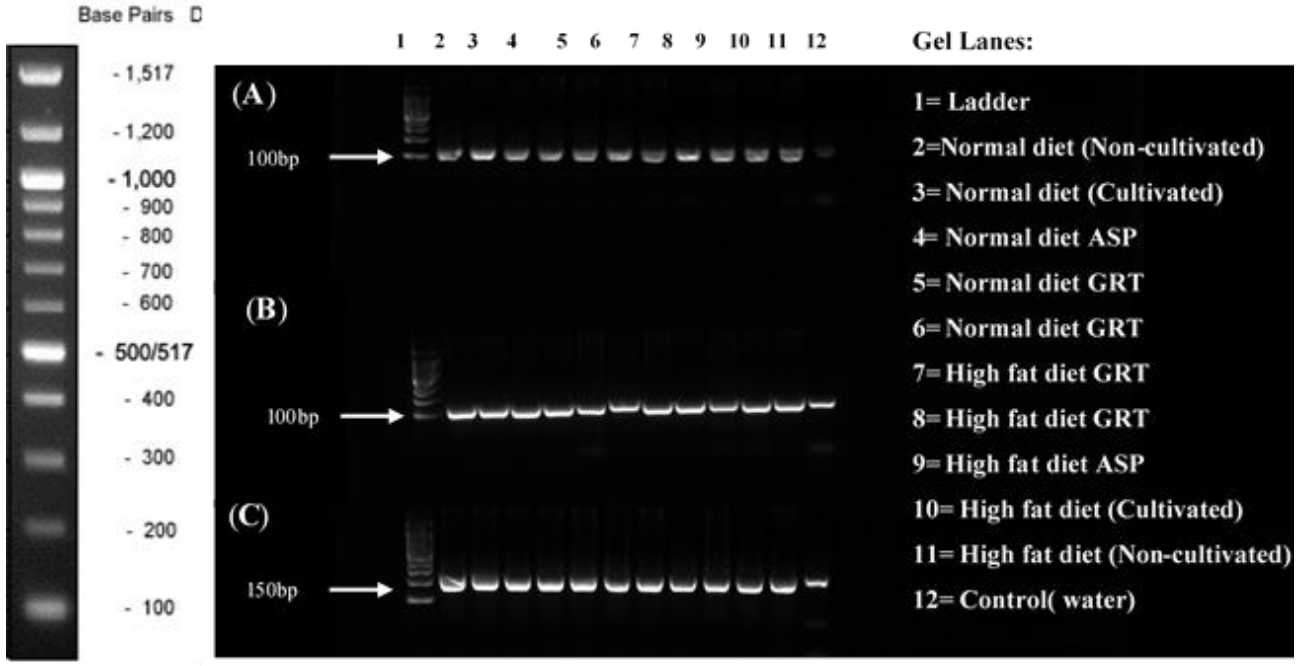


Figure 12: The 1.5% agarose gel electrophoresis. This shows the specificity of PCR amplification with three specific primer sets. PCR products amplified from the monkey's faecal DNA samples with group-targeted primers as follows: Gel A-*Bacteroidetes,* Gel B - *Firmicutes* and Gel C - *all bacteria.*

4.4. ALPHA DIVERSITY

Alpha diversity represents the number of different species that were detected in a sample. In this case, it detected the abundance of the species in a faecal sample obtained from the standard diet and Western diet-fed monkeys.

4.4.1. Preliminary optimization work

Two anaerobic culturing devices were proposed to be used for enumeration of the gut microbiota namely anaerobic chamber and Speedy Breedy culturing systems. Preliminary work was done to determine which culturing device would best suit our needs. Anaerobic chamber and Speedy Breedy were used for 48 hours and 10 hours respectively. DNA extraction and metagenomic sequencing were done. The figure below illustrates the number of species observed based on the alpha diversity of the species using the two anaerobic culturing systems used.

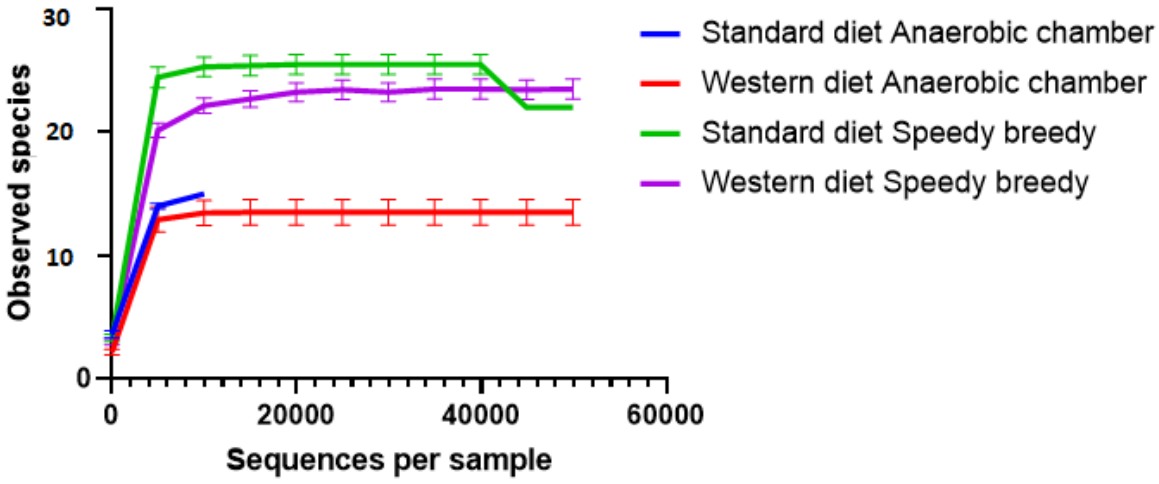


Figure 13: Comparison of two anaerobic culturing methods, anaerobic chamber and Speedy Breedy culturing systems. Speedy Breedy contains higher number of sequences per sample and higher number of species observed compared to anaerobic chamber. Stats presented as mean ±SEM.

Preliminary work revealed that the Speedy Breedy system performed better for anaerobic culture of bacteria from faecal samples as higher number of species diversity were detected from the cultures. The samples cultured using Speedy Breedy allowed deeper sequencing to be done, as compared to the samples from the anaerobic chamber. Speedy Breedy standard diet was allowed to be sequenced 49870 times per sample

while anaerobic chamber sample allowed 9982 sequences per sample. Therefore, the Speedy Breedy culturing system was used for further experimental work.

Speedy Breedy culturing system was used to determine optimum aspalathin concentration that could be used for anaerobic culture. The figure below compares aspalathin concentrations used which were 100 µM and 1 mM, respectively. When comparing the aspalathin concentrations on both diet groups, 1 mM concentration proved to promote higher species diversity than 100 µM. Therefore 1 mM concentration was used aspalathin in further studies.

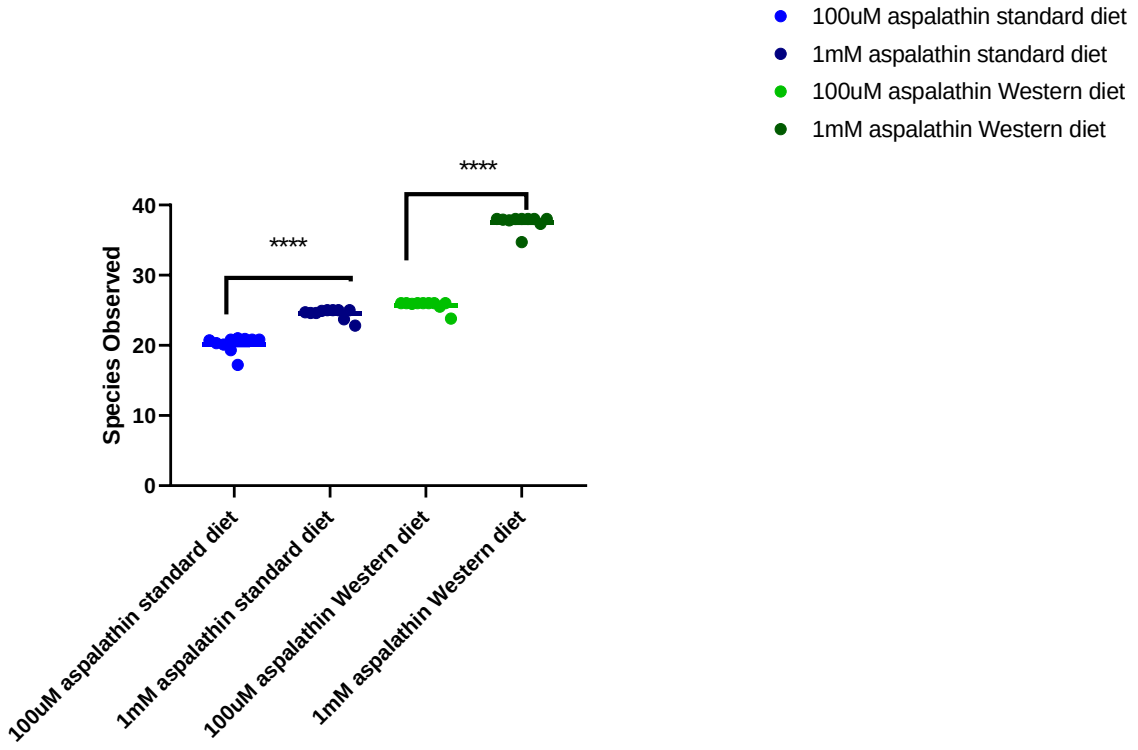
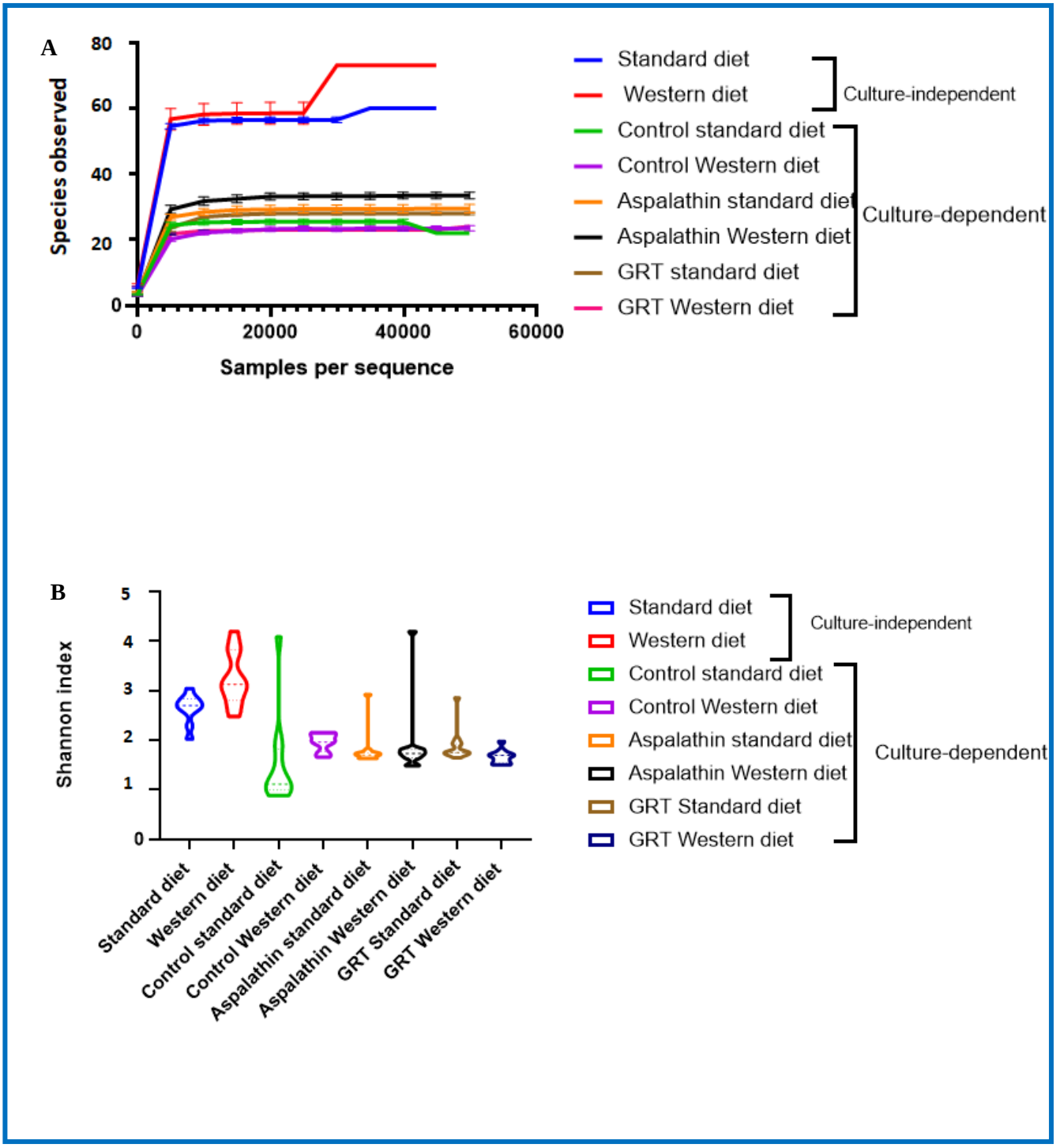


Figure 14: **Comparison of aspalathin concentrations.** 100 µM and 1mM for standard and Western diets. ****P < 0.001.

4.4.2. Results


A
Species observed
Samples per sequence
0
20
40
60
80
0
20000
40000
60000
Standard diet
Western diet
Control standard diet
Control Western diet
Aspalathin standard diet
Aspalathin Western diet
GRT standard diet
GRT Western diet
Culture-independent
Culture-dependent
B
Shannon index
0
1
2
3
4
5
Standard diet
Western diet
Control standard diet
Control Western diet
Aspalathin standard diet
Aspalathin Western diet
GRT Standard diet
GRT Western diet
Culture-independent
Culture-dependent

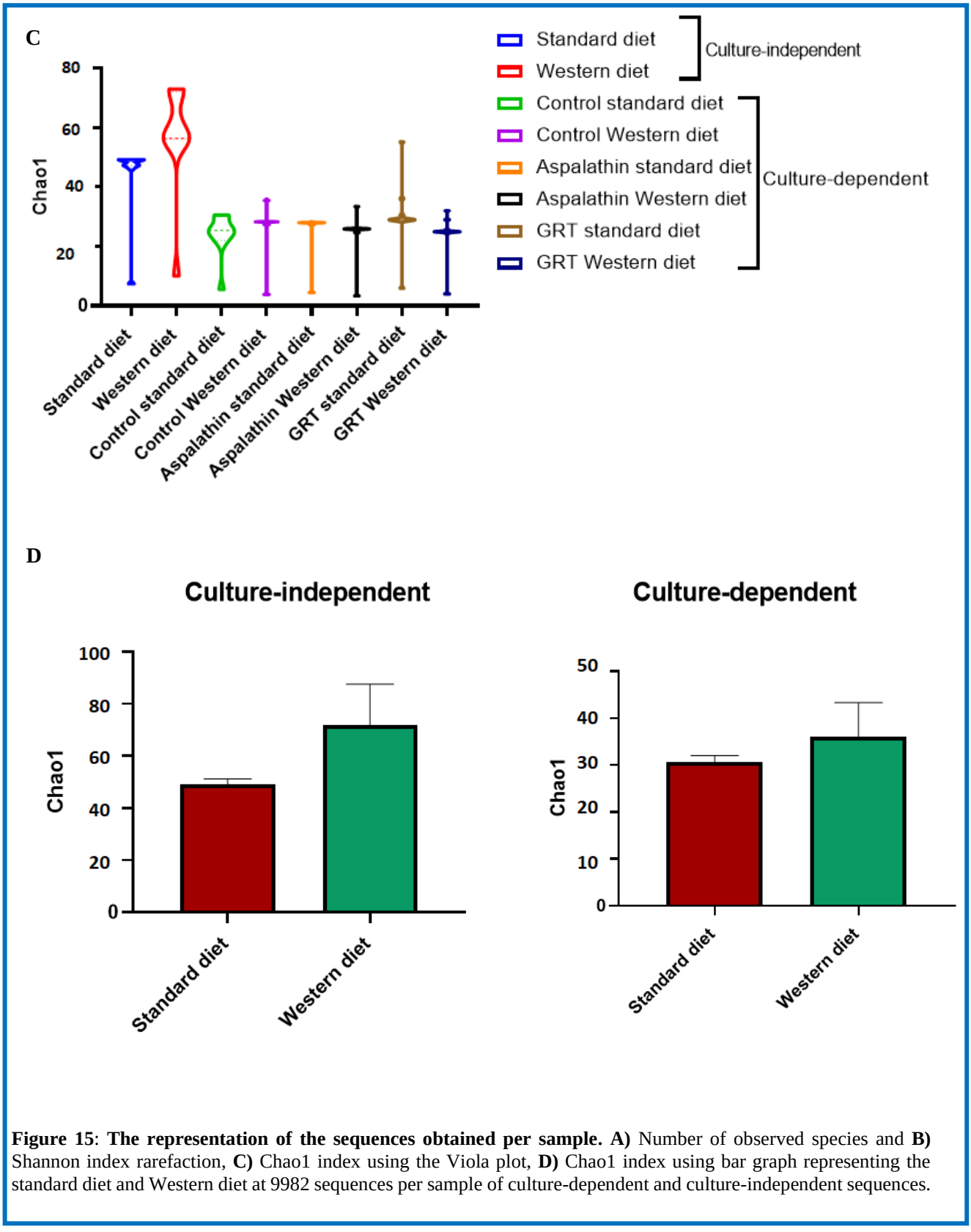


Figure 15: **The representation of the sequences obtained per sample. A)** Number of observed species and **B)** Shannon index rarefaction, **C)** Chao1 index using the Viola plot, **D)** Chao1 index using bar graph representing the standard diet and Western diet at 9982 sequences per sample of culture-dependent and culture-independent sequences.

Figure (15) represents alpha diversity of the culture-dependent and cultured-independent sequenced data using species observed, Chao1 and Shannon index estimator. Figure 15(A) represents the number of observed species which is the number of different species observed under each sample group. It shows that baseline samples had higher diversity compared to culture-dependent samples. Additionally, Western diet samples contained higher species diversity than the standard diet. Figure 15 (B) and (C) represents species diversity measured using the Shannon Index and Chao1 index abundance estimator, respectively. Looking at both alpha diversity graphs, we can conclude that they both show the same correlations that the baseline samples were more diverse than the culture-dependent samples. Figure 15(D) illustrates that the baseline samples have double the culture-dependent diversity using Chao1 index estimator.

4.5. BETA DIVERSITY

Thirty-two faecal samples were sent for 16S rRNA targeted metagenomic sequencing using the Ion Torrent Next Generation Sequencing platform at Central Analytical Facility (CAF), out of 32 samples, 6 were faecal samples cultured in the anaerobic chamber and 26 were those cultured using the Speedy Breedy culturing system. The figure below represents an overall distribution of 32 faecal samples that were sequenced. The PCoA analysis was classified based on diet using Bray-Curtis distance measure. Figure (16) represents samples classified based on diet fed, the red spheres represents Western diet fed monkeys and blue spheres represent standard diet fed monkeys group. Samples cultured with anaerobic chamber are clustered together and circled in red, whilst most of the samples cultured using Speedy Breedy are clustered in one area and they are circled in yellow.

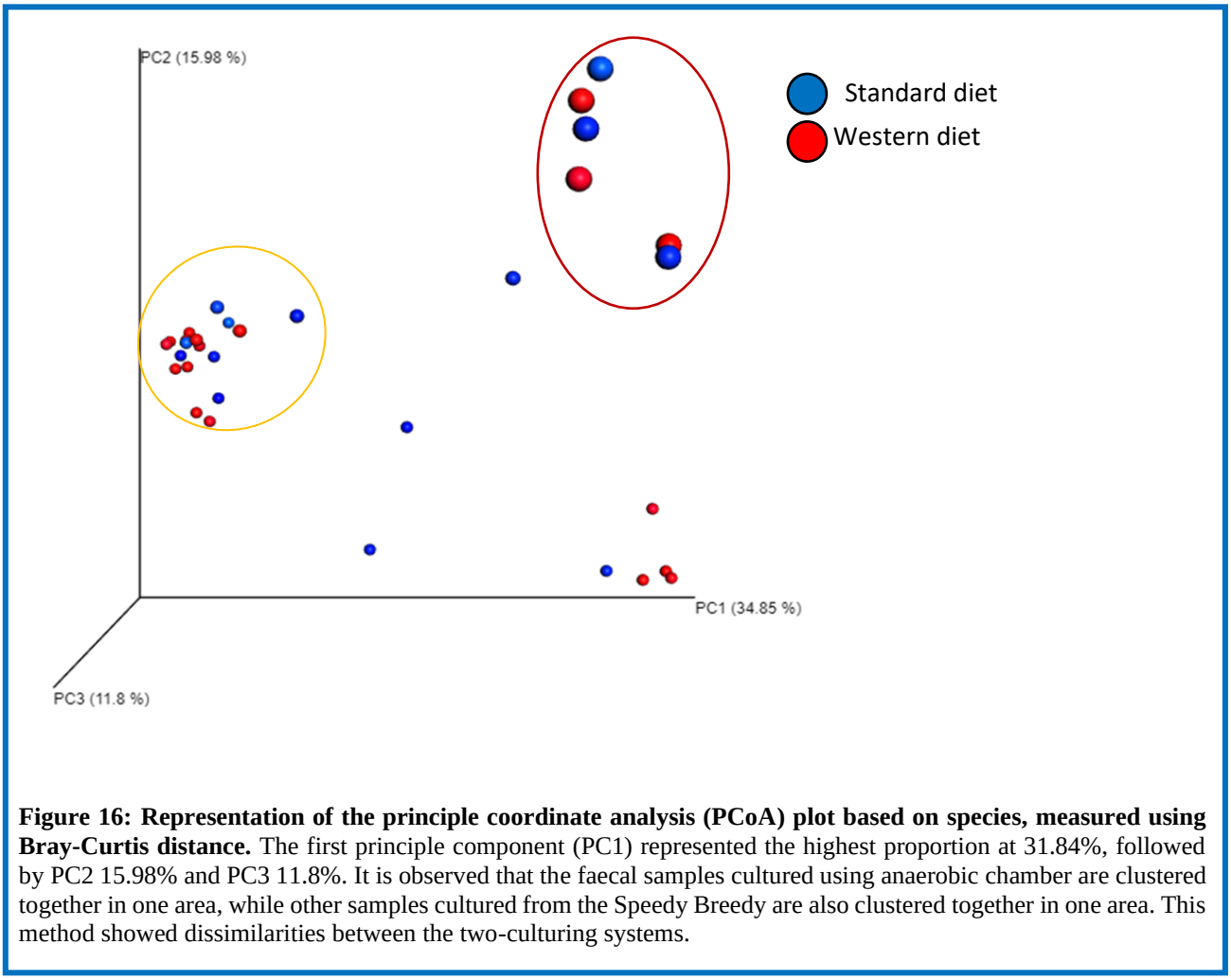


Figure 16: Representation of the principle coordinate analysis (PCoA) plot based on species, measured using Bray-Curtis distance. The first principle component (PC1) represented the highest proportion at 31.84%, followed by PC2 15.98% and PC3 11.8%. It is observed that the faecal samples cultured using anaerobic chamber are clustered together in one area, while other samples cultured from the Speedy Breedy are also clustered together in one area. This method showed dissimilarities between the two-culturing systems.

4.5.1. Effect of diet on faecal sample obtained from the standard diet fed monkeys

The PCoA below represents the 16S rRNA targeted metagenomic sequencing analysis data derived from standard diet, looking at all the treatment groups i.e. culture-independent, and culture-dependent which consists of samples cultured without rooibos phenolic compounds, aspalathin and Afriplex GRT™. The results obtained show that baseline samples clustered together in one area while the culture-dependent samples also clustered together in a separate area. Clustering is not as pronounced in standard diet.

