MONASH University

Interactions between *Haemonchus contortus* and the gut microbiota of sheep

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(MS in Parasitology)

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Abstract

Improved animal production is required to meet increasing global demands. One of the greatest impediments to small ruminant production is infection with the gastrointestinal parasite, *Haemonchus contortus*. In recent years there has been considerable interest in the gut microbiome and its impact on health, particularly human health. However, relatively little is known about interactions between the gut microbiota and gastrointestinal tract pathogens in sheep. Thus, this study was undertaken to investigate the link between the faecal microbiota of sheep, as a sample representing the gastrointestinal microbiota, and infection with *H. contortus*.

All 28 Merino wethers used in this study were housed undercover at the Monash University animal facility under the same conditions, were all the same age, and all provided (*ad libitum*) the same diet. Microbial analyses were conducted using automated ribosomal intergenic spacer analysis (ARISA) and 16S rRNA sequencings. First, ARISA was optimized and evaluated for use with sheep faeces. This preliminary study demonstrated that ARISA had sufficient discriminatory power to differentiate faecal microbial profiles of different sheep, and also showed excellent reproducibility in samples collected from the same three sheep on the same day, and the same sheep daily over one week. Based on this preliminary results, samples from 28 sheep collected over a 4 week period were analysed by using ARISA. The results demonstrated that the gut microbiome was stable over this period. The 16S rRNA analysis was conducted over a 2 week sampling period. Members of the phylum Firmicutes and Bacteriodetes were the predominant bacteria detected and constituted ~80% of the total population. Abundant taxa included genera belonging to the families Ruminococcaceae, Bacteriodales and Clostridiales.

Each of the 28 sheep was experimentally inoculated with 14,000 *H. contortus* infective larvae. Faecal samples were collected for 4 weeks prior and 4 weeks after infection. The difference in pre-infection faecal microbiota in sheep that went on to develop a high burden of *H. contortus* infection (n=5) to sheep that developed a low burden of infection (n=5) was investigated. This analysis revealed significant differences in the faecal microbiota between the two aforementioned groups. Differences were observed at the community level by both ARISA and 16S rRNA sequencing; and also at the taxa level, where key differences observed included statistically significant differences in relative abundance of Bacteriodetes (higher in high-burden sheep) and Firmicutes (lower in high-burden sheep).

A comparison of pre-infection microbiota to post-infection microbiota was also conducted. This analysis revealed that sheep with a high parasite burden underwent a greater change in community composition that sheep with a low parasite burden. Significant differences were observed in the relative abundances of Firmicutes and Bacteriodetes in high-burden sheep following infection, whereas in low-burden sheep the relative abundances of the most abundant phyla remain stable. Interestingly, in high burden sheep there appeared to be a shift after infection towards a microbiota with similarities to the low-burden microbiota.

This study reveals a correlation between faecal microbiota and susceptibility/resistance to *H. contortus* infection in sheep. These findings warrant further investigation to determine whether there is a causal link between gut microbial composition and resistance to infection of gastrointestinal parasites in sheep; and if so, how this can be best exploited for gains in animal health and production.

Commonly Used Abbreviations

Acronym	Definition
AGRF	Australian Genome Research Facility
ANOSIM	Analysis of similarity
ANOVA	Analysis of variance
ARISA	Automated ribosomal intergenic spacer analysis
cFEC	Cumulative faecal egg count
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
EBV	Estimated breeding value
EPG	Egg per gram of faeces
FEC	Faecal egg count
IBD	Inflammatory bowel disease
ITS	Internal transcribed spacer
LEfSe	Linear discriminant analysis effect size
nMDS	Non-metric multidimensional scaling
OTU	Operational taxonomic unit
PCoA	Principal coordinate analysis
PERMANOVA	Permutational multivariate analysis of variance
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SCFAs	Short chain fatty acids
SD	Standard deviation
T-RFLP	Terminal restriction fragment length polymorphism
WS	Window size

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Signature: 

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Date: 05.08.2017

Communications

2017

- Md. Abdullah Al Mamun, David Piedrafita, Phil Rayment, Mark Sandeman, Andrew R. Greenhill 2017. The gut microbiota of sheep and infection with the gastrointestinal parasite *Haemonchus contortus*. Australian Society for Microbiology Meeting, Hobart, Tasmania. **10 minutes oral talk**.
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Dedication

I was born and grew up in a village in rural northern Bangladesh. There was no road connection and no electricity. The village was on the bank of the one of the longest rivers in the world, Brahmaputra. My mother just finished year seven in the school when she got married with my father. My parents struggled in their life with six kids but never gave up. It was not easy to foresee coming to Australia and completing a higher academic qualification like a PhD from that childhood situation. It was mostly my mother, and accompanied by my father, who constantly inspired me and made me believe that nothing is impossible. I know it is them who will be the happiest people in the world once they hear of my PhD graduation.