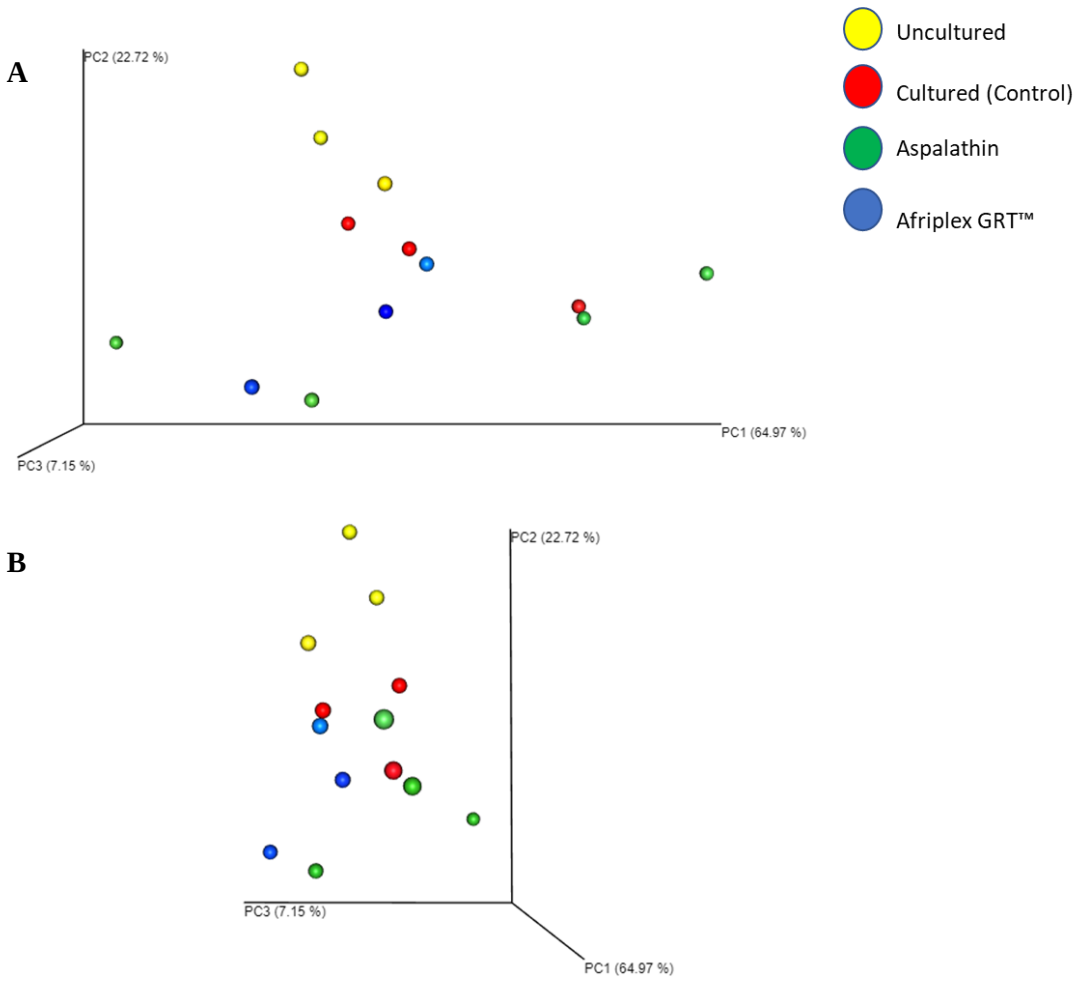


Figure 17: **Representation of the PCoA for baseline (culture-independent) and cultured-dependent samples obtained from the standard diet-fed monkeys**. Data measured using Bray-Curtis distance measure. (PC1) represented the highest proportion at 64.97%, followed by PC2 22.72% and PC3 7.15%. From the results obtained, it shows that the culture-independent samples are clustered together while the cultured samples are scattered. Both plots represent the same PCoA, the bottom plot is turned 90^{o} along the horizontal axis.

.

4.5.2. Effect of diet on faecal sample obtained from the Western diet fed monkeys.

Western diet-fed samples PCoA clustering was monitored. There was more pronounced clustering effect of the spheres between culture-dependent and culture-independent samples. Figure (18) shows that culture-independent samples clustered together in one area while the culture-dependent samples clustered together in another area.

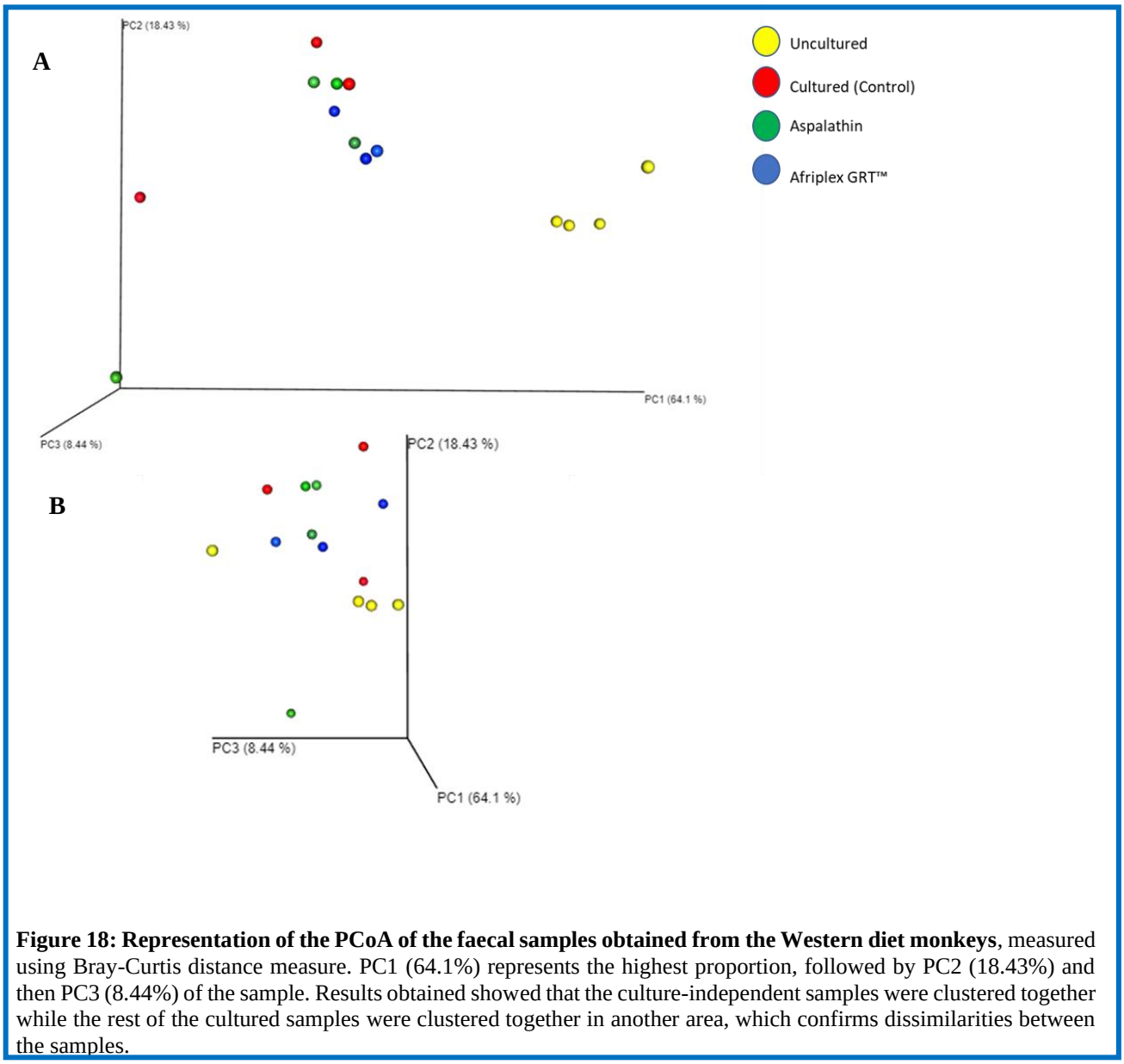


Figure 18: Representation of the PCoA of the faecal samples obtained from the Western diet monkeys, measured using Bray-Curtis distance measure. PC1 (64.1%) represents the highest proportion, followed by PC2 (18.43%) and then PC3 (8.44%) of the sample. Results obtained showed that the culture-independent samples were clustered together while the rest of the cultured samples were clustered together in another area, which confirms dissimilarities between the samples.

Principle of co-ordinate analysis allows us to arrange multi-variable data into clusters so that it is easy to associate the data. There are ten principle components affecting the principle co-ordinate analysis plot. The first 3 components are the most abundant, and those are the ones plotted on the 3D principle coordinate analysis. There are 4 distance measuring techniques which were used i.e. Manhattan, Bray-Curtis, Shannon index and CHISQ, with regards to this study analysis we used Bray-Curtis distance measuring tool.

Diet plays a role in the gut bacterial species that are observed per sample. Therefore, samples from each diet group are expected to cluster together. Figure (16) illustrates PCoA that is classified by diet with blue representing standard diet-fed monkey samples, while red represents the Western diet-fed monkeys. Principal components (PC) are ranged from the highest to the lowest with PC1 being the highest percentage. PC1 has 34%, PC2 at 15.98% and PC3 at 11.8%. Standard diet samples were distributed through-out the PCoA. Western diet samples were clustered in two regions, close to PC1 and other close to PC2.

There is a difference with samples cultured using the different culturing methods. Anaerobic chamber cultured samples were on the top right-hand corner of the PCoA plot. And they were clearly distinguished from the rest of the plot. They are marked with a red ring around them. This expressed that Speedy Breedy was more reliable source for culture. We were unable to conduct further experiments using the anaerobic chamber as it broke down and could not fix for a whole year since certain components required re-fabrication. Therefore, we do not have conclusive data.

Figure (17) illustrates distribution of standard diet-fed monkey samples and figure (18) illustrates sample distribution for Western diet-fed monkeys' samples. Both figures Bray-Curtis was used to investigate the beta diversity of the species. Samples used were that of Speedy Breedy culturing and culture-independent samples. On both diet groups culture-independent samples cluster together. Standard diet-fed monkey samples had no clear cluster or distribution among the cultured samples. Western diet samples had distinct clustering as we noted that culture-dependent samples; in the presence of Afriplex GRT™ and that of aspalathin are clustered together in the same area. The limiting factor is that we had 3-4 samples in each treatment group, therefore, we cannot make a conclusion on the cultured samples based on few data points.

4.6. EFFECT OF DIET ON NON-CULTURED SAMPLES

4.6.1. Gut microbiota phyla distribution

The gut microbiota is composed of numerous phyla such; *Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Verrucomicrobia, Tenericutes, Spirochaetes, Lentisphere* and *Synegistetes*. The figure below represents the distribution of the gut microbiota phyla under culture-independent conditions, classified by diet. A total of 10 phyla were detected as indicated by OTU count percentages from each diet as shown in figure (19).

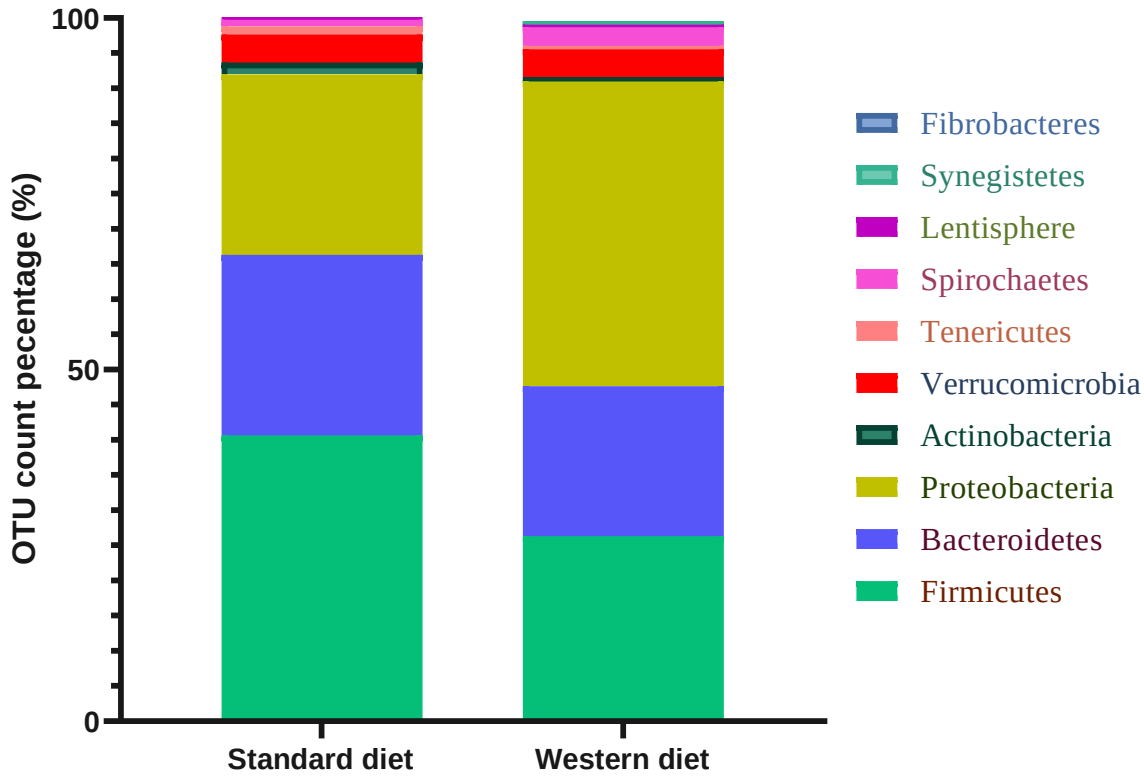


Figure 19: Distribution of the gut microbiota phyla. Representation of Bacterial phyla detected from faecal samples obtained from the standard diet-fed and Western diet.

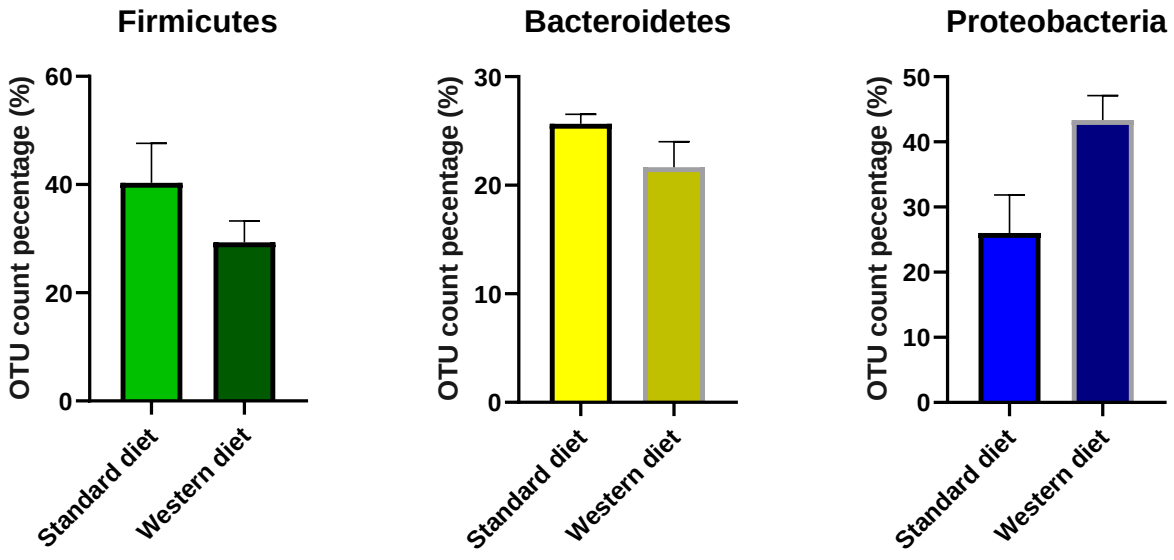


Figure 20: Representation of the three dominant gut microbiota phyla (*Firmicutes*, *Proteobacteria*, and *Bacteroidetes)* from the standard diet, compared to the Western diet**.** The results indicate that *Firmicutes* were decreased from 40.7% to 26.33%, and *Bacteroidetes* decreased from 25.67% to 21.33%, *Proteobacteria* increased from 25.67% to 43.33% respectively.

The Western diet affects the general distribution of bacterial phyla. In figure (19) under a standard diet, *Firmicutes* was the most abundant phylum, followed by *Bacteroidetes* and *Proteobacteria*. The Western diet decreases the abundance of *Firmicutes* and *Bacteroidetes* while it increases that of *Proteobacteria*.

The abundance of phyla in standard diet consisted of *Firmicutes* 40.67%, *Bacteroidetes* 25.66%, *Proteobacteria* 25.67%, *Actinobacteria* 1.67%, *Verrucomicrobia* 4%, T*enericutes* 1.167%, *Spirochaetes* 0.9%, *Lentisphere* 0.3%, *Synegistetes* 0.08%, and *Fibrobacters* 0.095%.

The phyla distribution of the Western diet showed that *Proteobacteria* was the most abundant phylum with 43.33%, followed by *Firmicutes* 26.33%, *Bacteroidetes* 21.33%, *Actinobacteria* 0.64%, *Verrucomicrobia* 3.39%, *Tenericutes* 0.48%, *Spirochaetes* 2.67%, *Lentisphere* 0.33% and *Synegistetes* 0.54%. Figure (20) compares the shift in the phyla due to diet. The Western diet decreased the *Firmicute* abundance by 11% ± 8.3% and *Bacteroidetes* by 4% ± 2.49% whilst *Proteobacteria* was increased by 17.33% ± 6.96%.

4.6.2. Gut microbiota genera distribution

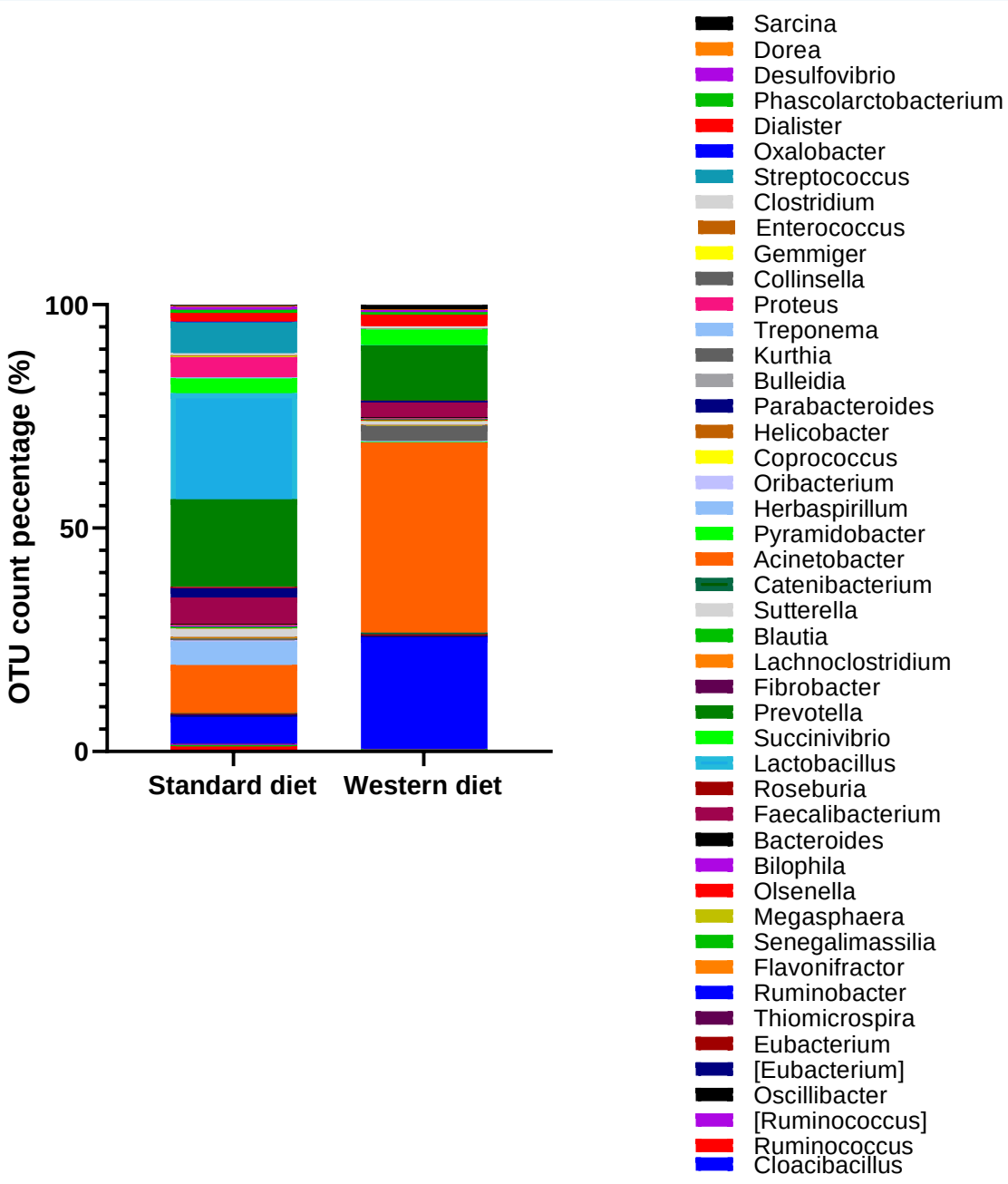


Figure 21: Representation of the overall genus distribution as classified by diet for faecal samples obtained from either standard diet-fed or Western diet monkeys.

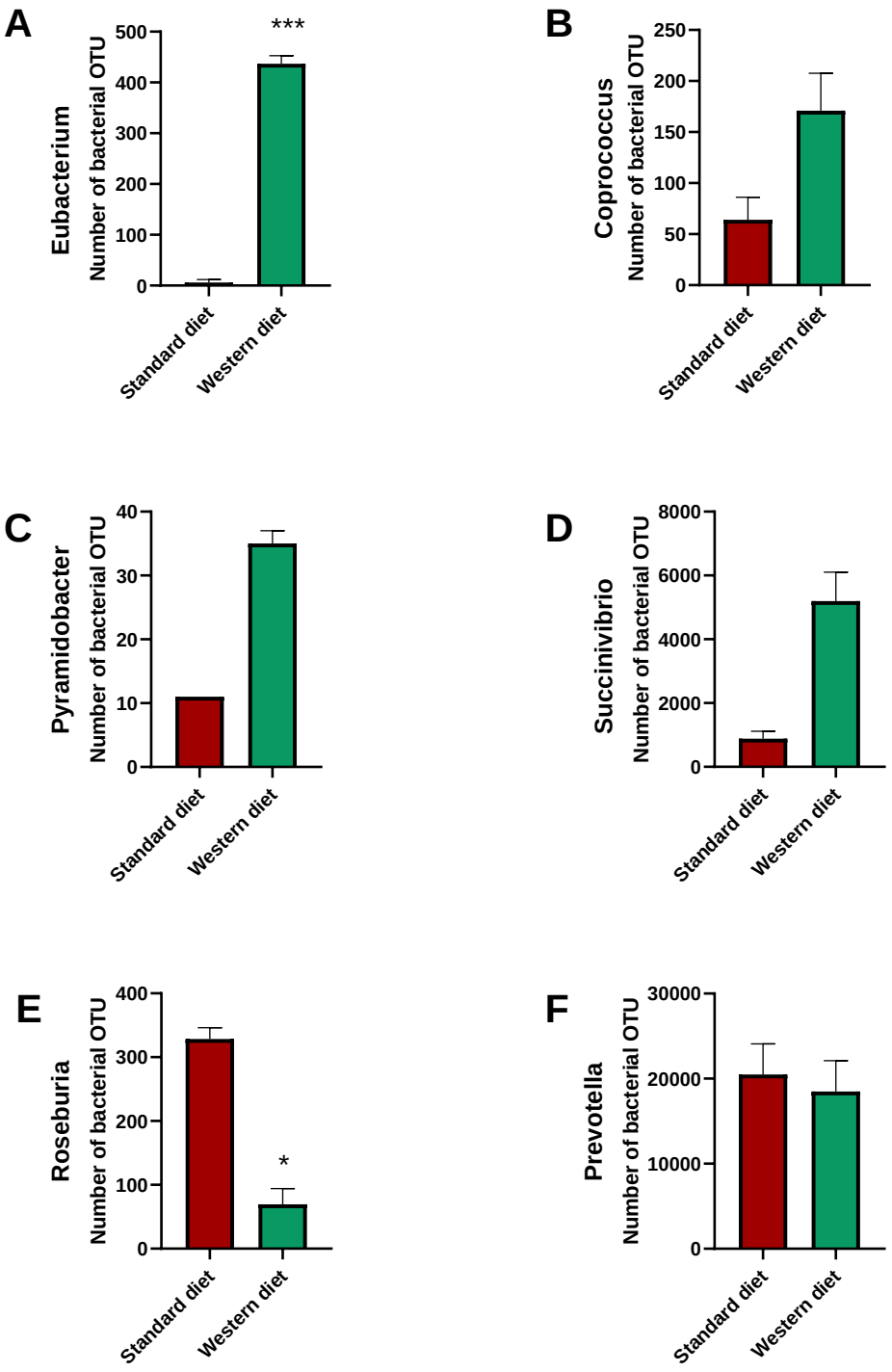
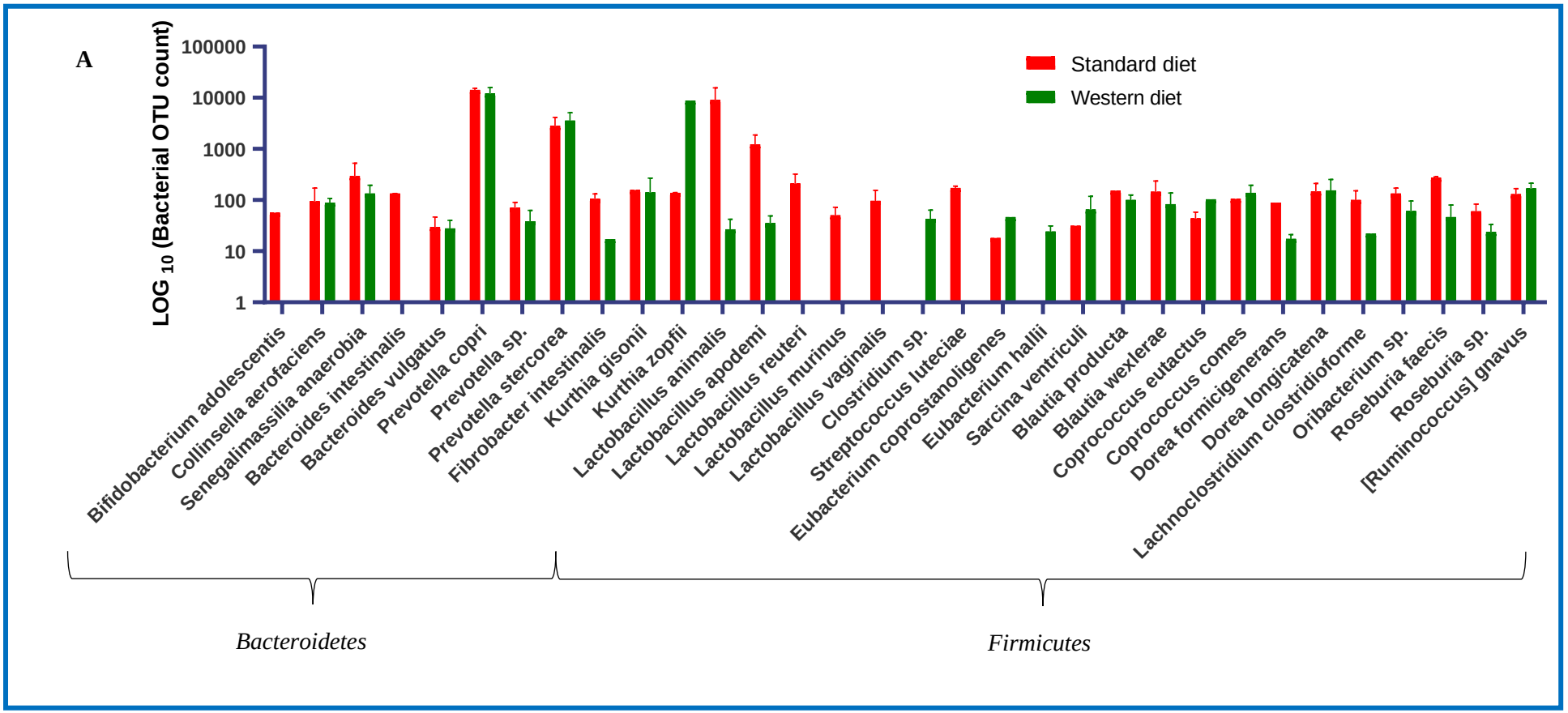


Figure 22: Representation of the gut microbiota genera obtained from culture-independent faecal samples classified by diet. **A**) *Eubacterium* (431±16.82, p=0.0004), **B**) *Coprococcus* (107±42.72, p=0.079), **C**) *Pyramidobacter* (24±2, p=0.053) and **D**) *Succinivibrio* (4311±934.4, p=0.114) were increased in high fat diet, while **E**) *Roseburia* (259±30.11, p=0.0178) and (F) *Prevotella* (1992±5110, p=0.72) were decreased. Data presented as mean ± SEM, p<0.05*, p<0.01** Stats analysed using Welch's t-test.

Baseline culture-independent data was used to determine the effect of diet on the gut microbiota genera. The Western diet caused a shift in the gut microbiota genera. The standard diet contained more diverse genera distribution than the Western diet's diversity as observed in figure (21). The Western diet caused a depletion of *Lactobacillus* and *Streptococcus* while it increased the abundance of *Acinetobacter, Ruminobacter* and *Succinivibrio* genera. The results obtained show that the most dominant families in both diet groups were; *Prevotella, Lactobacillus, Ruminobacter, Acinetobacter, and Faecalibacterium*. Genera which were found to be decreased by the Western diet were; *Prevotella, Lactobacillus, Faecalibacterium,* and *Roseburia*. However, *Eubacterium* and *Succinivibrio* were increased by the Western diet. The most prominent changes were of *Prevotella,* decreased from 19.56% to 12.35%, while *Acinetobacter* increased from 10.47% to 42.53% bacterial OTU percentage.

Figure (22) represents specific genera that were observed and showed some changes due to the Western diet intake, at culture-independent state. The genera *Coprococcus, Succinivibrio, Roseburia,* and *Eubacterium* were increased under the Western diet with *Eubacterium* being significantly increased. *Roseburia* and *Prevotella* were decreased with *Roseburia* being significantly decreased by the Western diet. The Western diet depleted several of the most beneficial species, while the abundance of many harmful bacteria increased.

4.6.3. Overall species distribution



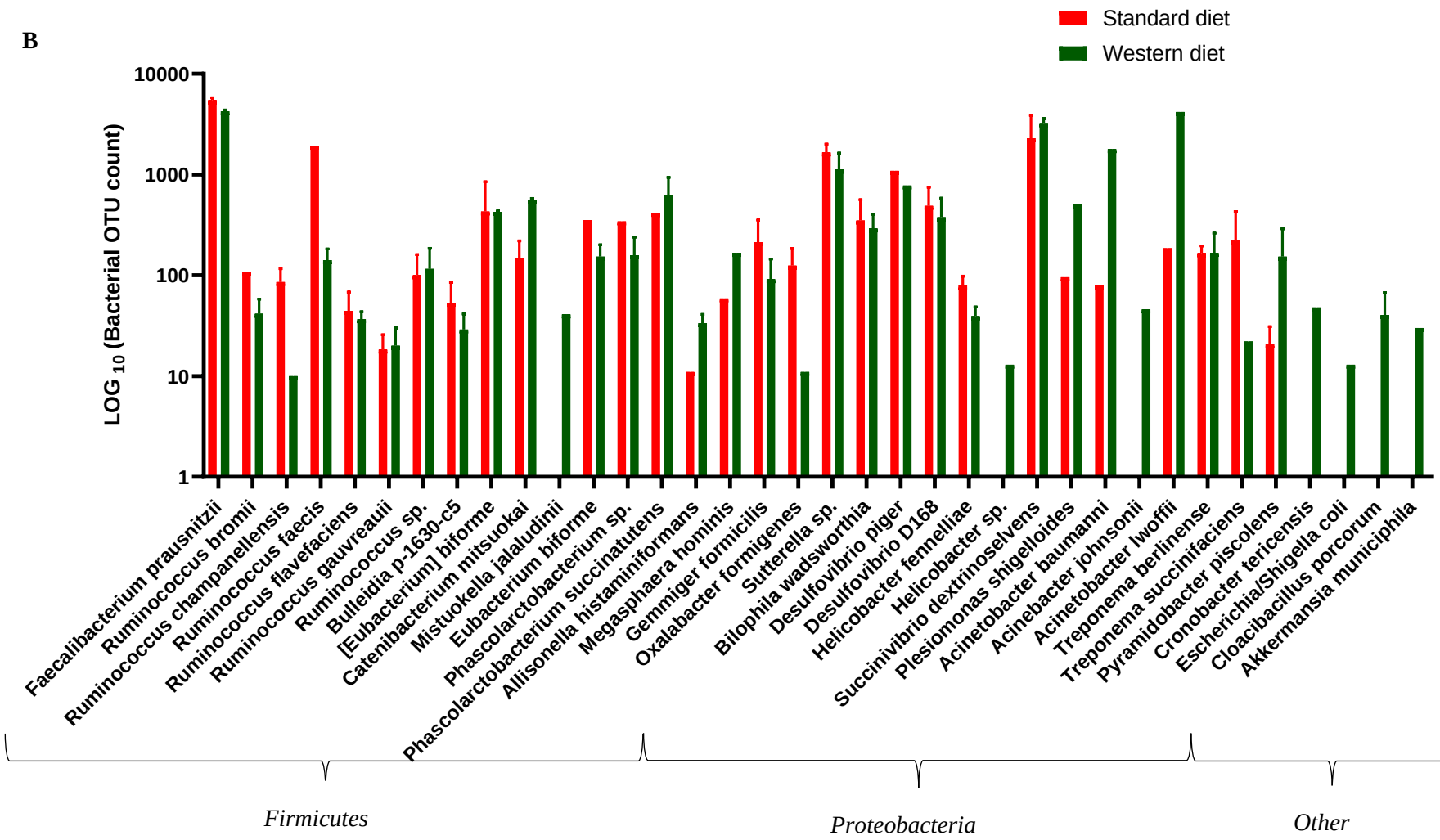


Figure 23: A-B) The Relative abundance of the overall distribution of the gut microbiota species at the species level. The species compared obtained from faecal samples from a standard diet and Western diet, respectively. Species belonging to the *Bacteroidetes* phylum were abundant at a standard diet as compared to the Western diet. Species belonging to the *Proteobacteria* phylum were more abundant on Western diet than on the standard diet. Data presented as mean ± SEM on a log10 scale.

4.6.4. Beneficial species on the gut microbiota

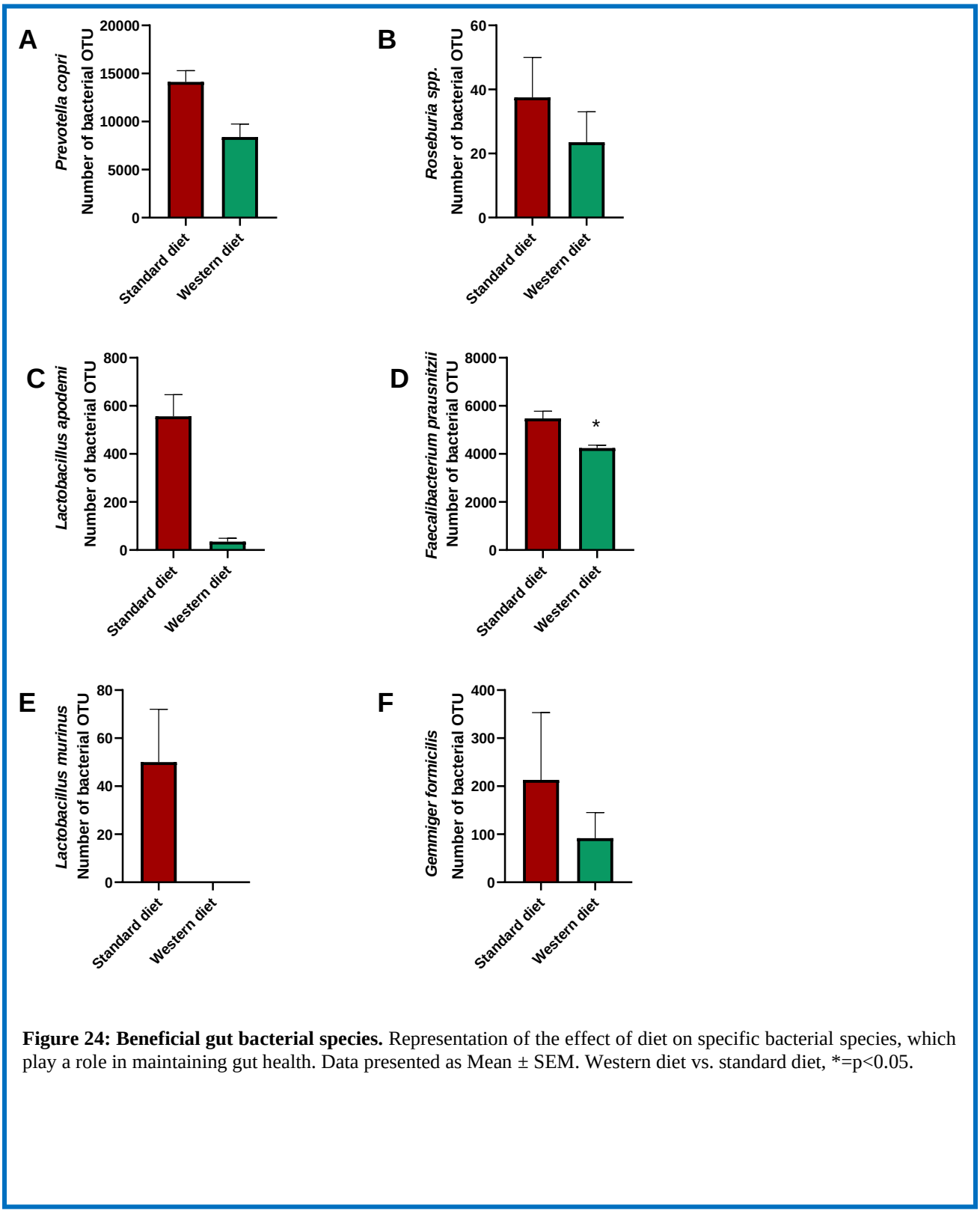


Figure 24: Beneficial gut bacterial species. Representation of the effect of diet on specific bacterial species, which play a role in maintaining gut health. Data presented as Mean ± SEM. Western diet vs. standard diet, *=p<0.05.

4.6.5. Harmful species on the gut microbiota

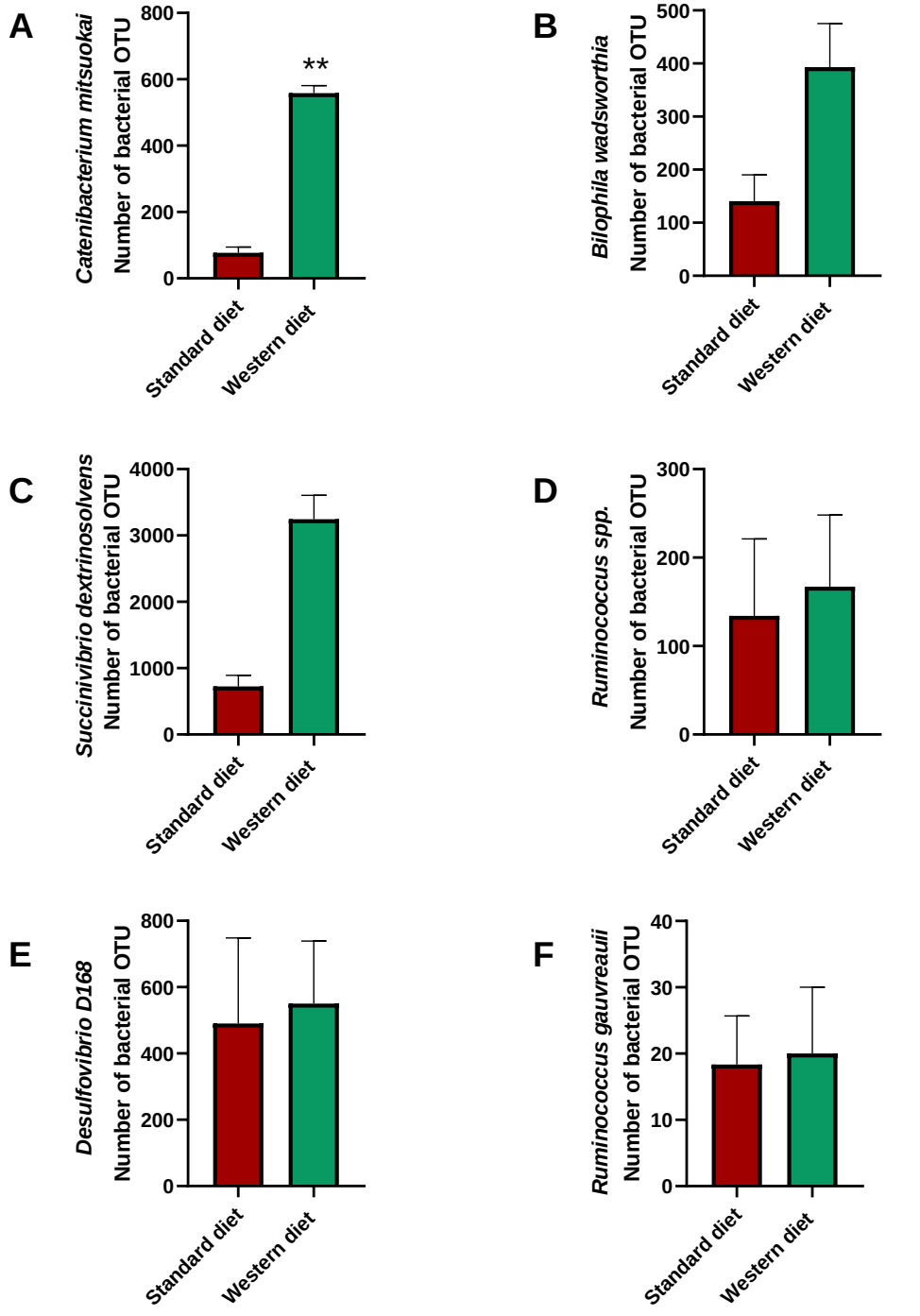


Figure 25: **Harmful gut bacterial species**. Representation of the effect of diet on specific bacterial species, regarded to be harmful to gut health were found to be increased in high fat fed monkeys. Data presented as mean ± SEM. Western diet vs. standard diet, **=p<0.005.

The overall representation of bacterial species based on culture-independent samples. Species found under the *Bacteroidetes* and *Firmicutes* phyla, were more abundant in the standard diet group, compared to the Western diet group. However, species under the *Proteobacteria* phyla were more abundant in the Western diet group compare to the standard diet group. Species that have a probiotic effect such as *Bifidobacterium adolescentis, Lactobacillus reuteri*, and *Lactobacilllus murinus* were depleted in the Western diet-fed monkeys. While species which are known to be potentially harmful to gut health, and with pro-inflammatory properties such as *Helicobacter spp., Succinivibrio dextrinosolvens,* and *Acinectobacter johnsonii* were enriched in Western diet-fed monkeys (figure 23).

Figure (24) illustrates that *Prevotella copri, Lactobacillus apodemi, Lactobacillus murinus*, and *Faecalinacterium prausnitzii* abundance were decreased with *Faecalibacterium prausnitzii* significantly decreased (1233 ±3283.8; p = 0.0411) by the Western diet. Figure (25) represents the increased abundance of potential harmful bacteria *Catenibacterium mistuokai, Succinivibrio dextrinosolvens, Bilophila waadsworthia,* and *Rumminococcus spp.,* with *Catenibacterium mistuokai* (481±28.6 with P= 0.005) being significantly increased on the Western diet.

4.7. EFFECT OF ANAEROBIC CULTURING

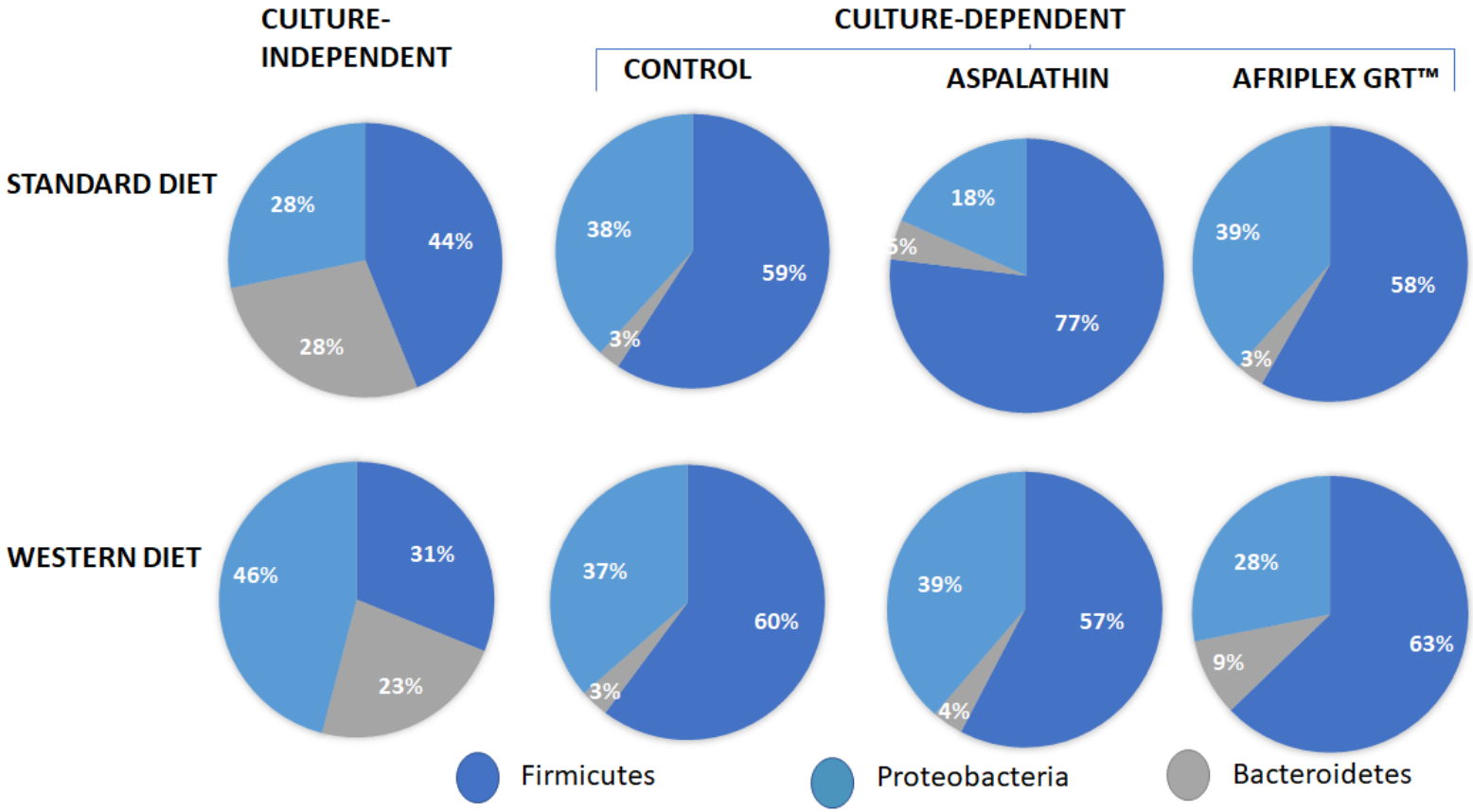


Figure 26: Representation of the most dominant phyla. Comparing the effects of treatment during culturing for both standard diet and high fat diet groups.