With utmost respect, I dedicate this thesis to

My parents

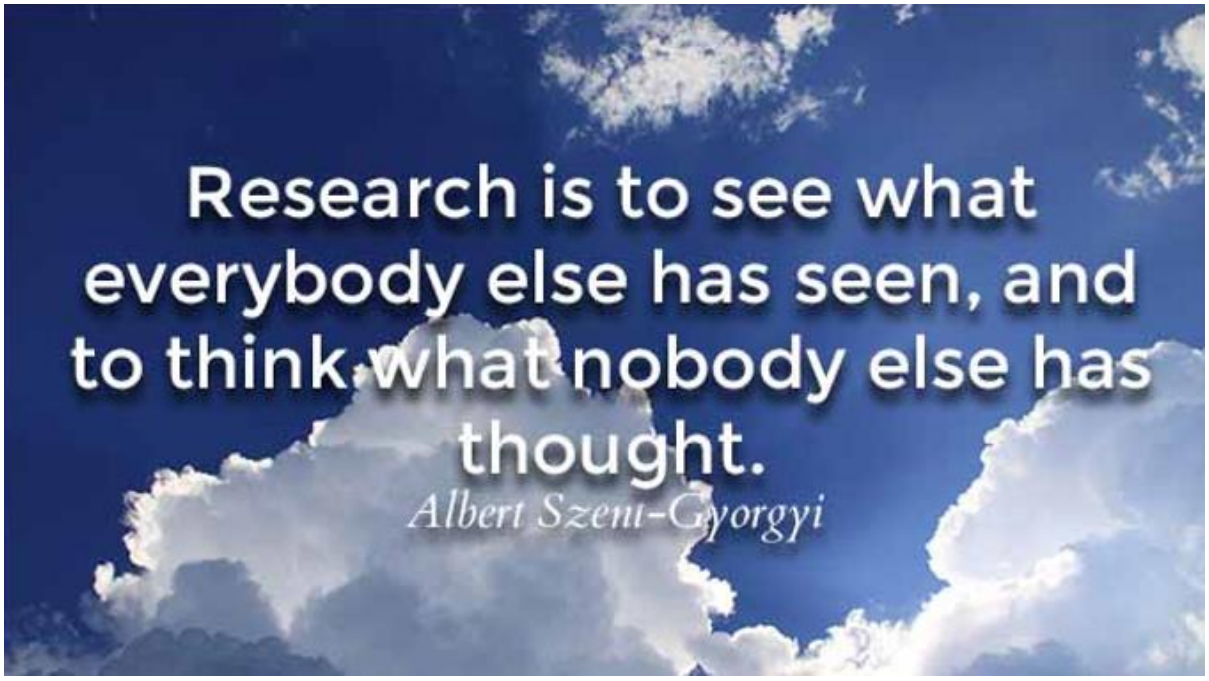

Research is to see what everybody else has seen, and to think what nobody else has thought.
Albert Szent-Gyorgyi