4.7.1. Effect of culturing and favoured species during culturing

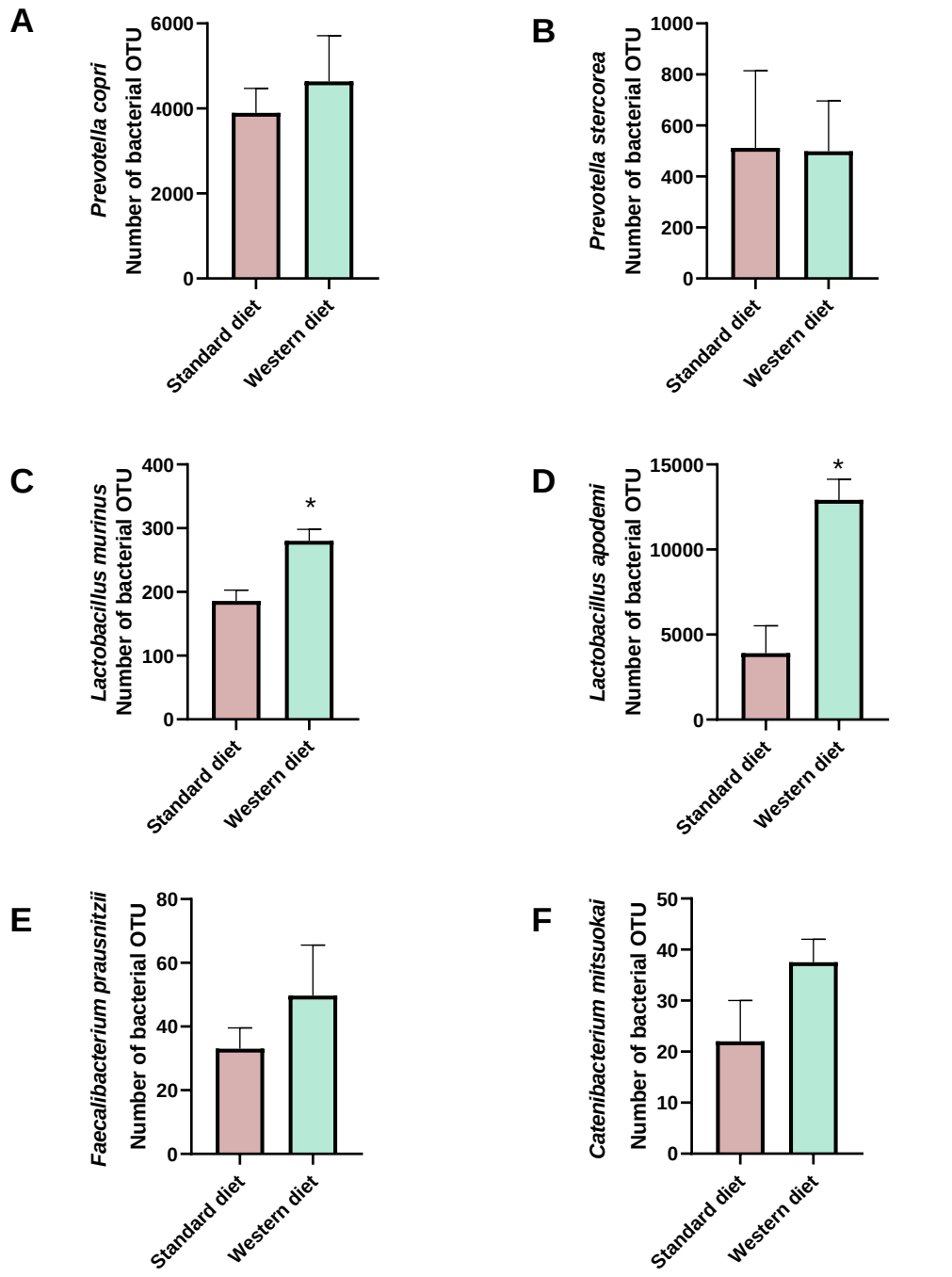


Figure 27: The effect of diet on cultured species. **A**) *Prevotella copri* (740.7±12.18, p=0.61), **B**) *Prevotella stercorea* (13±360, p=0.97), **C**) *Lactobacillus apodemi* (9008±2018, p=0.013), **D**) *Lactobacillus murinus* (94.3±24.8, p=0.041, **E**) *Faecalibacterium prausnitzii* (16±17.18, p=0.39) and **F**) *Catenibacterium mistuokai* (15.5±9.179, p=0.26) Data analysed using Welch's t-test, presented as mean ± SEM. Aspalathin and/or GRT vs control, * p<0.05.

Anaerobic culturing is known to enumerate selected microorganisms based on their environmental and nutritive requirements. Certain phyla are favoured during culturing and some are discriminated against. *Bacteroidetes* phylum is not favoured during anaerobic culturing. When comparing culture-dependent to culture-independent (baseline) samples in both standard diet and Western diet-fed samples, *Bacteroidetes* were decreased, while *Firmicutes* and *Proteobacteria* were increased. In the standard diet group, *Bacteroidetes* were decreased from 28% to 3%, *Proteobacteria* increased from 28% 38.5% and *Firmicutes* increased from 44% to 59%. In the Western diet group, *Bacteroidetes* were decreased from 23% to 3%, *Firmicutes* were increased from 31% to 60%, while *Proteobacteria* abundance decreased from 48% to 37% as illustrated in figure (26). Certain species are favoured during culturing, such as *Lactobacillus murinus* and *Lactobacillus apodemi* which were significantly increased in the Western diet culture-dependent samples, whilst these were depleted in the culture-independent state.

Figure (26) summarises the effect of culturing using baseline culture-independent samples compared to culture-dependent samples to assess the phyla percentage shifts. For culture-dependent samples, the control untreated samples were compared to aspalathin or Afriplex GRT™. For the standard diet, aspalathin increased *Bacteroidetes* abundance slightly from 3% to 5% (2% ±0.94%), *Firmicutes* increased from 59% to 77% (13.33% ± 19.82%) and *Proteobacteria* decreased from 38% to 18% (14.3% ± 20.4%). Comparing control to Afriplex GRT™ all the phyla abundance (*Firmicutes* (2.67% ±20.06%), *Bacteroidetes* (0.67% ± 1.2%) and *Proteobacteria* (5.3% ± 22.23%)) remained unchanged. Therefore, use of aspalathin and Afriplex GRT™ on standard diet had no major effect on the bacterial phyla derived from a standard diet.

Under a Western diet, aspalathin did not affect *Proteobacteria* as they only slightly increased from 37% to 39% (2.33% ± 22.76%), *Firmicutes* decreased from 60% to 57% (2.3% ± 21%) while *Bacteroidetes* slightly increased from 3% to 4% (0.33% ± 1.88%). However, Afriplex GRT™ decreased *Proteobacteria* from 37% to 28% (7.33% ± 22.5%), increased *Firmicutes* from 60% to 63% (4.67% ± 19.8%) and *Bacteroidetes* by 3% to 9% (2% ± 2.63%). Therefore, in Western diet-fed monkey faecal samples bacterial phyla were not changed by aspalathin. However, in the presence of Afriplex GRT™ there were notable changes, as it decreased the prevalence of *Proteobacteria* and increased the prevalence of *Bacteroidetes*.

4.8. EFFECT OF ROOIBOS PHENOLIC COMPOUNDS ON CULTURED GUT MICROBIAL DIVERSITY

4.8.1. Effect of aspalathin and Afriplex GRT™ on phyla

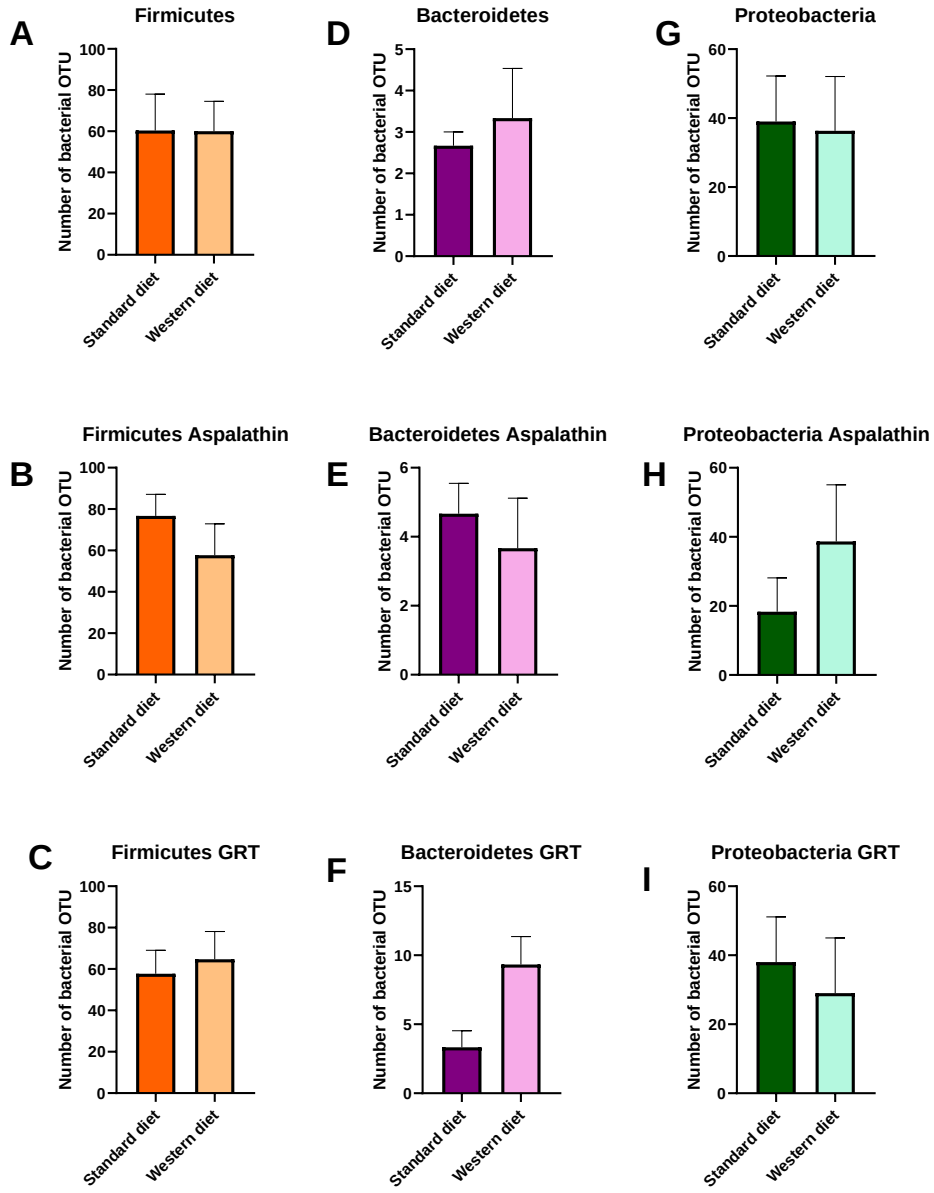


Figure 28: The effect of aspalathin and GRT on cultured gut microbiota phyla. Graphs represents **A**) *Firmicutes* cultured without aspalathin or GRT (0.33±23, p=0.98), **B**) F*irmicutes* with aspalathin (19±18.4, p=0.36), **C**) *Firmicutes* with GRT (7±17.6, p=0.71), **D**) *Bacteroidetes* without aspalathin or GRT (0.67±1.2, p=0.64), **E**) *Bacteroidetes* with Aspalathin (1±1.7, p=0.59), **F**) *Bacteroidetes* with GRT(6±2.4, p=0.078), **G**) *Proteobacteria* without rooibos phenolic compounds (2.67±20.56, p=0.64), **H**) *Proteobacteri*a with Aspalathin (20.33±19.1, p=0.36), and **I**) *Proteobacteria* with GRT (9±20.7, p=0.68). Statistics analysis was done using t-test with Welch's correction, data presented as mean± SEM.

4.8.2. Effect of Aspalathin and Afriplex GRT™ on the genera

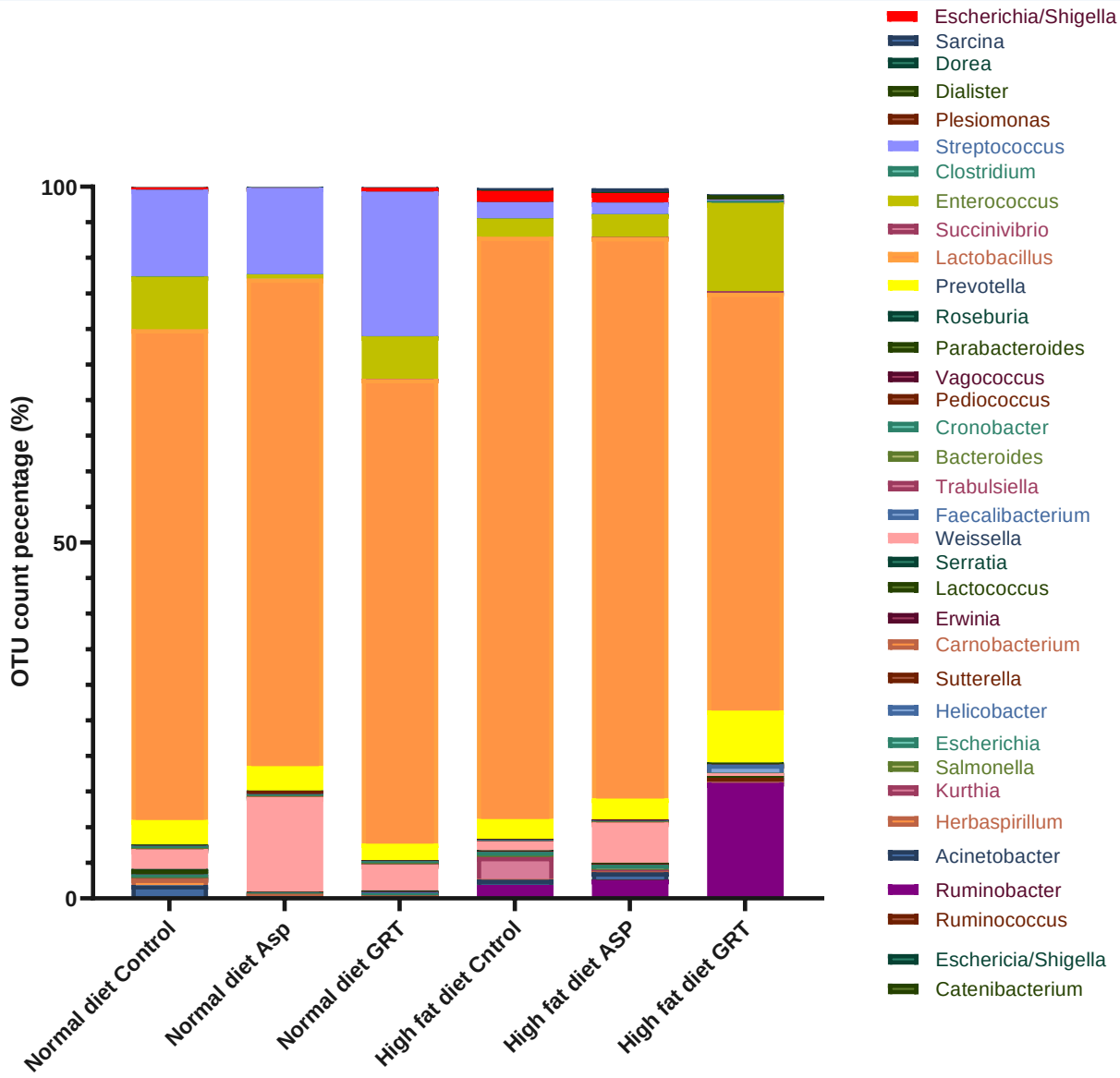


Figure 29: Representation of relative abundance of the cultured genera level, comparing control, aspalathin and GRT of standard diet and high fat diet faecal samples.

4.8.3. Effect of aspalathin and Afriplex GRT™ on relative abundance of specific genera of standard diet

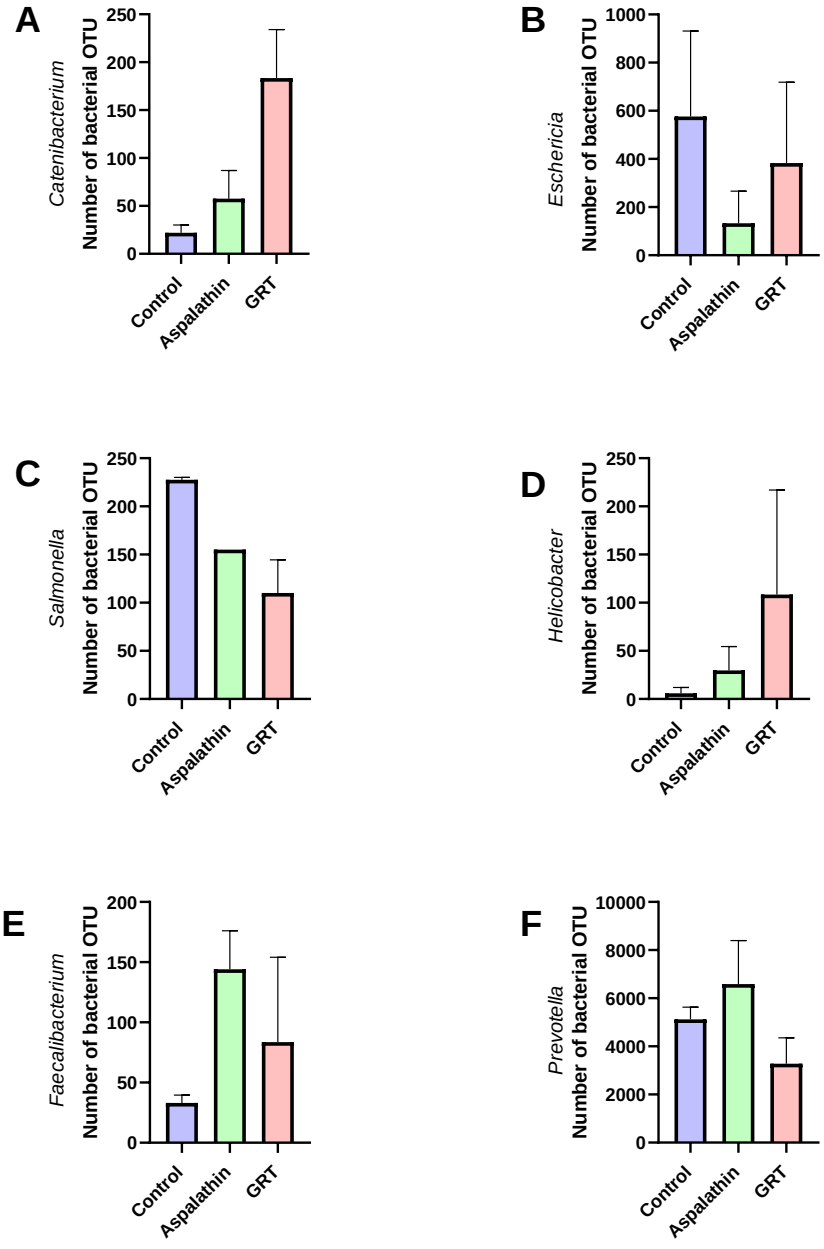


Figure 30: Relative abundance of standard diet cultured gut microbiota genera using aspalathin and GRT. **A**) *Catenibacterium* (aspalathin (35.67±30.31, p=0.35), GRT (161.5±51.13, p=0.19)), **B**) *Escherichia* (aspalathin (443 ± 379, p=0.42), GRT (193±488, p=0.72)), **C**) *Salmonella* (GRT (117±488, p= 0.52)), **D**) *Helicobacter* (aspalathin (23.67 ± 25.5), GRT (102.5± 108.7, p= 0.52)), **E**) *Faecalibacterium* (aspalathin (111± 32.6, p = 0.17)), **F**) *Prevotella* (aspalathin (1472±1875, p=0.51), GRT(1842±1195, p=0.22)) control vs aspalathin or Afriplex GRT™. Data presented as mean ± SEM.

4.8.4. Effect of aspalathin and Afriplex GRT™ on relative abundance of specific genera of Western diet

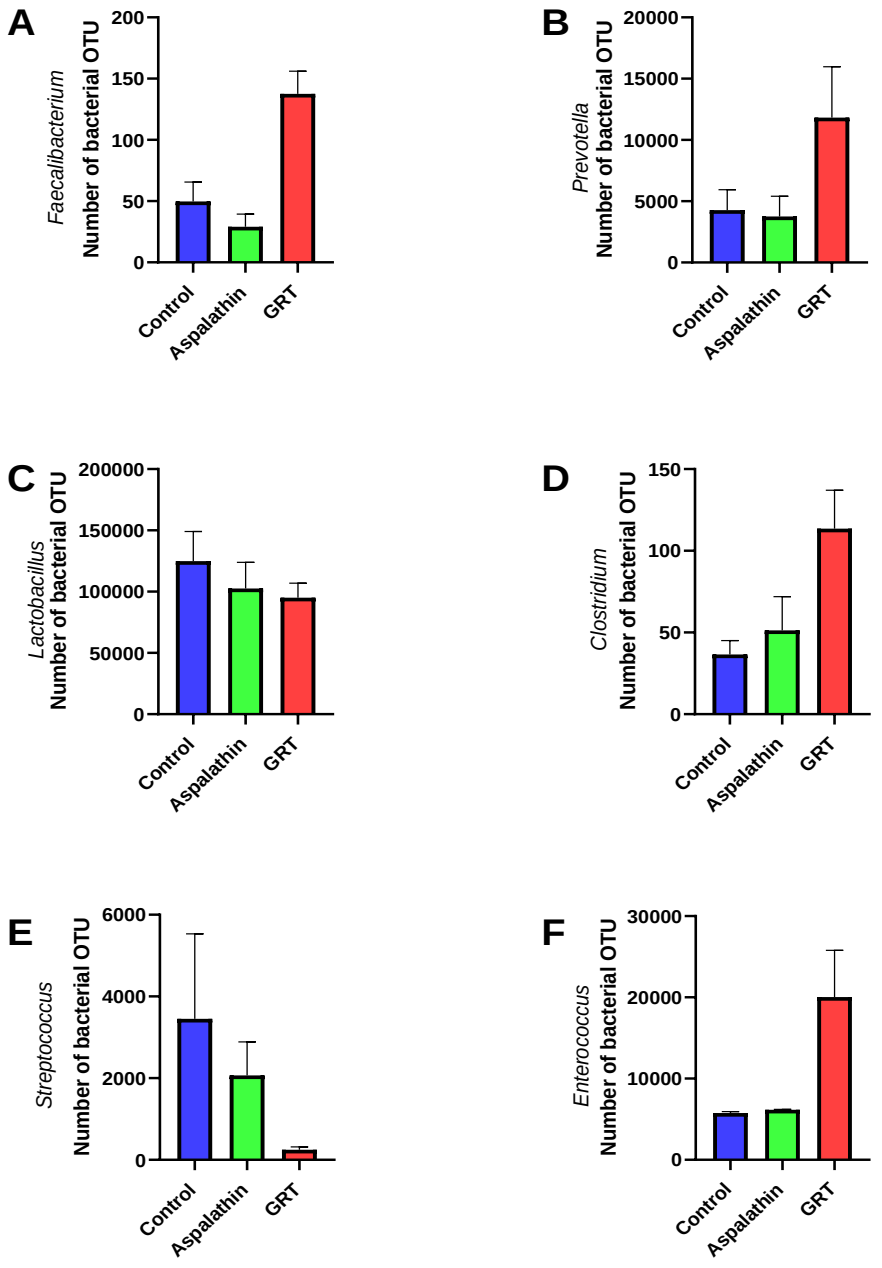


Figure 31: Relative abundance of Western diet cultured gut microbiota genera using aspalathin and GRT. **A**) *Faecalibacterium* (aspalathin 20.6±19, p=0.35), GRT (87.8±24, p=0.053)). **B**) *Prevotella* (aspalathin 502±2343, p=0.84), GRT (7560±446, p=0.20)). **C**) *Lactobacillus* (aspalathin 22336±32325, p=0.52), GRT (29746±26887, p=0.35)). **D**) *Enterococcus* (aspalathin (413.5±182.8, p=0.25), GRT (14290±5753, p=0.24)). **E**) *Clostridium* (aspalathin 14.8±22.3, p=0.16), GRT(1388±2237, p=0.63)) and **F**) *Enterococcus* (ASP (1388±2237, p=0.63), GRT(3208±2082, p=0.36)) Data presented as mean ± SEM. Aspalathin or GRT vs control.

Figure (30) represents data observed from cultured gut microbiota genera that were derived from the standard diet-fed monkey faecal samples. It was found that there was no correlation observed amongst the gut microbiota genera following treatment with aspalathin or Afriplex GRT™. However, in figure (31), the Western diet fed monkey faecal samples it was found that Afriplex GRT™ had an effect on the beneficial genera, increasing the abundance of "good" gut bacteria and decreasing "bad" bacteria. Figure (31) showed that *Faecalibacterium, Prevotella, Clostridium* and *Enterococcus* abundance increased in the presence of Afriplex GRT™ while *Streptococcu*s abundance decreased.