Table of Contents

CHAPTER 1 INTRODUCTION AND REVIEW OF THE LITERATURE 1

1.1 GENERAL INTRODUCTION 2

1.1.1 Global Food Security 2

1.1.2 Increasing Demand for Meat and Animal Products 3

1.1.3 Major Impediments to Agricultural Productivity 4

1.2 *HAEMONCHUS CONTORTUS* 5

1.2.1 The Parasite, H. contortus 5

1.2.2 Life Cycle of H. contortus 6

1.2.3 Lost Productivity and Economic Importance of H. contortus 7

1.2.4 Optimising Production Traits of Small Ruminants 8

1.3 GUT MICROBIOTA 11

1.3.1 An Overview of Gut Microbiota and its Purported Importance 11

1.3.2 Ecological Principles in the Composition of the Gut Microbiota 12

1.3.3 Composition of the Gut Microbiota in Animals 13

1.3.4 Factors Affecting the Stability of Gut Microbiota Composition 16

1.3.5 Microbiota in Nutrition and Metabolism 17

1.4 METHODS FOR THE INVESTIGATION OF GUT MICROBIAL COMMUNITIES 19

1.4.1 Detection of Microbes in Complex Samples 19

1.4.2 Community Fingerprinting 21

1.4.3 ARISA 22

1.4.4 Next-Generation Sequencing 24

1.5 ROLE OF GUT MICROBIOTA IN IMMUNITY AND RESISTANCE TO INFECTION 26

1.5.1 Gut Microbial Composition and Infectious Diseases 26

1.5.2 Studies in Ruminants 30

1.5.2.1 Microbial communities of the digestive tract of ruminants 30

1.5.2.2 Parasite infection and gut microbiota 30

1.5.2.3 Ruminant immunity and parasite infection 31

1.6 PROJECT AIMS 32

CHAPTER 2 OPTIMISATION OF ARISA FOR THE ANALYSIS OF MICROBIAL COMPOSITION WITHIN SHEEP FAECES 35

2.1 INTRODUCTION 36

2.2 MATERIALS AND METHODS 39

2.2.1 Animal Handling and Sampling 39

2.2.2 Primer Selection 40

2.2.3 Master Mix Selection 41

2.2.4 Genomic DNA Extraction, Quantification and Homogenization 42

2.2.5 PCR 42

2.2.6 Fragment Separation and ARISA Analysis 43

2.2.7 Statistical Analysis 44

2.3 RESULTS 46

2.3.1 PCR Profiles of Different Primer Sets 46

2.3.2 ARISA is Reproducible and Sensitive 47

2.3.3 Selection of the Best Combination of Master Mix, Primers and DNA Extraction 49

2.3.4 Preliminary ARISA Trial to Determine Reproducibility and Differential Capacity 51

2.4 DISCUSSION 54

CHAPTER 3 THE COMPOSITION AND STABILITY OF THE FAECAL MICROBIOTA OF SHEEP 59

3.1 INTRODUCTION 60

3.2 METHODS 62

3.2.1 Animal Handling and Sampling *62*

3.2.2 DNA Extraction, Quantification and Homogenization *63*

3.2.3 ARISA *63*

3.2.4 16S rRNA Sequencing *64*

3.2.5 Bioinformatics Methods *64*

3.2.6 Ecological and Statistical Analyses *65*

3.3 RESULTS 67

3.3.1 Community Structure and Composition of the Sheep Gut Microbiota *67*

3.3.1.1 Community structure determined by ARISA 67

3.3.1.2 Community composition determined by 16S rRNA gene sequencing 69

3.3.2 Similarity and Stability of Faecal Microbiota Over Time *71*

3.3.2.1 Community structure over 4 week period determined by ARISA 71

3.3.2.2 Community composition over 2 week period determined by 16S rRNA sequencing 74

3.3.2.3 Community composition over 6 month period determined by 16S rRNA sequencing 81

3.3.3 Comparison of Faecal Microbiota of Individual Sheep *88*

3.3.3.1 ARISA demonstrates that the sheep faecal microbiome is not homogenous 88

3.3.3.2 Diversity, relative abundance and bacterial species of individual sheep by 16S rRNA sequencing 89

3.4 DISCUSSION 91

CHAPTER 4 INTERACTIONS BETWEEN SHEEP GUT MICROBIOTA AND THE PARASITE *HAEMONCHUS CONTORTUS* **97**

4.1 INTRODUCTION 98

4.2 MATERIALS AND METHODS 100

4.2.1 Experimental Animals *100*

4.2.2 Experimental Infection with H. contortus *Parasites* *100*

4.2.3 Parasitological Examination *101*

4.2.4 Experimental Design and Sampling for ARISA and 16S Amplicon Sequencing *101*

4.2.5 DNA Extraction, Quantification and Homogenization *103*

4.2.6 ARISA *103*

4.2.7 16S rRNA Gene Illumina MiSeq Sequencing *103*

4.2.8 Bioinformatic Analysis *103*

4.2.9 Ecological and Statistical Analysis *104*

4.3 RESULTS 105

4.3.1 Faecal Egg Count of infected sheep *105*

4.3.2 The Community Structure of Faecal Microbiota of High- and Low-Burden Sheep Prior to Infection Determined by ARISA *107*

4.3.3 The Community Structure and Composition of Faecal Microbiota of High- and Low-Burden Sheep Prior to Infection Determined by 16S rRNA Sequencing *110*

4.3.3.1 Rarefaction 110

4.3.3.2 Cluster analysis of faecal microbiota in high and low burden sheep prior to infection 111

4.3.3.3 Diversity of microbial community prior to infection 112

4.3.3.4 Composition of microbial community prior to infection 113

4.3.4 Comparison of the Community Structure of Faecal Microbiota of High- and Low-Burden Sheep Before and After H. contortus *Infection Determined by ARISA 119*

4.3.5 Comparison of the Community Structure and Composition of Faecal Microbiota of High- and Low-Burden Sheep Before and After H. contortus *Infection Determined by 16S rRNA Sequencing* *123*

4.3.5.1 Rarefication 123

4.3.5.2 Cluster analysis for comparison of microbial composition before and after infection with *H. contortus* 123

4.3.5.3 The impact of *H. contortus* infection on microbial diversity 125

4.3.5.4 The impact of *H. contortus* infection on microbial community composition 126

4.4 DISCUSSION 133

4.4.1 Prior to Infection with H. contortus *133*

4.4.2 After Infection with H. contortus *137*

CHAPTER 5 GENERAL DISCUSSION 141

REFERENCES 151

APPENDICES 169

APPENDIX 1 170

APPENDIX 2 172

APPENDIX 3 173

APPENDIX 4 189

APPENDIX 5 207

List of Figures

FIGURE 1.1 INCREASING DEMAND FOR MEAT, AS DEMONSTRATED BY THE INCREASING MEAT PRODUCTION PER CAPITA IN THE LATER PART OF THE 20TH CENTURY.....4

FIGURE 1.2 A DIAGRAMMATIC REPRESENTATION OF THE *H. CONTORTUS* LIFE CYCLE. 7

FIGURE 1.3 DYNAMIC HOST–COMMENSAL BACTERIA–PATHOGEN INTERACTIONS IN THE INTESTINAL MICROENVIRONMENT.....29

FIGURE 2.1 INTERGENIC REGION BETWEEN 16S AND 23S GENES OF THE BACTERIAL GENOME. 37

FIGURE 2.2 EXPERIMENTAL SAMPLING DESIGN. THREE INDIVIDUAL SHEEP WERE SAMPLED SIX TIMES OVER A 5 DAY PERIOD. 40

FIGURE 2.3 PCR AMPLIFICATION OF FOUR PRIMER SETS WITH HOTSTARTAQ PLUS AND GOTAQ GREEN MASTER MIX VISIBLE IN 0.8% AGAROSE GEL.....47

FIGURE 2.4 ELECTROPHEROGRAMS OF THE TRIPLICATE SAMPLES OF ARISA FROM THE SAME DNA EXTRACTION DEMONSTRATES REPRODUCIBILITY. 48