4.8.5. Effect of aspalathin and Afriplex GRT™ on relative abundance of specific species of the standard diet

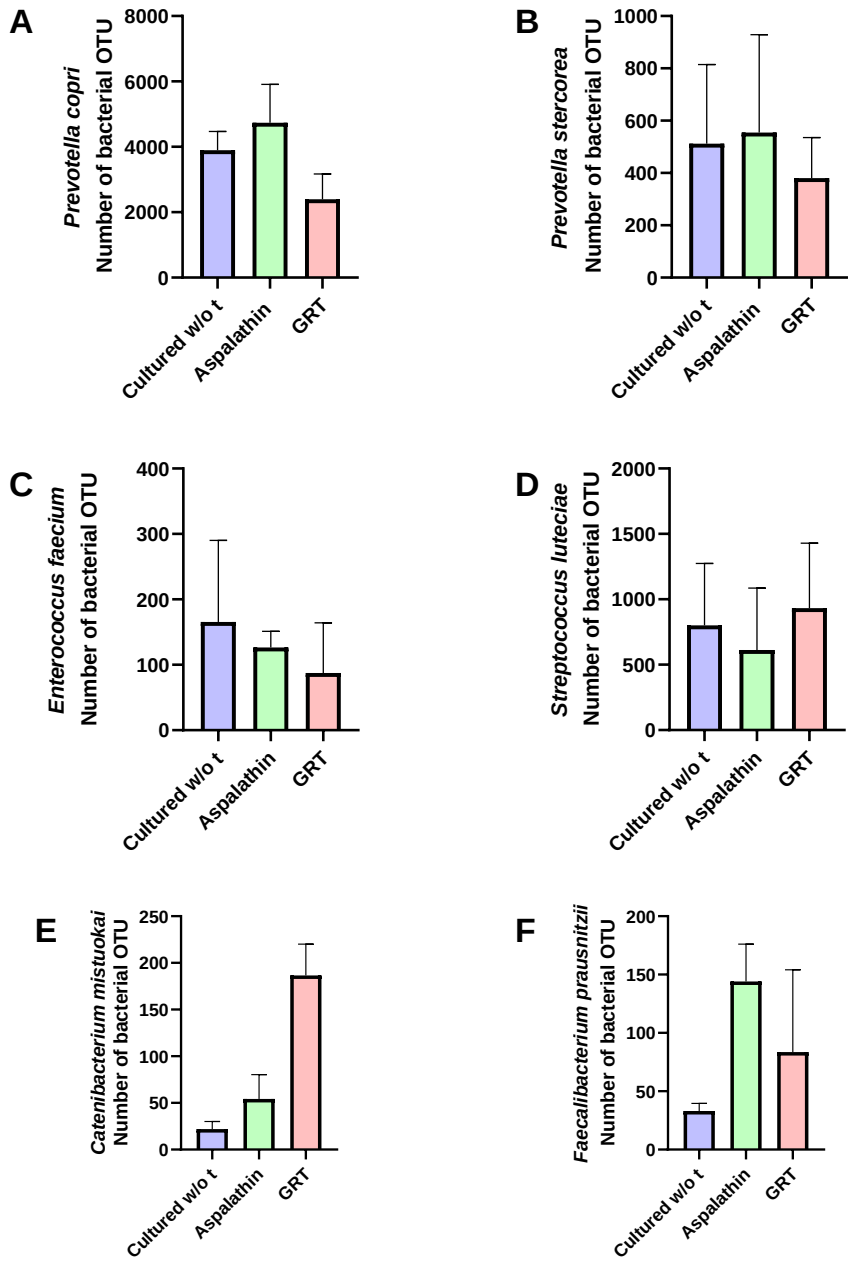


Figure 32: **The effect of rooibos phenolic compounds (aspalathin or GRT) on standard diet samples**. Data presented as mean ± SEM.

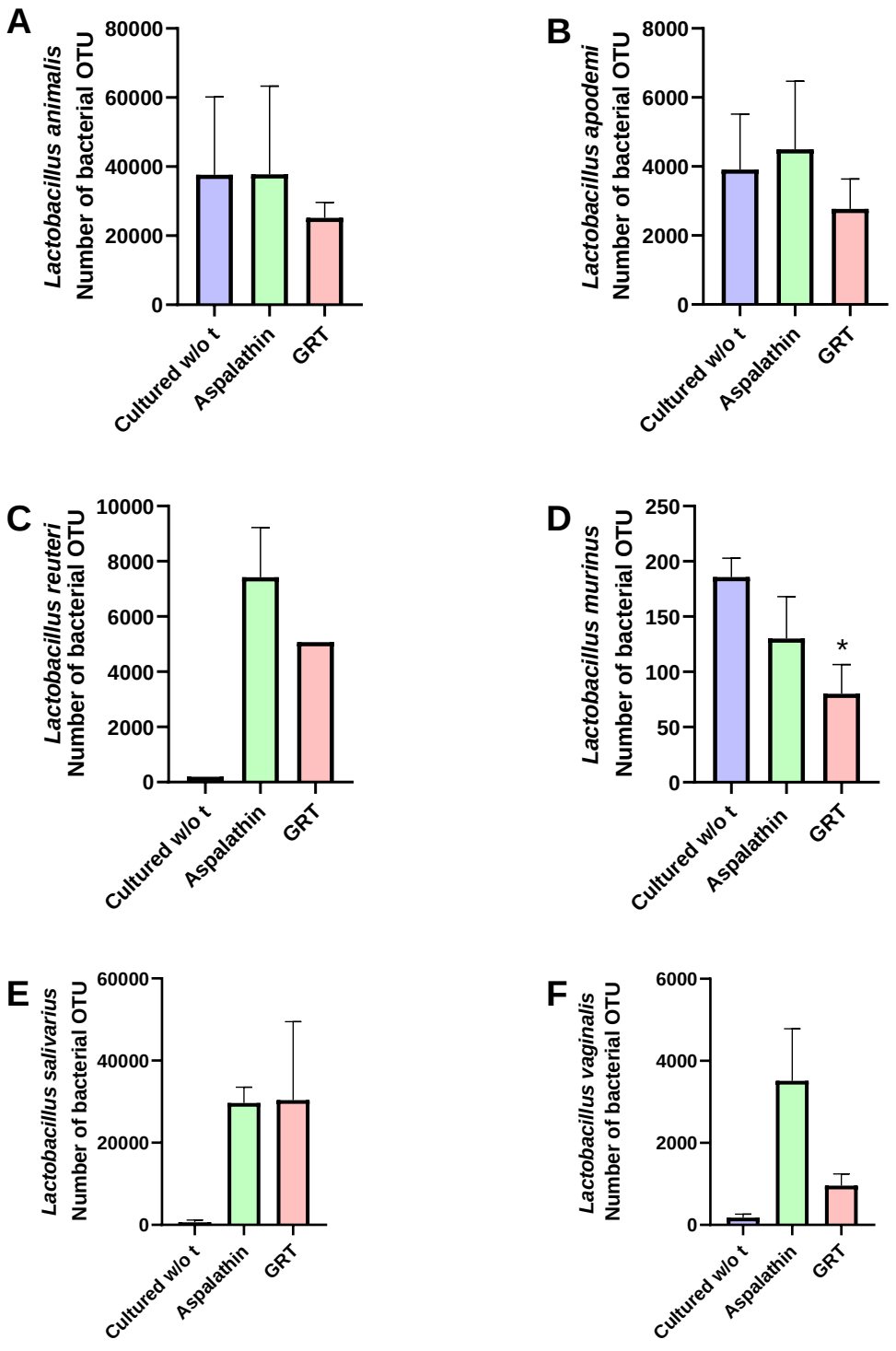


Figure 33: **The effect of aspalathin or GRT on standard diet faecal samples**. Data presented as mean ± SEM. Aspalathin and GRT vs control, * $p<0.05$.

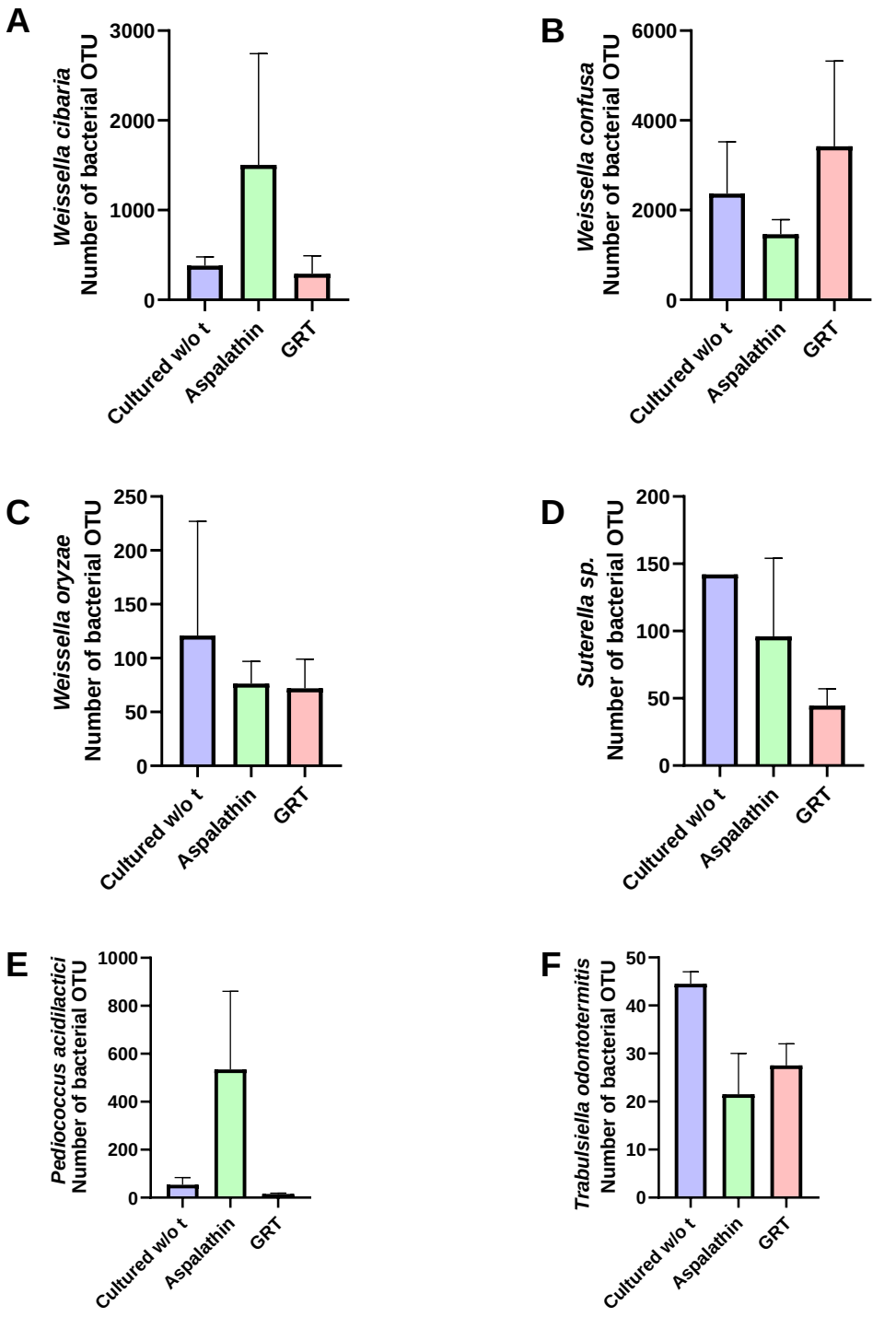


Figure 34: **The effect of aspalathin or GRT on standard diet faecal samples**. Aspalathin or GRT did not affect the abundance of the specific gut bacteria during culture. Data presented as mean ± SEM.

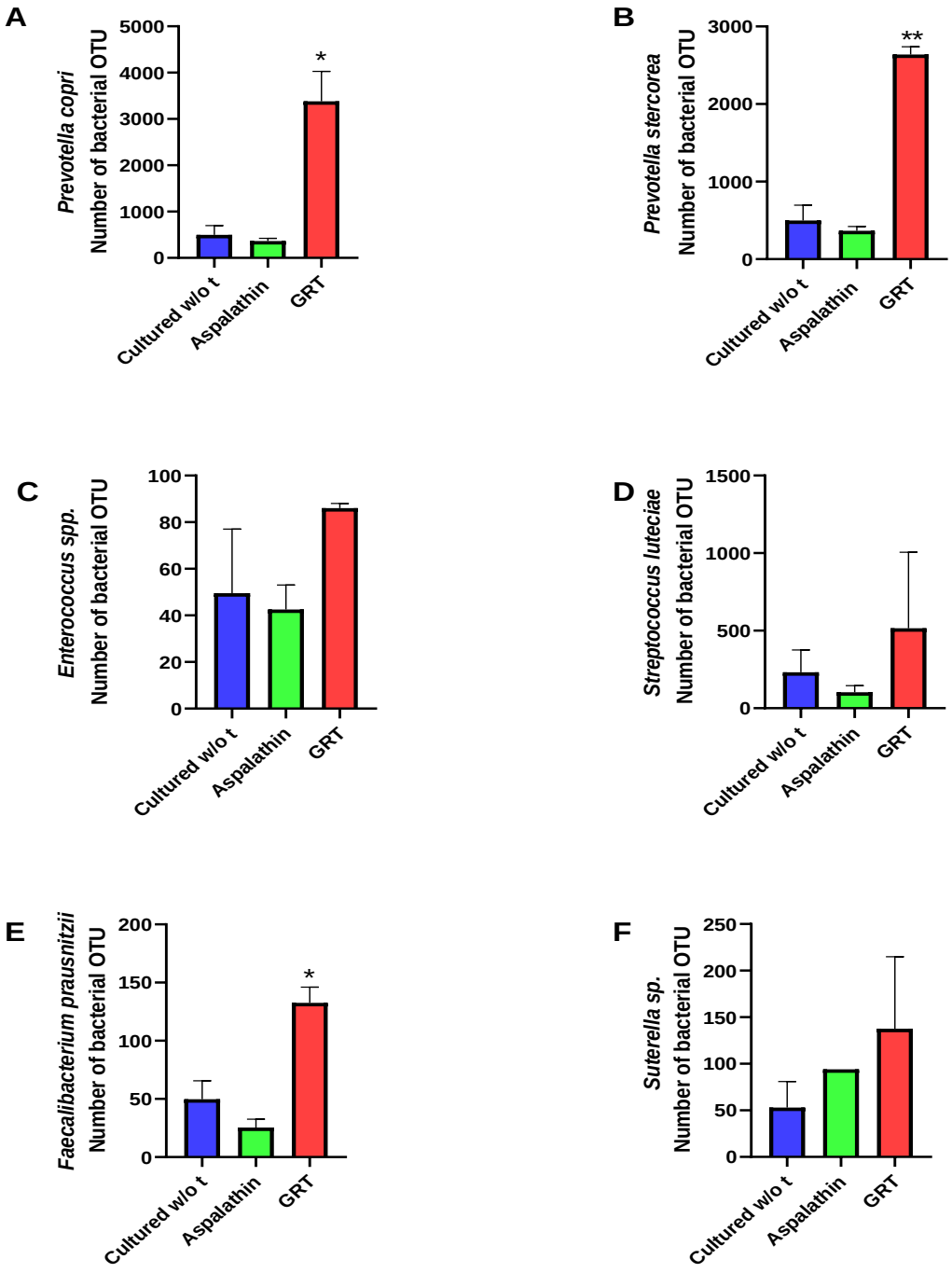


Figure 35: The effect of Aspalathin and GRT on Western diet monkey faecal samples. Data presented as mean ± SEM. Aspalathin and GRT compared to control samples, * $p<0.05$ and ** $p<0.001$, respectively.

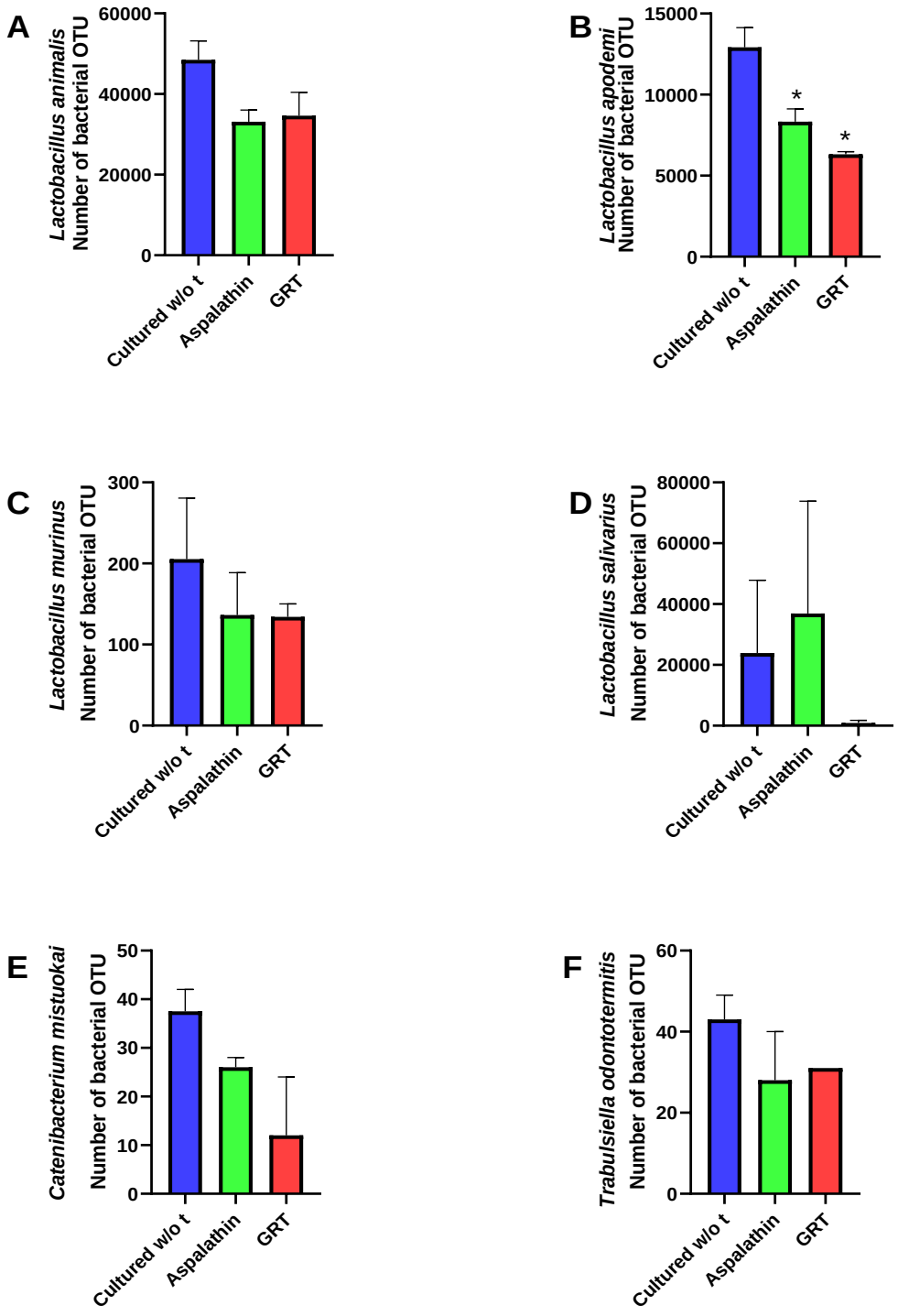


Figure 36: **The effect of aspalathin or GRT on Western diet monkey faecal samples**. Aspalathin, GRT compared to control samples, * $p<0.05$.

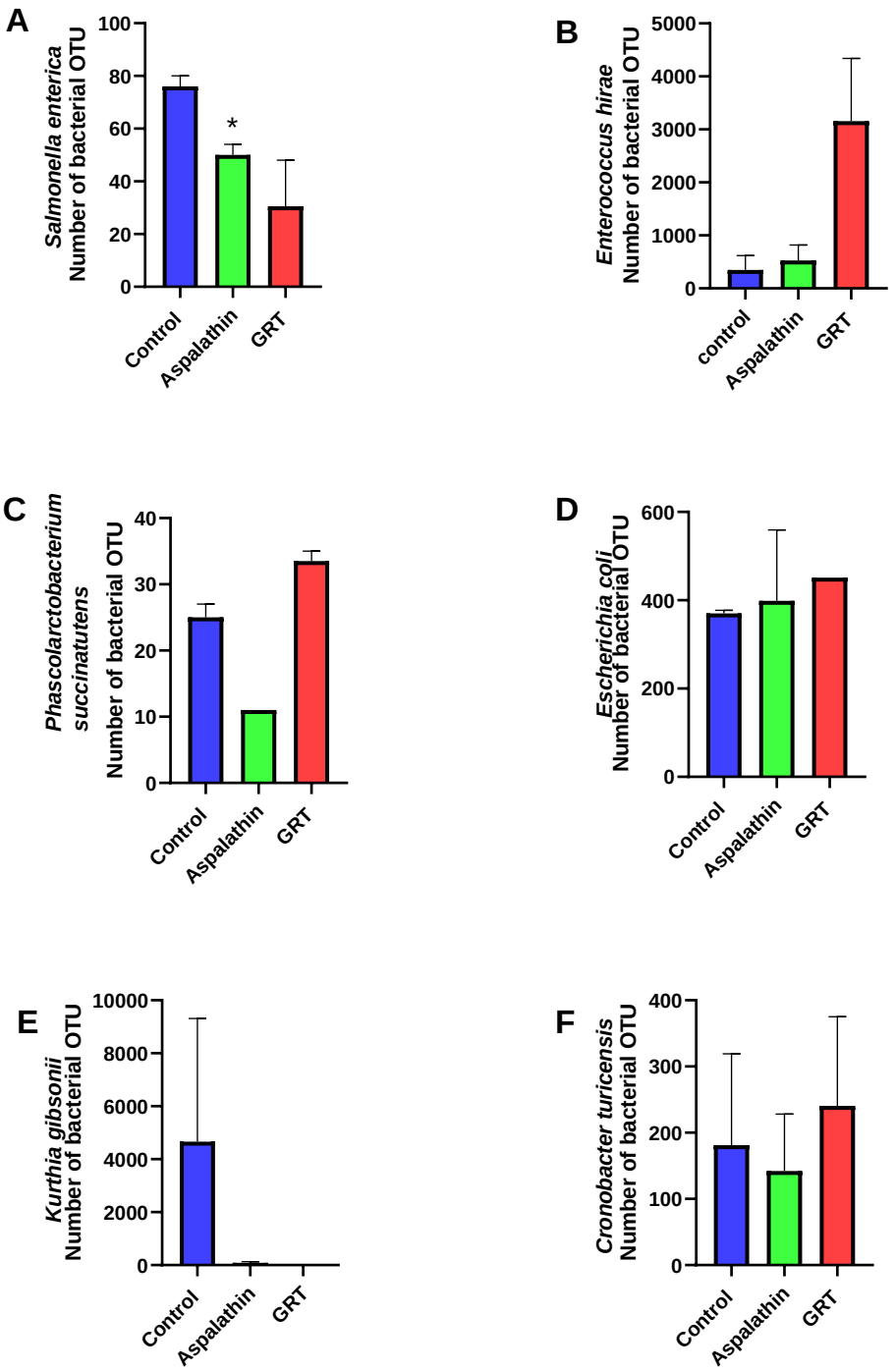


Figure 37: The effect of aspalathin or GRT on Western diet monkey faecal samples. Aspalathin, GRT compared to control samples, * $p<0.05$.

At a species level, due to the large diversity, only a few species were statistically affected by the treatment. From the faecal samples of standard diet monkeys, under culture, GRT (figure 34) decreased the abundance of *Lactobacillus murinus* (GRT (105±31.5, p=0.036). Western diet monkey faecal samples, cultured with either aspalathin or GRT (figures 35 to 37) GRT increased the abundance of *Prevotella copri, Prevotella stercorea* and *Faecalibacterium prausnitzi* significantly. While the presence of GRT decreased the abundance of *Lactobacillus apodemi* species. Whilst aspalathin decreased the presence of *Lactobacillus apodemi* and *Salmonella enterica* significantly.

4.9. SUMMARY TABLE

The summary table compares the effect of a Western diet on baseline culture-independent samples, the effect of Western diet on culture-dependent samples and the effect Afriplex GRT™ extract.

Table 6: Effect of Western diet before and after culturing, and GRT™ treatment

Species	Culture-independent Western diet	Culture-dependent Western diet	Culture-dependent effect of Afriplex GRT™
Faecalibacterium prausnitzii	↓	↑	↑
Prevotella copri	↓	↑	↑
Prevotella stercorea	↓	No change	↑
Lactobacillus murinus	↓	↑	↓
Catenibacterium mitsuokai	↑	↑	↓
Lactobacillus apodemi	↓	↑	↓
Lactobacillus animalis	↓	↑	↓

CHAPTER V

5. DISCUSSION

5.1. ALPHA DIVERSITY RICHNESS OF THE VERVET MONKEYS

Western diet plays a major role in altering the diversity of the gut microbiota. A study conducted by Amato and colleagues (2015) showed that the alpha diversity of monkey fed a Western-diet fed was higher compared to the non-western (standard) diet-fed monkeys (Amato *et al.*, 2015). Amato's findings correlates with the data observed in our study, in which we show that the Western diet-fed monkeys had higher species diversity than the standard diet-fed monkeys. However, human studies conducted by Yatsunenko and colleagues (2012), and Schorr and colleagues (2014), showed that there are higher species diversity in humans on a non-Western diet (Schnorr *et al*., 2014, Yatsunenko *et al.*, 2012).

An *in vivo* study conducted by David and colleagues (2014) examined if dietary interventions in humans alters gut microbial communities in a rapid, diet-specific manner. They compared plant and animal-based diets, they found that changing the macronutrients to animal-based diet decreases *Firmicute* abundance whilst bile tolerant microorganisms were increased (David *et al*., 2014). Our findings are in support of this, as both *Firmicute* and *Bacteroidete*s abundance was decreased in the Western diet fed monkeys whist *Proteobacteria* were increased in the both baseline culture-independent and cultured and samples. However, species diversity was reduced approximately by half in the culture-dependent samples, and regardless of culture, the Western diet was found to have higher species diversity than the standard diet.

5.2. EFFECT OF DIET ON THE GUT MICROBIOTA

Baseline culture-independent samples were collected so that we could assess the role diet plays in reshaping the gut microbiota phyla, genera, and species. The data showed that the diet played an essential role in reshaping the gut microbiota and this data correlates with numerous studies reported in the literature (Collins *et al*., 2016, Mastrocola *et al*., 2018). From our study, we observed that the *Firmicutes* phylum of the standard diet-fed monkey faecal samples was the most dominant followed by *Bacteroidetes* then *Proteobacteria*. Our results are similar to the vervet monkey study results conducted by Amato and colleagues (2015). However, according to a study conducted by Yatsunenko and colleagues (2012) on humans consuming a plant-based diet, *Bacteroidetes* were more abundant, followed by *Firmicutes* with *Proteobacteri*a being less dominant but still detectable (Hildebrandt *et al.*, 2009, Yastunenko *et al*., 2012, Amato *et al*., 2015).

In our study, *Proteobacteria* was found to be the most abundant phylum followed by *Firmicutes* and then *Bacteroidetes*. Suggesting that in vervet monkeys a Western diet decreases *Bacteroidetes* and *Firmicutes*

abundance while *Proteobacteria* increases. However, in the study reported by Turnbaugh and colleagues (2008) the Western diet-associated faecal community had a significantly higher relative abundance of the *Firmicutes* and a significantly lower relative abundance of the *Bacteroidetes*, (Turnbaugh *et al*, 2008). In the mice study reported by Hildebrandt and colleagues (2009), high-fat feeding of 40% fat as energy increased the *Proteobacteria* and decreases *Bacteroidetes* phylum. Supporting our findings that Western diet changes gut microbiota which leads to an increase in the prevalence of *Proteobacteria* in mice (Carmody *et al*., 2015).

At a genus level, our study showed that the *Acinetobacter, Ruminobacter, Eubacterium, Succinivibrio*, and *Pyramidobacter* were increased with Western diet-feeding, with *Eubacterium* being significantly increased as compared to a normal diet. *Acinetobacter* is a known pathogenic species reported to be increased by high-fat feeding (Ou *et al*., 2013). It is reported that *Roseburia, Prevotella, Lactobacillus,* and *Ruminobacter* are decreased by a Western diet with *Roseburia* being significantly decreased and *Lactobacillus* being depleted.

A study conducted by Russell and colleagues (2011) showed that subjects with a high protein/low carbohydrate diet have reduced *Roseburia* and *Eubacterium rectale* in their gut microbiota and a decreased proportion of butyrate in their faeces (Russell *et al*., 2011). A study by Million and colleagues (2012) reported that certain species in the *Lactobacillus* genera were decreased in obese individuals. Whilst *Lactobacillus* and *Bifidobacterium* species are associated with normal weight (Million *et al*., 2012). Our findings showed that the *Prevotella* genus was decreased on the Western diet, however this not statistically significant. This data correlates with a study reported by Schnorr (2014), where they looked at the gut microbiota from the Hadza people of Tanzania on a standard diet compared to the Italians who were on a Mediterranean diet. It was found that the Hadza people had higher *Prevotella, Eubacterium,* and *Succinivibrio* compared to the Italians (Schnorr *et al.*, 2014). In addition, the study (Ou *et al*., 2013) also confirmed that humans on a high fibre diet were found to have higher *Prevotella, Succinivibrio,* and *Oscillospira spp*. The study by Amato and colleagues (2015) contradicted our findings in terms of the abundance of *Prevotella*. They found decreased *Prevotella* in monkeys on a standard diet whilst our study shows that *Prevotella* was increased in the standard diet and decreased in the Western diet. However, our findings are in agreement with the findings of De Filippis and colleagues (2019). Our study showed that the *Succinivibrio* genus was low in a standard diet and increased by the Western diet. However, other studies (Ou *et al*., 2013, and Schnorr *et al.*, 2014) in humans was not in support of our study in terms of *Succinivibrio genus.*

At the genus level, our study showed that *Roseburia spp*. and *Faecalibacterium prausnitzii* were decreased in the Western diet-fed monkey faecal samples and this correlates with the data reported by both Neyrinck (2012) and Tamanai-Shacoori (2017). *Faecalibacterium prausnitzii* is known to be a butyrate-producing bacterium that plays an essential role in preventing metabolic diseases and has been used to treat dysbiosis. The abundance of *Faecalibacterium prausnitzii* promotes a healthy gut and it is known to have anti-inflammatory properties (Ganesan *et al*., 2018).

Bilophila wadsworthia was found to be increased in the Western diet-fed monkey's faecal samples. This species causes acute inflammation and generates hydrogen sulphide via taurine respiration (O'Keefe *et al*., 2015), which leads to intestinal barrier dysfunction (Natividad *et al*., 2018). Similarly, to our findings, *Bilophila wasworthia* was also increased in Western diet-fed monkey's faecal samples (Amato *et al*., 2015). In addition, *Catenibacterium mistuokai* was found to be increased in the Western diet-fed monkey. A study reported by Clarke and colleagues (2012), on humanized mice fed high-fat diet showed that *Catenibacterium mistuokai* was also increased along with other species such as *Clostridium innocuum* and *Eubacterium dolichum* (Clarke *et al*., 2012) and this report correlates with the data observed in our study.

5.3. IMPACT OF CULTURING ON THE GUT MICROBIOTA DIVERSITY SHIFTS OR GROWTH

5.3.1. Choice of anaerobic culturing system

Anaerobic culturing enumerates bacterial species based on their environmental and nutritive needs. Strict anaerobic bacteria tend to be difficult to grow under simulated anaerobic conditions. There were two anaerobic culturing systems i.e. Speedy Breedy and anaerobic chamber culturing systems used in our study. In both methods, samples were enumerated on broth media. Our results showed that the Speedy Breedy was a better culturing system to use as we got higher species diversity and we got higher sequences per sample. There is not much-published data on the Speedy Breedy as mostly it is used by the food industry to detect contamination in their food samples (SpeedyBreedy.com, 2018). The anaerobic chamber we used broke down while we were still doing a pilot study, we couldn't do any further analysis and we do not have enough data to properly compare the two systems. However, conclusions made were based on the pilot study data Beta diversity showed that samples cultured using anaerobic chamber and the Speedy Breedy clustered in different areas indicating that the results achieved by the two systems were dissimilar.

5.3.2. Comparing culture-independent and culture-independent metagenomics techniques

The baseline culture-independent sequenced data is mostly used by researchers because it is relates to the *in vivo* microbiota diversity, and it's not labour intensive, easy to characterize and profile complex communities, it enables taxonomic composition and functional metagenomics studies (Cocolin *et al*., 2013, Hiergeist *et al.,* 2015). Culture-independent metagenomics technique compared to culture-dependent methods can provide direct and in-depth insight into the composition of the microbiota. While culture-dependent techniques do not detect organisms with low abundance (Hiergeist *et al*., 2015). From our findings it was noted that that there were differences in bacterial species alpha and beta microbial diversity when comparing culture-dependent and culture-independent techniques. The culture-independent samples had higher species diversity compared to culture-dependent techniques. Our study showed that the culture-independent samples on beta diversity were clustered together in one area while the culture-dependent also clustered together in a separate area.

5.3.3. Species favoured due to culturing

Culturing is known to favour certain microorganisms due to their environmental, nutritive and oxygen requirements. In this study, it was noted that the *Bacteroidetes* phylum was not favoured with culturing, as most of the species abundance was decreased and depleted. Based on our findings this suggests that many of the species belonging to the *Bacteroidetes* phylum are either unculturable or slow to grow as the culturing period for the Speedy Breedy culturing system was shorter. Our findings showed that bacterial species such as *Bifidobacterium adolescentis, Collinsella aerofaciens*, *Bacteroides intestinalis, Bacteroides vulgatus*, and *Prevotella spp*. classified under the *Bacteroidetes* phylum detected at baseline culture-independent samples were depleted during culturing. However, most species belonging to the *Firmicutes* phyla were favoured during culturing as the number of species (OTU) detected increased when compared to those of the baseline culture-independent data. The *Lactobacillus* genera were highly favoured during culturing as it increased the number of species OTU detected. When comparing the standard diet to the Western diet data, *Lactobacillus murinus* and *Lactobacillus apodemi* species were significantly increased. However, *Lactobacillus vaginalis* abundance was depleted during anaerobic culturing. *Lactobacillus* genus is a facultative anaerobe; hence it grows at a faster rate than other strict anaerobe bacteria. A study conducted by Vartoukian (2016) reported on the *in vitro* culture of unculturable bacteria; showed that *Prevotella spp*. and other unculturable bacterial species need siderophore-like/iron-chelating agents to stimulate their growth. (Vartoukian *et al*., 2016).

5.4. SHIFTS IN THE MICROBIAL COMMUNITY INFLUENCED BY ROOIBOS PHENOLIC COMPOUNDS, i.e. ASPALATHIN AND AFRIPLEX GRT™

Anaerobic culturing shifts the phyla distribution. Our findings showed that the OTU percentage for *Firmicutes* and *Proteobacteria* phyla in both Western and the standard diet groups was at a similar abundance level. However, the Bacteroidetes phyla showed a slight increase in the Western diet as compared to the standard diet. This data allowed us to determine the effect of aspalathin and Afriplex GRT™ on the gut microbiota *in vitro*. Aspalathin and Afriplex GRT™ did not have a significant effect on the phyla of the gut microbiome species. Although, Afriplex GRT™ decreased the prevalence of *Proteobacteria* diversity in the Western diet-fed monkeys and increased the prevalence of the *Firmicutes* in the standard diet fed monkeys, however the changes were not significant. *Faecalibacterium* and *Prevotella* genus. were found to be increased when treated with Afriplex GRT™. This was supported by a study reported by Popa and colleagues (2019) that showed that *Prevotella* levels were higher in diets high in polyphenolic compounds (Popa *et al.*, 2019).

Aspalathin and Afriplex GRT™ suppressed the growth of *Lactobacillus* genus and this is supported by a study reported by (Parkar *et al*., 2008) where polyphenol compounds were tested with *Lactobacillus rhamnosus* species and it was found that out of all tested polyphenols, quercetin and naringenin gave the highest inhibitory effects on the growth of the *Lactobacillus rhamnosus* species. Both aspalathin and Afriplex GRT™ in the standard diet and Western diet showed an inhibitory effect on *Lactobacillus* species, with *Lactobacillus murinus* being significantly decreased by Afriplex GRT™ in the standard diet fed monkey samples and *Lactobacillus apodemi* significantly decreased by both aspalathin and Afriplex GRT™ in the high fat fed monkey samples.

In our study, we show the "good" gut microbiota species were increased in the presence of Afriplex GRT™ while decreases the prevalence of the pathogenic species. *Prevotella* genus, in faecal samples from Western diet-fed monkeys, were increased by Afriplex GRT™ with *Prevotella copri* and *Prevotella stercorea* significantly increased by Afriplex GRT™ whilst the growth of pathogenic species such as *Salmonella enterica* was inhibited by Afriplex GRT™, and aspalathin. These findings are in support of Tzounis (2007) showing that polyphenols benefited the growth of species such as *Lactobacillus* and *Bifidobacterium* and inhibited the growth of pathogenic bacteria (Tzounis *et al*., 2007).

Furthermore, the results obtained demonstrate that aspalathin did not invoke changes in the gut microbiota diversity while Afriplex GRT™ invoked changes. This may be due to aspalathin being a single phenolic compound that is not easily broken down by the bacteria due to its stable *C-C* glycosidic bond. A study by

Courts & Williamson (2009), showed that aspalathin needs to be methylated before it can be taken up by the cells. This might be the case in this study (Courts & Williamson, 2009). However, as Afriplex GRT™ is an aspalathin-rich unfermented rooibos extract that contains a complex mixture of polyphenols, its effect was more pronounced and was shown to suppress the pathogenic bacteria while it enumerated the beneficial bacteria.

CHAPTER VI

6. CONCLUSION

6.1. CONCLUSION

The gut microbiota has been termed the hidden organ as it protects the host against pathogenic bacteria, maintains the gut barrier function and has a number of metabolic functions. The growing interest in the role of the intestinal microbiota metabolic diseases development has gained interest, although it remains a relatively new area of research. Diet is linked to altered gut microbiota diversity, and therefore has been amongst the factors of interest in the field. Our study was able to show that the Western diet decreases the prevalence of *Firmicutes* and *Bacteroidetes* phyla which are associated with beneficial microbes. Whilst it also increased the prevalence of *Proteobacteria* phylum, associated with pathogenic gut bacterial species, supporting the notion that diet alters the gut microbiota distribution. The study also suggests that culturing decreases the microbial richness as demonstrated by the decreased abundance of *Bacteroidetes,* enriched *Firmicutes* and *Proteobacteria* phylum.

Natural compounds, such as medicinal plants have gained attention due to their ability to modulate the gut microbiota for beneficial effects. The study also evaluated the potential prebiotic effect of rooibos phenolic compounds using aspalathin and aspalathin-rich green rooibos extract Afriplex GRT™ on the gut microbiota regulation. Here we showed that the treatment of *in vitro* cultures with Afriplex GRT™ increases the prevalence of beneficial bacteria such as *Faecalibacterium prausnitzii* and *Prevotella copri*. This then suggests that Afriplex GRT™ was able to reverse Western diet induced gut microbiota changes, and that Afriplex GRT™ could be used as a beneficial prebiotic on gut health.

6.2. STUDY LIMITATIONS

- The biggest limitation was the number of animals used. We used three animals in each study group instead of the five planned in the study due to the unavailability of the Speedy Breedy culturing vessels.
- The second limitation for this study was equipment failure. The anaerobic chamber kept on leaking and breaking down, and the repair process for it is still ongoing. Therefore, we could not conclude our work on it.
- The third limitation was the closure of the Speedy Breedy company in 2018. The vessels we used for anaerobic culturing were unavailable which limited the number of samples we were able to do.

6.3. FUTURE WORK

- We are planning to increase the number of animals used for this study.
- We have to look at the breakdown products of aspalathin by action of microbial species.
- We are also planning to do an *in vitro* study using human samples of obese and normal weight subjects to elucidate the effect that the microbiome phenotype has on metabolic disease.

CHAPTER VII

7. REFERENCES

Agyemang, C., Meeks, K., Beune, E., Owusu-Dabo, E., Mockenhaupt, F., Addo, J., de Graft Aikins, A., Bahendeka, S., Danquah, I., Schulze, M., Spranger, J., Burr, T., Agyei-Baffour, P., Amoah, S., Galbete, C., Henneman, P., Klipstein-Grobusch, K., Nicolaou, M., Adeyemo, A., van Straalen, J., Smeeth, L. & Stronks, K. 2016. Obesity and type 2 diabetes in sub-Saharan Africans – Is the burden in today's Africa similar to African migrants in Europe? The RODAM study. *BMC Medicine*, 14(1).

Alberti, K., Eckel, R., Grundy, S., Zimmet, P., Cleeman, J., Donato, K., Fruchart, J., James, W., Loria, C. & Smith, S. 2010. Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Forceon Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World HeartFederation; International Atherosclerosis Society; and International Association for the Study of Obesity. *Obesity and metabolism*, (1): 63.

Allin, K., Nielsen, T. & Pedersen, O. 2015. Mechanisms in Endocrinology: Gut microbiota in patients with type 2 diabetes mellitus. *European Journal of Endocrinology*, 172(4): R167-R177.

Al-Qawasmeh, R. & Tayyem, r. 2018. Dietary and Lifestyle Risk Factors and Metabolic Syndrome: Literature Review. *Current Research in Nutrition and Food Science Journal*, 6(3): 594-608.

Amato, K., Yeoman, C., Cerda, G., A. Schmitt, C., Cramer, J., Miller, M., Gomez, A., R. Turner, T., Wilson, B., Stumpf, R., Nelson, K., White, B., Knight, R. & Leigh, S. 2015. Variable responses of human and non-human primate gut microbiomes to a Western diet. *Microbiome*, 3(1).

Animal welfare institute. 2018. Non-Human Primates. *Awionline.org*. https://awionline.org/content/non-human-primates 16 May 2019.

Anon. 2018. CM0733, Maximum Recovery Diluent (Peptone Saline Broth) | Oxoid - Product Detail. *Oxoid.com*. http://www.oxoid.com/UK/BLUE/prod_detail/prod_detail.asp?pr=CM0733&c=UK&lang=EN 21 November 2018.

Anon.2018. *Bvascientific.com*. http://bvascientific.com/Customer/miscne/specpages/299070.pdf 20 November 2018

Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D., Fernandes, G., Tap, J., Bruls, T., Batto, J., Bertalan, M., Borruel, N., Casellas, F., Fernandez, L., Gautier, L., Hansen, T., Hattori, M.,

Hayashi, T., Kleerebezem, M., Kurokawa, K., Leclerc, M., Levenez, F., Manichanh, C., Nielsen, H., Nielsen, T., Pons, N., Poulain, J., Qin, J., Sicheritz-Ponten, T., Tims, S., Torrents, D., Ugarte, E., Zoetendal, E., Wang, J., Guarner, F., Pedersen, O., de Vos, W., Brunak, S., Doré, J., Antolín, M., Artiguenave, F., Blottiere, H., Almeida, M., Brechot, C., Cara, C., Chervaux, C., Cultrone, A., Delorme, C., Denariaz, G., Dervyn, R., Foerstner, K., Friss, C., van de Guchte, M., Guedon, E., Haimet, F., Huber, W., van Hylckama-Vlieg, J., Jamet, A., Juste, C., Kaci, G., Knol, J., Lakhdari, O., Layec, S., Le Roux, K., Maguin, E., Mérieux, A., Melo Minardi, R., M'rini, C., Muller, J., Oozeer, R., Parkhill, J., Renault, P., Rescigno, M., Sanchez, N., Sunagawa, S., Torrejon, A., Turner, K., Vandemeulebrouck, G., Varela, E., Winogradsky, Y., Zeller, G., Weissenbach, J., Ehrlich, S. & Bork, P. 2011. Enterotypes of the human gut microbiome. *Nature*, 473(7346): 174-180.

Assimakopoulos, S., Triantos, C., Maroulis, I. & Gogos, C. 2018. The Role of the Gut Barrier Function in Health and Disease. *Gastroenterology Research*, 11(4): 261-263.

Backhed, F., Ding, H., Wang, T., Hooper, L., Koh, G., Nagy, A., Semenkovich, C. & Gordon, J. 2004. The gut microbiota as an environmental factor that regulates fat storage. *Proceedings of the National Academy of Sciences*, 101(44): 15718-15723.

Balamurugan, R., George, G., Kabeerdoss, J., Hepsiba, J., Chandragunasekaran, A. & Ramakrishna, B. 2009. Quantitative differences in intestinal Faecalibacterium prausnitzii in obese Indian children. *British Journal of Nutrition*, 103(3): 335-338.

Berg, R. 1996. The indigenous gastrointestinal microflora. *Trends in Microbiology*, 4(11): 430-435.

Berings, M., Karaaslan, C., Altunbulakli, C., Gevaert, P., Akdis, M., Bachert, C. & Akdis, C. 2017. Advances and highlights in allergen immunotherapy: On the way to sustained clinical and immunologic tolerance. *Journal of Allergy and Clinical Immunology*, 140(5): 1250-1267.

Binns, N. 2013. *Probiotics, prebiotics and the gut microbiota*. Brussels: ILSI Europe.

Biology discussions. 2019. Culture Media for Cultivation of Anaerobic Bacteria: 4 Types. *Biology Discussion*. http://www.biologydiscussion.com/microorganisms/culture-media-for-cultivation-of-anaerobic-bacteria-4-types/55049 16 May 2019.

Blaut, M. & Clavel, T. 2007. Metabolic Diversity of the Intestinal Microbiota: Implications for Health and Disease. *The Journal of Nutrition*, 137(3): 751S-755S.

Braune, A. & Blaut, M. 2016. Bacterial species involved in the conversion of dietary flavonoids in the human gut. *Gut Microbes*, 7(3): 216-234.

Brial, F., Le Lay, A., Dumas, M. & Gauguier, D. 2018. Implication of gut microbiota metabolites in cardiovascular and metabolic diseases. *Cellular and Molecular Life Sciences*, 75(21): 3977-3990.

Brown, J. & Hazen, S. 2018. Microbial modulation of cardiovascular disease. *Nature Reviews Microbiology*, 16(3): 171-181.

Byrne, C., Chambers, E., Morrison, D. & Frost, G. 2015. The role of short-chain fatty acids in appetite regulation and energy homeostasis. *International Journal of Obesity*, 39(9): 1331-1338.

Cani, P. (2019). Microbiota and metabolites in metabolic diseases. *Nature Reviews Endocrinology*, 15(2), pp.69-70.

Cani, P., Amar, J., Iglesias, M., Poggi, M., Knauf, C., Bastelica, D., Neyrinck, A., Fava, F., Tuohy, K., Chabo, C., Waget, A., Delmee, E., Cousin, B., Sulpice, T., Chamontin, B., Ferrieres, J., Tanti, J., Gibson, G., Casteilla, L., Delzenne, N., Alessi, M. & Burcelin, R. 2007. Metabolic Endotoxemia Initiates Obesity and Insulin Resistance. *Diabetes*, 56(7): 1761-1772.

Cani, P., Neyrinck, A., Fava, F., Knauf, C., Burcelin, R., Tuohy, K., Gibson, G. & Delzenne, N. 2007. Selective increases of bifidobacteria in gut microflora improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxaemia. *Diabetologia*, 50(11): 2374-2383.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walter, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods. 7(5), 335-336.

Carmody, R., Gerber, G., Luevano, J., Gatti, D., Somes, L., Svenson, K. & Turnbaugh, P. 2015. Diet Dominates Host Genotype in Shaping the Murine Gut Microbiota. *Cell Host & Microbe*, 17(1): 72-84.

Chao, A., 1984. Nonparametric estimation of the number of classes in a population. Scand. J. Statist. 11(4), 265-270.

Chen, G., Qiu, Y., Zhuang, Q., Wang, S., Wang, T., Chen, J. & Wang, K. 2018. Next-generation sequencing library preparation method for identification of RNA viruses on the Ion Torrent Sequencing Platform. *Virus Genes*, 54(4): 536-542.

Cheng, L., O'Grady, G., Du, P., Egbuji, J., Windsor, J. & Pullan, A. 2010. Gastrointestinal system. *Wiley Interdisciplinary Reviews: Systems Biology and Medicine*, 2(1): 65-79.

Clarke, S., Murphy, E., Nilaweera, K., Ross, P., Shanahan, F., O'Toole, P. & Cotter, P. 2012. The gut microbiota and its relationship to diet and obesity. *Gut Microbes*, 3(3): 186-202.

Clayton, J., Gomez, A., Amato, K., Knights, D., Travis, D., Blekhman, R., Knight, R., Leigh, S., Stumpf, R., Wolf, T., Glander, K., Cabana, F. & Johnson, T. 2018. The gut microbiome of nonhuman primates: Lessons in ecology and evolution. *American Journal of Primatology*, 80(6): e22867.

Clayton, J., Vangay, P., Huang, H., Ward, T., Hillmann, B., Al-Ghalith, G., Travis, D., Long, H., Tuan, B., Minh, V., Cabana, F., Nadler, T., Toddes, B., Murphy, T., Glander, K., Johnson, T. & Knights, D. 2016. Captivity humanizes the primate microbiome. *Proceedings of the National Academy of Sciences*, 113(37): 10376-10381.

Cocolin, L., Alessandria, V., Dolci, P., Gorra, R. & Rantsiou, K. 2013. Culture independent methods to assess the diversity and dynamics of microbiota during food fermentation. *International Journal of Food Microbiology*, 167(1): 29-43.

Collins, K., Paul, H., Hart, D., Reimer, R., Smith, I., Rios, J., Seerattan, R. & Herzog, W. 2016. A High-Fat High-Sucrose Diet Rapidly Alters Muscle Integrity, Inflammation and Gut Microbiota in Male Rats. *Scientific Reports*, 6(1).

Collins, M. & Gibson, G. 1999. Probiotics, prebiotics, and synbiotics: approaches for modulating the microbial ecology of the gut. *The American Journal of Clinical Nutrition*, 69(5): 1052s-1057s.

Courts, F. & Williamson, G. 2009. The C-glycosyl flavonoid, aspalathin, is absorbed, methylated and glucuronidated intact in humans. *Molecular Nutrition & Food Research*, 53(9): 1104-1111.

Dahlgren, R. (1968). Revision of the genus *Aspalathus*. II. The species with the ericoid and pinoid leaflets. 7. Subgenus *Nortiera*. With remarks on rooibos tea cultivation. *Botaniska Notiser*, 121, 165–208.

David, L., Maurice, C., Carmody, R., Gootenberg, D., Button, J., Wolfe, B., Ling, A., Devlin, A., Varma, Y., Fischbach, M., Biddinger, S., Dutton, R. & Turnbaugh, P. 2014. Diet rapidly and reproducibly alters the human gut microbiome. *Nature*, 505(7484): 559-563.

De Filippis, F., Pasolli, E., Tett, A., Tarallo, S., Naccarati, A., De Angelis, M., Neviani, E., Cocolin, L., Gobbetti, M., Segata, N. & Ercolini, D. 2019. Distinct Genetic and Functional Traits of Human Intestinal Prevotella copri Strains Are Associated with Different Habitual Diets. *Cell Host & Microbe*, 25(3): 444-453.e3.

De Filippo, C., Cavalieri, D., Di Paola, M., Ramazzotti, M., Poullet, J., Massart, S., Collini, S., Pieraccini, G. & Lionetti, P. 2010. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proceedings of the National Academy of Sciences*, 107(33): 14691-14696.

De Vadder, F., Kovatcheva-Datchary, P., Goncalves, D., Vinera, J., Zitoun, C., Duchampt, A., Bäckhed, F. & Mithieux, G. 2014. Microbiota-Generated Metabolites Promote Metabolic Benefits via Gut-Brain Neural Circuits. *Cell*, 156(1-2): 84-96.

Deliorman Orhan, D., Aslan, M., Aktay, G., Ergun, E., Yesilada, E. & Ergun, F. (2003). Evaluation of hepatoprotective effect of *Gentiana olivieri* herbs on subacute administration and isolation of active principle. *Life Sciences*, **72**, 2273–2283.

Diamant, M., Blaak, E. & de Vos, W. 2010. Do nutrient-gut-microbiota interactions play a role in human obesity, insulin resistance and type 2 diabetes? *Obesity Reviews*, 12(4): 272-281.

Donaldson, G., Lee, S. & Mazmanian, S. 2015. Gut biogeography of the bacterial microbiota. *Nature Reviews Microbiology*, 14(1): 20-32.

Everard, A. & Cani, P. 2013. Diabetes, obesity and gut microbiota. *Best Practice & Research Clinical Gastroenterology*, 27(1): 73-83.

Fairbanks, L., Blau, K. & Jorgensen, M. 2009. The high-fibre diet promotes weight loss and affects maternal behaviour in vervet monkeys. *American Journal of Primatology*, 72(3): 234-241.

Fåk, F. & Bäckhed, F. 2012. *Lactobacillus* reuteri Prevents Diet-Induced Obesity, but not Atherosclerosis, in a Strain Dependent Fashion in Apoe−/− Mice. *PLoS ONE,* 7(10): e46837.

Fincham BVSc, J., Benadé, A., Kruger MNUTR, M., Smuts, C., Gobregts, E., Chalton, D. & Kritchevsky, D. 1998. Atherosclerosis: Aortic Lipid Changes Induced by Diets Suggest Diffuse Disease with Focal Severity in Primates That Model Human Atheromas. *Nutrition*, 14(1): 17-22.

Fuller, R. 1992. History and Development of Probiotics. *Probiotics*, Springer, Netherlands, 1-8.

Ganesan, K., Chung, S., Vanamala, J. & Xu, B. 2018. Causal Relationship between Diet-Induced Gut Microbiota Changes and Diabetes: A Novel Strategy to Transplant Faecalibacterium prausnitzii in Preventing Diabetes. *International Journal of Molecular Sciences*, 19(12): 3720.

Gentile, C. & Weir, T. 2018. The gut microbiota at the intersection of diet and human health. *Science*, 362(6416): 776-780.

Ghosh, A., Mehta, A. & Khan, A. 2019. Metagenomic Analysis and its Applications. *Encyclopedia of Bioinformatics and Computational Biology*: 184-193.

Gibson, G., Hutkins, R., Sanders, M., Prescott, S., Reimer, R., Salminen, S., Scott, K., Stanton, C., Swanson, K., Cani, P., Verbeke, K. & Reid, G. 2017. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nature Reviews Gastroenterology & Hepatology*.

Gibson, G., Scott, K., Rastall, R., Tuohy, K., Hotchkiss, A., Dubert-Ferrandon, A., Gareau, M., Murphy, E., Saulnier, D., Loh, G., Macfarlane, S., Delzenne, N., Ringel, Y., Kozianowski, G., Dickmann, R., Lenoir-Wijnkoop, I., Walker, C. & Buddington, R. 2010. Dietary prebiotics: current status and new definition. *Food Science & Technology Bulletin: Functional Foods*, 7(1): 1-19.

Gregoire, C. 2019. How the Body Maintains A Healthy Balance Of 'Friendly' Gut Bacteria. *Huffpost*. https://www.huffpost.com/entry/gut-bacteria-health_n_6473198 14 November 2019.

Groves, C. 2001. Why taxonomic stability is a bad idea, or why are there so few species of primates (or are there?). *Evolutionary Anthropology: Issues, News, and Reviews*, 10(6): 192-198.

Grundy, S. 2018. Metabolic Syndrome. *Endocrinology*: 71-107.

Hanhineva, K., Törrönen, R., Bondia-Pons, I., Pekkinen, J., Kolehmainen, M., Mykkänen, H. & Poutanen, K. 2010. Impact of Dietary Polyphenols on Carbohydrate Metabolism. *International Journal of Molecular Sciences*, 11(4): 1365-1402.

Hayashi, H., Shibata, K., Sakamoto, M., Tomita, S. & Benno, Y. 2007. Prevotella copri sp. nov. and Prevotella stercorea sp. nov., isolated from human faeces. *International Journal of Systematic and Evolutionary Microbiology*, 57(5): 941-946.

Hespell, R. 1992. The Genera Succinivibrio and Succinimonas. *The Prokaryotes*: 3979-3982.

Hiergeist, A., Gläsner, J., Reischl, U. & Gessner, A. 2015. Analyses of Intestinal Microbiota: Culture versus Sequencing: Figure 1. *ILAR Journal*, 56(2): 228-240.

Hildebrandt, M., Hoffmann, C., Sherrill–Mix, S., Keilbaugh, S., Hamady, M., Chen, Y., Knight, R., Ahima, R., Bushman, F. & Wu, G. 2009. High-Fat Diet Determines the Composition of the Murine Gut Microbiome Independently of Obesity. *Gastroenterology*, 137(5): 1716-1724.e2.

Hills, R., Pontefract, B., Mishcon, H., Black, C., Sutton, S. & Theberge, C. 2019. Gut Microbiome: Profound Implications for Diet and Disease. *Nutrients*, 11(7): 1613.

Holmes, E., Li, J., Marchesi, J. & Nicholson, J. 2012. Gut Microbiota Composition and Activity in Relation to Host Metabolic Phenotype and Disease Risk. *Cell Metabolism*, 16(5): 559-564.

Huang, M., du Plessis, J., du Preez, J., Hamman, J. & Viljoen, A. 2008. Transport of aspalathin, a Rooibos tea flavonoid, across the skin and intestinal epithelium. *Phytotherapy Research*, 22(5): 699-704.

Isani, M., Bell, B., Delaplain, P., Bowling, J., Golden, J., Elizee, M., Illingworth, L., Wang, J., Gayer, C., Grishin, A. & Ford, H. 2018. Lactobacillus murinus HF12 colonizes neonatal gut and protects rats from necrotizing enterocolitis. *PLOS ONE*, 13(6): e0196710.

Islam, K., Fukiya, S., Hagio, M., Fujii, N., Ishizuka, S., Ooka, T., Ogura, Y., Hayashi, T. & Yokota, A. 2011. Bile Acid Is a Host Factor That Regulates the Composition of the Cecal Microbiota in Rats. *Gastroenterology*, 141(5): 1773-1781.

Johansson, M., Ambort, D., Pelaseyed, T., Schütte, A., Gustafsson, J., Ermund, A., Subramani, D., Holmén-Larsson, J., Thomsson, K., Bergström, J., van der Post, S., Rodriguez-Piñeiro, A., Sjövall, H., Bäckström, M. & Hansson, G. 2011. Composition and functional role of the mucus layers in the intestine. *Cellular and Molecular Life Sciences*, 68(22): 3635-3641.

Johansson, M., Jakobsson, H., Holmén-Larsson, J., Schütte, A., Ermund, A., Rodríguez-Piñeiro, A., Arike, L., Wising, C., Svensson, F., Bäckhed, F. & Hansson, G. 2015. Normalization of Host Intestinal Mucus Layers Requires Long-Term Microbial Colonization. *Cell Host & Microbe*, 18(5): 582-592.

Johnson, R., Beer, D., Dludla, P., Ferreira, D., Muller, C. & Joubert, E. 2018. Aspalathin from Rooibos (Aspalathus linearis): A Bioactive C-glucosyl Dihydrochalcone with Potential to Target the Metabolic Syndrome. *Planta Medica*, 84(09/10): 568-583.

Joubert, E., de Beer, D., Malherbe, C., Muller, N., Bonnet, S., van der Westhuizen, J. & Ferreira, D. 2013. Occurrence and sensory perception of Z-2-(β-d-glucopyranosyloxy)-3-phenylpropenoic acid in rooibos (Aspalathus linearis). *Food Chemistry*, 136(2): 1078-1085.

Joubert, E., Gelderblom, W., Louw, A. & de Beer, D. 2008. South African herbal teas: Aspalathus linearis, Cyclopia spp. and Athrixia phylicoides—A review. *Journal of Ethnopharmacology*, 119(3): 376-412.

Kavanagh, K., Fairbanks, L., Bailey, J., Jorgensen, M., Wilson, M., Zhang, L., Rudel, L. & Wagner, J. 2007. Characterization and Heritability of Obesity and Associated Risk Factors in Vervet Monkeys*. *Obesity*, 15(7): 1666-1674.

Khosravi, A. & Mazmanian, S. 2013. Disruption of the gut microbiome as a risk factor for microbial infections. *Current Opinion in Microbiology*, 16(2): 221-227.

Kim, M., Lee, J. & Han, J. 2014. Deglycosylation of isoflavoneC-glycosides by newly isolated human intestinal bacteria. *Journal of the Science of Food and Agriculture*, 95(9): 1925-1931.

Kim, T., Lee, J., Chae, Y., Jung, I. & Kim, M. 2018. Additive effects of evogliptin in combination with pioglitazone on fasting glucose control through direct and indirect hepatic effects in diabetic mice. *European Journal of Pharmacology*, 830: 95-104.

Koeppen, B. & Roux, D. 1966. C-glycosylflavonoids. The chemistry of aspalathin. *Biochemical Journal*, 99(3): 604-609.

Koh, A., Molinaro, A., Ståhlman, M., Khan, M., Schmidt, C., Mannerås-Holm, L., Wu, H., Carreras, A., Jeong, H., Olofsson, L., Bergh, P., Gerdes, V., Hartstra, A., de Brauw, M., Perkins, R., Nieuwdorp, M., Bergström, G. and Bäckhed, F. (2018). Microbially Produced Imidazole Propionate Impairs Insulin Signaling through mTORC1. *Cell*, 175(4), pp.947-961.e17.

Kovatcheva-Datchary, P., Nilsson, A., Akrami, R., Lee, Y., De Vadder, F., Arora, T., Hallen, A., Martens, E., Björck, I. & Bäckhed, F. 2015. Dietary Fiber-Induced Improvement in Glucose Metabolism Is Associated with Increased Abundance of Prevotella. *Cell Metabolism*, 22(6): 971-982.

Krafczyk, N. & Glomb, M.A. (2008). Characterization of phenolic compounds in rooibos tea. *Journal of Agricultural and Food Chemistry*, **56**, 3368–3376.

Kreuz, S., Joubert, E., Waldmann, K. & Ternes, W. 2008. Aspalathin, a flavonoid in Aspalathus linearis (rooibos), is absorbed by pig intestine as a C-glycoside. *Nutrition Research*, 28(10): 690-701.

Küpeli, E., Aslan, M., Gürbüz, I. & Yesilada, E. (2004). Evaluation of in vivo biological activity profile of isoorientin. *Zeitschrift für Naturforschung*, **59c**, *787–790.*

LeBlanc, J., Chain, F., Martín, R., Bermúdez-Humarán, L., Courau, S. & Langella, P. 2017. Beneficial effects on host energy metabolism of short-chain fatty acids and vitamins produced by commensal and probiotic bacteria. Microbial Cell Factories, 16(1).

Lee, H., Jenner, A., Low, C. & Lee, Y. 2006. Effect of tea phenolics and their aromatic fecal bacterial metabolites on intestinal microbiota. *Research in Microbiology*, 157(9): 876-884.

Lee, P., Yacyshyn, B. & Yacyshyn, M. 2018. Gut microbiota and obesity: An opportunity to alter obesity through faecal microbiota transplant (FMT). *Diabetes, Obesity and Metabolism*, 21(3): 479-490.

Ley, R. 2016. Prevotella in the gut: choose carefully. *Nature Reviews Gastroenterology & Hepatology*, 13(2): 69-70.

Lopez-Siles, M., Duncan, S., Garcia-Gil, L. & Martinez-Medina, M. 2017. Faecalibacterium prausnitzii: from microbiology to diagnostics and prognostics. *The ISME Journal*, 11(4): 841-852.

Louw, J., Woodroof, C., Seier, J. & Wolfe-Coote, S. 1997. The effect of diet on the Vervet monkey endocrine pancreas. *Journal of Medical Primatology*, 26(6): 307-311.

Madhavan, A., Sindhu, R., Parameswaran, B., Sukumaran, R. & Pandey, A. 2017. Metagenome Analysis: A Powerful Tool for Enzyme Bioprospecting. *Applied Biochemistry and Biotechnology*, 183(2): 636-651.

Makki, K., Deehan, E., Walter, J. & Bäckhed, F. 2018. The Impact of Dietary Fiber on Gut Microbiota in Host Health and Disease. *Cell Host & Microbe*, 23(6): 705-715.

Marques, A., Paludo, K., Dallagassa, C., Surek, M., Pedrosa, F., Souza, E., Cruz, L., LiPuma, J., Zanata, S., Rego, F. & Fadel-Picheth, C. 2014. Biochemical Characteristics, Adhesion, and Cytotoxicity of Environmental and Clinical Isolates of Herbaspirillum spp. *Journal of Clinical Microbiology*, 53(1): 302-308.

Martín, R., Bermúdez-Humarán, L. & Langella, P. 2018. Searching for the Bacterial Effector: The Example of the Multi-Skilled Commensal Bacterium Faecalibacterium prausnitzii. *Frontiers in Microbiology*, 9.

Martín, R., Miquel, S., Chain, F., Natividad, J., Jury, J., Lu, J., Sokol, H., Theodorou, V., Bercik, P., Verdu, E., Langella, P. & Bermúdez-Humarán, L. 2015. Faecalibacterium prausnitzii prevents physiological damages in a chronic low-grade inflammation murine model. *BMC Microbiology*, 15(1).

Martin, S., Palmour, R.M., Goldwater, R., Gutkowsa, J., Hughes, C., Hamet, P., Ervin, F.R., 1990. Characterization of a primate model of hypertension. The response of hypertensive and normotensive male Vervets (*Cercopithecus aethiops*) to cold pressor stress, captopril administration, and an acute bolus of atrial natriuretic factor. *American Journal of Hypertension*. 3, 27–32.

Mastrocola, R., Ferrocino, I., Liberto, E., Chiazza, F., Cento, A., Collotta, D., Querio, G., Nigro, D., Bitonto, V., Cutrin, J., Rantsiou, K., Durante, M., Masini, E., Aragno, M., Cordero, C., Cocolin, L. & Collino, M. 2018. Fructose liquid and solid formulations differently affect gut integrity, microbiota composition and related liver toxicity: a comparative in vivo study. *The Journal of Nutritional Biochemistry*, 55: 185-199.

McCracken, E., Monaghan, M. & Sreenivasan, S. 2018. Pathophysiology of the metabolic syndrome. *Clinics in Dermatology*, 36(1): 14-20.

Million, M., Maraninchi, M., Henry, M., Armougom, F., Richet, H., Carrieri, P., Valero, R., Raccah, D., Vialettes, B. & Raoult, D. 2012. Obesity-associated gut microbiota is enriched in Lactobacillus reuteri and depleted in Bifidobacterium animalis and Methanobrevibacter smithii. *International Journal of Obesity*, 36(6): 817-825.

Mobini, R., Tremaroli, V., Ståhlman, M., Karlsson, F., Levin, M., Ljungberg, M., Sohlin, M., Bertéus Forslund, H., Perkins, R., Bäckhed, F. & Jansson, P. 2017. Metabolic effects ofLactobacillus reuteriDSM 17938 in people with type 2 diabetes: A randomized controlled trial. *Diabetes, Obesity and Metabolism*, 19(4): 579-589.

Morgan, X. & Huttenhower, C. 2012. Chapter 12: Human Microbiome Analysis. *PLoS Computational Biology*, 8(12): e1002808.

Morton, J. 1983. Rooibos tea, aspalathus linearis, a caffeine less, low-tannin beverage. *Economic Botany*, 37(2): 164-173.

Mu, Q., Tavella, V. & Luo, X. 2018. Role of Lactobacillus reuteri in Human Health and Diseases. *Frontiers in Microbiology*, 9.

Muller, C. 2017. Effects of Rooibos on microbiota dysbiosis: implications for diet-induced metabolic dysfunction. In *11th World Congress on Endocrinology and Metabolic Disorders*. (https://www.omicsonline.org/conference-proceedings/2161-1017-C2-025-002.pdf 15 February 2019.)

Muller, C., Joubert, E., de Beer, D., Sanderson, M., Malherbe, C., Fey, S. & Louw, J. 2012. Acute assessment of an aspalathin-enriched green rooibos (Aspalathus linearis) extract with hypoglycemic potential. *Phytomedicine*, 20(1): 32-39.

Muller, C., Malherbe, C., Chellan, N., Yagasaki, K., Miura, Y. & Joubert, E. 2017. Potential of rooibos, its major C-glucosyl flavonoids, and Z-2-(β-D-glucopyranosyloxy)-3-phenylpropenoic acid in prevention of metabolic syndrome. *Critical Reviews in Food Science and Nutrition*, 58(2): 227-246.

Munukka, E., Wiklund, P., Pekkala, S., Völgyi, E., Xu, L., Cheng, S., Lyytikäinen, A., Marjomäki, V., Alen, M., Vaahtovuo, J., Keinänen-Kiukaanniemi, S. & Cheng, S. 2012. Women With and Without Metabolic Disorder Differ in Their Gut Microbiota Composition. *Obesity*, 20(5): 1082-1087.

Murphy, E., Velazquez, K. & Herbert, K. 2015. Influence of high-fat diet on gut microbiota. *Current Opinion in Clinical Nutrition and Metabolic Care*, 18(5): 515-520.

Nakamura, K.T.N., Jin, J-S., Ma, C-M., Komatsu, K., Iwill behima, M., and Hattori, M. 2011. The C-glucosyl bond of puerarin will be cleaved hydrolytically by a human intestinal bacterium strain PUE to yield its aglycone daidzein and an intact glucose. *Chemical and Pharmaceutical Bulletin* 59: 23-27.

Natividad, J., Lamas, B., Pham, H., Michel, M., Rainteau, D., Bridonneau, C., da Costa, G., van Hylckama Vlieg, J., Sovran, B., Chamignon, C., Planchais, J., Richard, M., Langella, P., Veiga, P. & Sokol, H. 2018. Bilophila wadsworthia aggravates high fat diet induced metabolic dysfunctions in mice. *Nature Communications*, 9(1).

Newberry, S. 2012. Probiotics for the Prevention and Treatment of Antibiotic-Associated Diarrhea. *JAMA*, 307(18): 1959.

Neyrinck, A., Possemiers, S., Verstraete, W., De Backer, F., Cani, P. & Delzenne, N. 2012. Dietary modulation of clostridial cluster XIVa gut bacteria (Roseburia spp.) by chitin–glucan fiber improves host metabolic alterations induced by high-fat diet in mice. *The Journal of Nutritional Biochemistry*, 23(1): 51-59.

O'Keefe, S., Li, J., Lahti, L., Ou, J., Carbonero, F., Mohammed, K., Posma, J., Kinross, J., Wahl, E., Ruder, E., Vipperla, K., Naidoo, V., Mtshali, L., Tims, S., Puylaert, P., DeLany, J., Krasinskas, A., Benefiel, A., Kaseb, H., Newton, K., Nicholson, J., de Vos, W., Gaskins, H. & Zoetendal, E. 2015. Fat, fibre and cancer risk in African Americans and rural Africans. *Nature Communications*, 6(1).

Oikonomou, E. & Antoniades, C. 2018. The role of adipose tissue in cardiovascular health and disease. *Nature Reviews Cardiology*, 16(2): 83-99.

Orlando, P., Chellan, N., Louw, J., Tiano, L., Cirilli, I., Dludla, P., Joubert, E. & Muller, C. 2019. Aspalathin-Rich Green Rooibos Extract Lowers LDL-Cholesterol and Oxidative Status in High-Fat Diet-Induced Diabetic Vervet Monkeys. *Molecules*, 24(9): 1713.

Osawa, R. 2006. Lactobacillus apodemi sp. nov., a tannase-producing species isolated from wild mouse faeces. *INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY*, 56(7): 1693-1696.

Ou, J., Carbonero, F., Zoetendal, E., DeLany, J., Wang, M., Newton, K., Gaskins, H. & O'Keefe, S. 2013. Diet, microbiota, and microbial metabolites in colon cancer risk in rural Africans and African Americans. *The American Journal of Clinical Nutrition*, 98(1): 111-120.

Pan, F., Zhang, L., Li, M., Hu, Y., Zeng, B., Yuan, H., Zhao, L. & Zhang, C. 2018. Predominant gut Lactobacillus murinus strain mediates anti-inflammaging effects in calorie-restricted mice. *Microbiome*, 6(1).

Parkar, S., Stevenson, D. & Skinner, M. 2008. Erratum to 'The potential influence of fruit polyphenols on colonic microflora and human gut health' [Int. J. of Food Microbiol. 124 (2008) 295–298]. *International Journal of Food Microbiology*, 128(2): 416.

Parkar, S., Trower, T. & Stevenson, D. 2013. Faecal microbial metabolism of polyphenols and its effects on human gut microbiota. *Anaerobe*, 23: 12-19.

Parks, B., Nam, E., Org, E., Kostem, E., Norheim, F., Hui, S., Pan, C., Civelek, M., Rau, C., Bennett, B., Mehrabian, M., Ursell, L., He, A., Castellani, L., Zinker, B., Kirby, M., Drake, T., Drevon, C., Knight, R., Gargalovic, P., Kirchgessner, T., Eskin, E. & Lusis, A. 2013. Genetic Control of Obesity and Gut Microbiota Composition in Response to High-Fat, High-Sucrose Diet in Mice. *Cell Metabolism*, 17(1): 141-152.

Parrott, T. 2019. Overview of Nonhuman Primates - Exotic and Laboratory Animals - Veterinary Manual. *Veterinary Manual*. https://www.msdvetmanual.com/exotic-and-laboratory-animals/nonhuman-primates/overview-of-nonhuman-primates 16 May 2019.

Pennisi, E. 2010. Semiconductors Inspire New Sequencing Technologies. *Science*, 327(5970): 1190-1190.

Phillips, J. & Everling, S. 2014. Event-related potentials associated with performance monitoring in non-human primates. *NeuroImage*, 97: 308-320.

Plaza-Díaz, J., Ruiz-Ojeda, F., Gil-Campos, M. & Gil, A. 2018. Immune-Mediated Mechanisms of Action of Probiotics and Synbiotics in Treating Pediatric Intestinal Diseases. *Nutrients*, 10(1): 42.

Plaza-Diaz, J., Ruiz-Ojeda, F., Gil-Campos, M. & Gil, A. 2019. Mechanisms of Action of Probiotics. *Advances in Nutrition*, 10(suppl_1): S49-S66.

Popa, D., Dragoi, C., Arsene, A., Dumitrescu, I., Nicolae, A. & Burcea-Dragomiroiu, G. 2019. The Relationship Between Phenolic Compounds from Diet and Microbiota.

Possemiers, S., Bolca, S., Verstraete, W., and Heyerick, A. 2011. The intestinal microbiome: a separate organ inside the body with the metabolic potential to influence the bioactivity of botanicals. *Fitoterapia*82: 53-66.

Precup, G. & Vodnar, D. 2019. Gut Prevotella as a possible biomarker of diet and its eubiotic versus dysbiotic roles: a comprehensive literature review. *British Journal of Nutrition*, 122(2): 131-140.

Puoane, T., Steyn, K., Bradshaw, D., Laubscher, R., Fourie, J., Lambert, V. & Mbananga, N. 2002. Obesity in South Africa: The South African Demographic and Health Survey. *Obesity Research*, 10(10): 1038-1048.

Ramulondi, E. 2014. Vervet monkey - SANBI. *SANBI*. https://www.sanbi.org/animal-of-the-week/vervet-monkey/ 15 May 2019.

Ranjan, R., Rani, A., Metwally, A., McGee, H. & Perkins, D. 2016. Analysis of the microbiome: Advantages of whole genome shotgun versus 16S amplicon sequencing. *Biochemical and Biophysical Research Communications*, 469(4): 967-977.

Rather, I., Bajpai, V., Kumar, S., Lim, J., Paek, W. & Park, Y. 2016. Probiotics and Atopic Dermatitis: An Overview. *Frontiers in Microbiology*, 7.

Rein, M., Renouf, M., Cruz-Hernandez, C., Actis-Goretta, L., Thakkar, S. & da Silva Pinto, M. 2013. Bioavailability of bioactive food compounds: a challenging journey to bioefficacy. *British Journal of Clinical Pharmacology*, 75(3): 588-602.

Rinninella, E., Raoul, P., Cintoni, M., Franceschi, F., Miggiano, G., Gasbarrini, A. & Mele, M. 2019. What is the Healthy Gut Microbiota Composition? A Changing Ecosystem across Age, Environment, Diet, and Diseases. *Microorganisms*, 7(1): 14.

Rudel, L.L., Reynolds, J.A., Bullock, B.C., 1981. Nutritional effects on blood lipid and HDL cholesterol concentrations in two subspecies of African green monkeys (*Cercopithecus aethiops*). *J. Lipid Res*. 22, 278–286.

Rusk, N. 2010. Torrents of sequence. *Nature Methods*, 8(1): 44-44.

Russell, W., Gratz, S., Duncan, S., Holtrop, G., Ince, J., Scobbie, L., Duncan, G., Johnstone, A., Lobley, G., Wallace, R., Duthie, G. & Flint, H. 2011. High-protein, reduced-carbohydrate weight-loss diets promote metabolite profiles likely to be detrimental to colonic health. *The American Journal of Clinical Nutrition*, 93(5): 1062-1072.

Sanderson, M., Mazibuko, S., Joubert, E., de Beer, D., Johnson, R., Pheiffer, C., Louw, J. & Muller, C. 2014. Effects of fermented rooibos (Aspalathuslinearis) on adipocyte differentiation. *Phytomedicine*, 21(2): 109-117.

Sandven, P., høiby, E. & Vorland, L. 2009. Evaluation of two vacuum bottle blood culture media - supplemented peptone broth and brain heart infusion broth. *Acta Pathologica Microbiologica Scandinavica Series B: Microbiology*, 95B (1-6): 257-259.

Schaedler, R. 1965. The development of the bacterial flora in the gastrointestinal tract of micE. *Journal of Experimental Medicine*, 122(1): 59-66.

Schmid, R., van Wyk, B., van Oudtshoorn, B. & Gericke, N. 1998. Medicinal Plants of South Africa. *Taxon*, 47(3): 787.

Schnorr, S., Candela, M., Rampelli, S., Centanni, M., Consolandi, C., Basaglia, G., Turroni, S., Biagi, E., Peano, C., Severgnini, M., Fiori, J., Gotti, R., De Bellis, G., Luiselli, D., Brigidi, P., Mabulla, A., Marlowe, F., Henry, A. & Crittenden, A. 2014. Gut microbiome of the Hadza hunter-gatherers. *Nature Communications*, 5(1).

Seck, E., Senghor, B., Merhej, V., Bachar, D., Cadoret, F., Robert, C., Azhar, E., Yasir, M., Bibi, F., Jiman-Fatani, A., Konate, D., Musso, D., Doumbo, O., Sokhna, C., Levasseur, A., Lagier, J., Khelaifia, S., Million, M. and Raoult, D. (2018). Salt in stools is associated with obesity, gut halophilic microbiota and Akkermansia muciniphila depletion in humans. *International Journal of Obesity*, 43(4), pp.862-871

Sezik, E., Aslan, M., Yesilada, E. & Ito, S. (2005). Hypoglycaemic activity of *Gentiana olivieri* and isolation of the active constituent through bioassay-directed fractionation techniques. *Life Sciences*, 76, 1223–1238.

Shannon, C.E., 1948. A mathematical theory of communication. Bell Syst. Tech. J. 27(3), 379-423, 623-656.

Sharafedtinov, K., Plotnikova, O., Alexeeva, R., Sentsova, T., Songisepp, E., Stsepetova, J., Smidt, I. & Mikelsaar, M. 2013. A hypocaloric diet supplemented with probiotic cheese improves body mass index and blood pressure indices of obese hypertensive patients - a randomized double-blind placebo-controlled pilot study. *Nutrition Journal*, 12(1).

Sircana, A., Framarin, L., Leone, N., Berrutti, M., Castellino, F., Parente, R., De Michieli, F., Paschetta, E. & Musso, G. 2018. Altered Gut Microbiota in Type 2 Diabetes: Just a Coincidence? *Current Diabetes Reports*, 18(10).

Smithsonian institution archives. 2019. Smithsonian Institution Archives. *Siarchives.si.edu*. http://siarchives.si.edu/research/videohistory_catalog9577.html 12 June 2019.

Smuts, C.M., Kruger, M., van Jaarsveld, P.J., Fincham, J.E., Schall, R., van der Merwe, K.J., Benadé, A.J., 1992. The influence of fish oil supplementation on plasma lipoproteins and arterial lipids in Vervet monkeys with established atherosclerosis. Prostaglandins Leukot. *Essent. Fatty Acids* 47, 129–138.

Snijman, P., Joubert, E., Ferreira, D., Li, X., Ding, Y., Green, I. & Gelderblom, W. 2009. Antioxidant Activity of the Dihydrochalcones Aspalathin and Nothofagin and Their Corresponding Flavones in Relation to Other Rooibos (Aspalathus linearis) Flavonoids, Epigallocatechin Gallate, and Trolox. *Journal of Agricultural and Food Chemistry*, 57(15): 6678-6684.

Snijman, P.W., Swanevelder, S., Joubert, E., Green, I.R. & Gelderblom, W.C.A. 2007. The antimutagenic activity of the major flavonoids of rooibos (*Aspalathus linearis*): Some dose-response effects on mutagen activation-flavonoid interactions. *Mutation Research,* **631**, 111–123.

Sokol, H., Lay, C., Seksik, P. & Tannock, G. 2008. Analysis of bacterial bowel communities of IBD patients: What has it revealed? *Inflammatory Bowel Diseases*, 14(6): 858-867.

Sonnenburg, E., Smits, S., Tikhonov, M., Higginbottom, S., Wingreen, N. & Sonnenburg, J. 2016. Diet-induced extinctions in the gut microbiota compound over generations. *Nature*, 529(7585): 212-215.

Speedy Breedy. 2018. How Speedy Breedy Works – Speedy Breedy. *Speedybreedy.com*. http://speedybreedy.com/about-speedy-breedy-blank/how-speedy-breedy-works/ 16 May 2019.

St Clair, R.W., Wood, L.L., Clarkson, T.B., 1981. Effect of sucrose polyester on plasma lipids with cholesterol absorption in African green monkeys with a variable hypercholesterolemic response to dietary cholesterol. *Metabolism*. 30, 176–183.

Stalmach, A., Mullen, W., Pecorari, M., Serafini, M. & Crozier, A. 2009. Bioavailability of *C*-Linked Dihydrochalcone and Flavanone Glucosides in Humans Following Ingestion of Unfermented and Fermented Rooibos Teas. *Journal of Agricultural and Food Chemistry*, 57(15): 7104-7111.

Suckling, K.E., Jackson, B., 1993. Animal models of human lipid metabolism. *Prog. Lipid Res*. 32, 1–24.

Sweeney, T. & Morton, J. 2013. The Human Gut Microbiome. *JAMA Surgery*, 148(6): 563.

Tamanai-Shacoori, Z., Smida, I., Bousarghin, L., Loreal, O., Meuric, V., Fong, S., Bonnaure-Mallet, M. & Jolivet-Gougeon, A. 2017. Roseburiaspp.: a marker of health?. *Future Microbiology*, 12(2): 157-170.

Tap, J., Mondot, S., Levenez, F., Pelletier, E., Caron, C., Furet, J., Ugarte, E., Muñoz-Tamayo, R., Paslier, D., Nalin, R., Dore, J. & Leclerc, M. 2009. Towards the human intestinal microbiota phylogenetic core. *Environmental Microbiology*, 11(10): 2574-2584.

Thursby, E. & Juge, N. 2017. Introduction to the human gut microbiota. *Biochemical Journal*, 474(11): 1823-1836.

Tuomi, T. 2005. Type 1 and Type 2 Diabetes: What Do They Have in Common?. *Diabetes*, 54(Supplement 2): S40-S45.

Turnbaugh, P., Hamady, M., Yatsunenko, T., Cantarel, B., Duncan, A., Ley, R., Sogin, M., Jones, W., Roe, B., Affourtit, J., Egholm, M., Henrissat, B., Heath, A., Knight, R. & Gordon, J. 2008. A core gut microbiome in obese and lean twins. *Nature*, 457(7228): 480-484.

Tzounis, X., Vulevic, J., Kuhnle, G., George, T., Leonczak, J., Gibson, G., Kwik-Uribe, C. & Spencer, J. 2007. Flavanol monomer-induced changes to the human faecal microflora. British Journal of Nutrition, 99(4): 782-792.

Urbańska, M., Gieruszczak-Białek, D. & Szajewska, H. 2016. Systematic review with meta-analysis: Lactobacillus reuteriDSM 17938 for diarrhoeal diseases in children. *Alimentary Pharmacology & Therapeutics*, 43(10): 1025-1034.

Vartoukian, S., Adamowska, A., Lawlor, M., Moazzez, R., Dewhirst, F. & Wade, W. 2016. In Vitro Cultivation of 'Unculturable' Oral Bacteria, Facilitated by Community Culture and Media Supplementation with Siderophores. *PLOS ONE*, 11(1): e0146926.

Wallace, J.M., Schwarz, M., Coward, P., Houze, J., Sawyer, J.K., Kelley, K.L., Chai, A., Rudel, L.L., 2005. Effects of peroxisome proliferator-activated receptor alpha/delta agonists on HDL-cholesterol in Vervet monkeys. *J. Lipid Res*. 46, 1009–1016. doi:10.1194/jlr.M500002-JLR200

WHO. 2018. Obesity and overweight. *Who.int*. https://www.who.int/en/news-room/fact-sheets/detail/obesity-and-overweight 26 April 2019.

Willing, B. & Jansson, J. 2010. *The Gut Microbiota*. Berkeley, Calif.: Lawrence Berkeley National Laboratory.

Wu, X., Ma, C., Han, L., Nawaz, M., Gao, F., Zhang, X., Yu, P., Zhao, C., Li, L., Zhou, A., Wang, J., Moore, J., Cherie Millar, B. & Xu, J. 2010. Molecular Characterization of the Faecal Microbiota in Patients with Type II Diabetes. *Current Microbiology*, 61(1): 69-78.

www.IDF.org, 2018. International Diabetes Federation - Facts & figures. *Idf.org*. https://www.idf.org/aboutdiabetes/what-is-diabetes/facts-figures.html 8 August 2018.

Yatsunenko, T., Rey, F., Manary, M., Trehan, I., Dominguez-Bello, M., Contreras, M., Magris, M., Hidalgo, G., Baldassano, R., Anokhin, A., Heath, A., Warner, B., Reeder, J., Kuczynski, J., Caporaso, J., Lozupone, C., Lauber, C., Clemente, J., Knights, D., Knight, R. & Gordon, J. 2012. Human gut microbiome viewed across age and geography. *Nature*, 486(7402): 222-227.

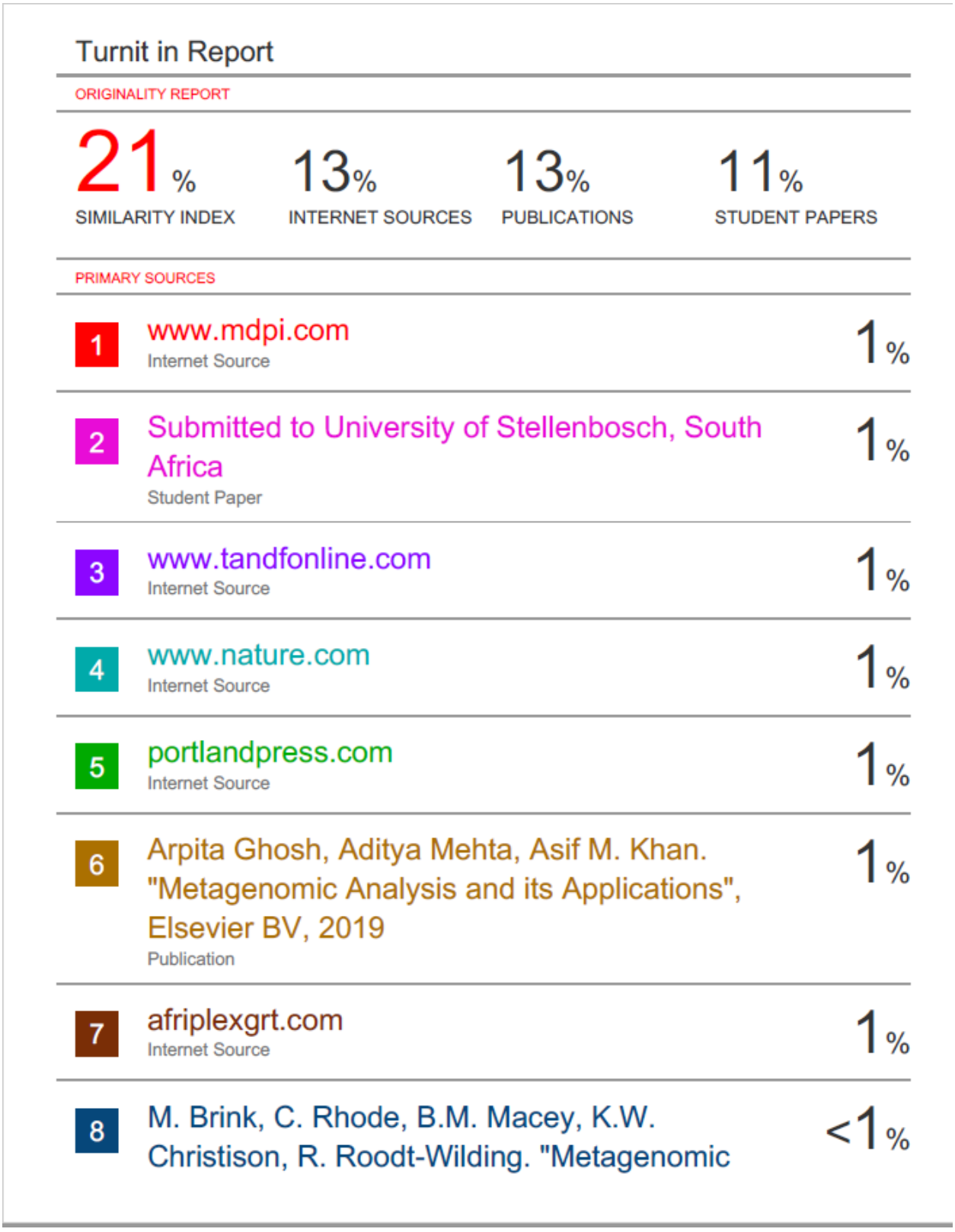
Zhang, C., Zhang, M., Pang, X., Zhao, Y., Wang, L. & Zhao, L. 2012. Structural resilience of the gut microbiota in adult mice under high-fat dietary perturbations. *The ISME Journal*, 6(10): 1848-1857.

Zhang, Y., Xiaowei, T., Bili, B., Xiaoqin, W., & Ying, Z. 2007. Metabolism of flavone C-glucosides and p-coumaric acid from antioxidant of bamboo leaves (AOB) in rats. British J. of Nutrition 97: 484-49.

Zucolotto, S.M., Goulart, S., Montanher, A.B., Reginatto, F.H., Schenkel, E.P. & Fröde, T.S. (2009). Bioassay-guided isolation of anti-inflammatory *C*-glucosylflavones from *Passiflora edulis*. *Planta Medica*, **75**, 1221–1226.

APPENDICES

Appendix A: Similarity reports and ethics approval



Turnit in Report

ORIGINALITY REPORT

21%	13%	13%	11%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	www.mdpi.com Internet Source	1%
2	Submitted to University of Stellenbosch, South Africa Student Paper	1%
3	www.tandfonline.com Internet Source	1%
4	www.nature.com Internet Source	1%
5	portlandpress.com Internet Source	1%
6	Arpita Ghosh, Aditya Mehta, Asif M. Khan. "Metagenomic Analysis and its Applications", Elsevier BV, 2019 Publication	1%
7	afriplexgrt.com Internet Source	1%
8	M. Brink, C. Rhode, B.M. Macey, K.W. Christison, R. Roodt-Wilding. "Metagenomic	<1%

ETHICS COMMITTEE FOR RESEARCH ON ANIMALS (ECRA)

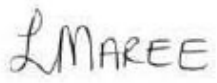
Animal Ethics Approval Certificate

APPROVAL PERIOD: October 2018 – July 2019

Decision of the Animal Ethics Committee for the use of living vertebrates for research, diagnostic procedures and product development

PROJECT NUMBER:	08/18			
PROJECT TITLE:	**The in-vitro faecal evaluation of prebiotic effects of Rooibos Phenolic Compounds in the microbiota of the Vervet monkey**			
PROJECT LEADER:	Ms Noluxabiso Mangwana			
DIVISION:	BRIP, SAMRC, Cape Town			
CATEGORY:	Biomedical technology (training)			
SPECIES OF ANIMAL:	Vervet monkeys (*Chlorocebus aethiops*)			
NUMBER OF ANIMALS:	10	Male and female	3-6kg	>20 years
NOT APPROVED:	n/a			
APPROVED:	4 October 2018			

PLEASE NOTE: Should the number or species of animal(s) required, or the experimental procedure(s) change, please submit a written request to the ECRA for approval before commencing with the experiment.



Dr L Maree **DATE** **4 October 2018**

CHAIRPERSON : ETHICS COMMITTEE FOR RESEARCH ON ANIMALS

Cape Peninsula
University of Technology

CAPE PENINSULA UNIVERSITY OF TECHNOLOGY ANIMAL ETHICS COMMITTEE (CPUT-AEC)
Registration Number NHREC: To be confirmed

P.O. Box 1906 • Bellville 7535 South Africa
Symphony Road Bellville 7535
Tel: +27 21 959 6917
Email: besterd@cput.ac.za

DATE 05 February 2020
REC Approval Reference No:
CPUT/AEC 2019/05

Dear Dr Mundembe

Re: APPLICATION TO THE CPUT-AEC FOR ETHICS CLEARANCE

Approval was granted by the Cape Peninsula University of Technology Animal Ethics Committee to Noluxabiso Mangwana (under your supervision) for ethical clearance on 16 November 2019. This approval is for research activities related to research in the Faculty of Applied Sciences at the Cape Peninsula University of Technology.

TITLE: **The *in vitro* faecal evaluation of prebiotic effects of rooibos phenolic compounds on the gut microbiota of vervet monkeys (*Chlorocebus pygerythrus*)**

Comment: The issuing of this permission letter is conditional to the obtaining of permission to use samples and ethics clearance from the relevant stakeholders at the South African Medical Research Council.

Approval will not extend beyond 06 February 2021. An extension should be applied for 6 weeks before this expiry date should data collection and use/analysis of data, information and/or samples for this study continue beyond this date.

The investigator(s) should understand the ethical conditions under which they are authorized to carry out this study and they should be compliant to these conditions. It is required that the investigator(s) complete an **annual progress report** that should be submitted to the CPUT-AEC in December of that particular year, for the CPUT-AEC to be kept informed of the progress and of any problems you may have encountered.

Kind Regards

Dr *Dirk Bester*
Chairperson – Animal Ethics Committee
Cape Peninsula University of Technology

Appendix B: Output

SYMPOSIA

Oral Presentation

N Mangwana C.J.F. Muller, R. Mundembe and N Thovhogi. The effects of rooibos phenolic compounds on the gut microbiota of vervet monkeys. South African Medical Research Council, Conference Centre. 9th annual Biomedical Research and Innovation Platform Symposium. 21October 2019.

Poster presentations

N Mangwana C.J.F. Muller, R. Mundembe and N Thovhogi. The effects of rooibos phenolic compounds on the gut microbiota of Vervet Monkeys. Stellenbosch University African Microbiome Institute Workshop and Sympossium, Stellenbosch Institute for Advanced Study (STIAS) Conference Centre. 28-30 November 2019.

CONFERENCES

Poster presentation

N Mangwana C.J.F. Muller, R. Mundembe and N Thovhogi. The effects of rooibos phenolic compounds on the gut microbiota of vervet monkeys. South African Medical Research Council, Conference Centre,13th Annual Early Career Scientist Convention: Western Cape, South Africa, 9-11October 2018.

Workshop and Convention

N Mangwana, AfricaBio Convention 2019, Durban ICC, Durban South Africa 26 – 28 August 2019.

Appendix C: Additional tables

Table S1: DNA concentrations and ratio.

Sample no	Sample name	Sample ID (Normal diet (ND)/ Western diet (WD)	Conc. ng/µL	A260/A280	A260/A230
SB-1	238 Uncultured	ND	190.1	1.85	2.14
SB-2	238 Control	ND	250.3	1.85	2.35
SB-3	238 Aspalathin 1mM	ND	118.4	1.84	1.69
SB-4	238 GRT	ND	13.5	2.13	1.56
SB-5	249 Uncultured	ND	170.5	1.84	2.07
SB-6	249 Control	ND	190.1	1.84	1.87
SB-7	249 Aspalathin 1mM	ND	166.8	1.85	2.39
SB-8	249 GRT	ND	133.8	1.85	2.71
SB-9	268 Uncultured	ND	193.6	1.83	2.10
SB-10	268 Control	ND	147.4	1.85	2.15
SB-11	268 Aspalathin 100uM	ND	214.6	1.85	2.25
SB-12	268 Aspalathin 1mM	ND	164.1	1.85	2.20
SB-13	268 GRT	ND	123.9	1.85	2.41
SB-14	281 Uncultured	WD	131.8	1.85	2.20
SB-15	281 Control	WD	158.5	1.84	2.18
SB-16	281 Aspalathin 1mM	WD	149.1	1.83	1.69
SB-17	281 GRT	WD	116.2	1.83	2.67
SB-18	343 Uncultured	WD	137.5	1.77	1.42
SB-19	343 Control	WD	228.1	1.83	2.10
SB-20	343 Aspalathin 100uM	WD	177.3	1.82	1.83
SB-21	343 Aspalathin 1mM	WD	250.4	1.87	2.13
SB-22	343 GRT	WD	198.4	1.85	2.71
SB-23	403 Uncultured	WD	193.6	1.86	2.60
SB-24	403 Control	WD	196.5	1.85	2.13
SB-25	403 Aspalathin 1mM	WD	268.1	1.85	1.84
SB-26	403 GRT	WD	155.5	1.87	2.47
AC-27	268 Uncultured	ND	195.2	1.82	1.69
AC-28	268 Control	ND	146.4	1.82	1.88
AC-29	268 Re-cultured	ND	123.5	1.80	1.46

AC-30	343 Uncultured	WD	216.5	1.83	1.94
AC-31	343 Control	WD	257.1	1.83	2.11
AC-32	343 Re-cultured	WD	228.6	1.82	2.02