FIGURE 2.5 ARISA CAN DIFFERENTIATE FAECAL MICROBIAL COMMUNITIES IN DIFFERENT INDIVIDUAL SHEEP. 49

FIGURE 2.6 AN NMDS PLOT REPRESENTING THE FAECAL MICROBIOAL COMPOSITION OF THREE INDIVIDUAL SHEEP. 53

FIGURE 3.1 AVERAGE RELATIVE ABUNDANCE OF THE 14 MOST PREVALENT OTUS DETECTED BY ARISA OVER 4 WEEKS OF SAMPLING. 68

FIGURE 3.2 HEATMAP+ ILLUSTRATING THE AVERAGE RELATIVE ABUNDANCES OF IDENTIFIED PHYLA.....70

FIGURE 3.3 DIVERSITY OF FAECAL MICROBIOTA OF SHEEP ACROSS 4 WEEKS OF SAMPLING BY ARISA 72

FIGURE 3.4 RELATIVE ABUNDANCES OF DOMINANT OTUS AS DETECTED BY ARISA. 73

FIGURE 3.5 CLUSTERING OF FAECAL MICROBIOTA OF WEEK 24 AND WEEK 25 SHEEP SAMPLES.....75

FIGURE 3.6 DIVERSITY OF GUT MICROBIOTA OF SHEEP ACROSS 2 WEEKS.....76

FIGURE 3.7 THE STABILITY OF THE CORE MICROBIOTA OF SHEEP OVER A 2-WEEK PERIOD. 78

FIGURE 3.8 AVERAGE RELATIVE ABUNDANCES OF THE 30 MOST ABUNDANT GENERA FROM 11 SHEEP.....80

FIGURE 3.9 SIGNIFICANT DIFFERENCES IN RELATIVE ABUNDANCES OF GENERA OF FAECAL BACTERIA FROM 11 SHEEP SAMPLES.....81

FIGURE 3.10 NMDS PLOT REPRESENTING THE FAECAL MICROBIAL COMPOSITION OF TWO SHEEP 83

FIGURE 3.11 THE STABILITY OF THE CORE MICROBIOTA OF SHEEP OVER A 6 MONTHS PERIOD. 84

FIGURE 3.12 AVERAGE RELATIVE ABUNDANCES OF 30 DOMINANT GENERA FROM TWO SHEEP. 86

FIGURE 3.13 SIGNIFICANT DIFFERENCES IN RELATIVE ABUNDANCES OF GENERA OF FAECAL BACTERIA FROM TWO SHEEP SAMPLES. 87

FIGURE 3.14 AN NMDS REPRESENTING THE GUT MICROBIAL COMPOSITION OF DIFFERENT SHEEP SAMPLES 89

FIGURE 4.1 DIAGRAMMATIC REPRESENTATION OF EXPERIMENTAL DESIGN. 102

FIGURE 4.2 CUMULATIVE EGGS PER GRAM FOR EACH OF THE 28 SHEEP. 106

FIGURE 4.3 COMPARISON OF CEPG OF HIGH AND LOW PARASITE BURDEN SHEEP. UNPAIRED T-TEST. 106

FIGURE 4.4 DISTINCT CLUSTERING OF MICROBIOME BETWEEN HIGH AND LOW PARASITE BURDEN SHEEP. 108

FIGURE 4.5 VENN DIAGRAM REPRESENTING THE SHARED AND UNIQUE OTUS IN HIGH AND LOW PARASITE BURDEN SHEEP. 109

Figure 4.6 Average relative abundances of 10 most dominant OTUs of high-burden and low-burden sheep 110

FIGURE 4.7 DISTINCT CLUSTERING OF FAECAL MICROBIOTA BETWEEN HIGH AND LOW PARASITE BURDEN SHEEP 112

FIGURE 4.8 AVERAGE RELATIVE ABUNDANCES OF IDENTIFIED PHYLA. 114

FIGURE 4.9 AVERAGE RELATIVE ABUNDANCES OF THE DOMINANT FAMILIES. 115

FIGURE 4.10 GENERA WITH ABSOLUTE LDA SCORE ≥ 2.0. 118

FIGURE 4.11 VENN DIAGRAM REPRESENTING THE SHARED AND UNIQUE OTUS IN SHEEP WITH HIGH AND LOW BURDENS OF PARASITE. 119

FIGURE 4.12 CLUSTERING OF GUT MICROBIOME OF UNINFECTED AND INFECTED SHEEP USING ARISA.... 121

FIGURE 4.13 AVERAGE RELATIVE ABUNDANCES OF COMMONLY DETECTED OTUS OF UNINFECTED AND INFECTED SHEEP DETECTED BY ARISA. 122

FIGURE 4.14 CLUSTERING OF FAECAL MICROBIOTA OF UNINFECTED AND INFECTED SHEEP BASED ON 16S DATA 124

FIGURE 4.15 THE BACTERIAL PHYLA WITH SIGNIFICANT DIFFERENCES IN RELATIVE ABUNDANCES BEFORE AND AFTER INFECTION. 127

FIGURE 4.16 THE BACTERIAL FAMILIES WITH SIGNIFICANT DIFFERENCES IN RELATIVE ABUNDANCES BEFORE AND AFTER INFECTION. 129

FIGURE 4.17 SIGNIFICANTLY DISCRIMINATIVE GENERA WITH ABSOLUTE LDA SCORE ≥ 2.0 BETWEEN UNINFECTED AND INFECTED GROUP OF SHEEP USING LEFSE 132

FIGURE A3.1 OTU ACCUMULATION PLOT. WEEKLY SAMPLES CURVES ARE OVERLAPPING EACH OTHER. .. 173

FIGURE A3.2 VISUALIZATION OF MICROBIAL COMMUNITY COMPOSITION AT GENUS LEVEL USING HIERARCHICAL TREES SHOWN AS PIE CHARTS (KRONA CHARTS)..... 174

FIGURE A3.3 RAREFACTION CURVE BASED ON OTU RICHNESS VALUES 175

FIGURE A3.4 CLUSTERING OF MICROBIOME BETWEEN WEEK 24 AND WEEK 25 SHEEP SAMPLES..... 176

FIGURE A3.5 THE STABILITY OF THE CORE MICROBIOTA OF SHEEP OVER A 2-WEEK PERIOD. 177

FIGURE A3.6 AVERAGE RELATIVE ABUNDANCES OF 30 MOST ABUNDANT TAXA FROM 11 SHEEP 178

FIGURE A3.7 SIGNIFICANT DIFFERENCES IN RELATIVE ABUNDANCES OF TAXA OF FAECAL BACTERIA FROM 11 SHEEP SAMPLES..... 179

FIGURE A3.8 NMDS PLOT REPRESENTING THE FAECAL MICROBIAL COMPOSITION OF DIFFERENT SHEEP SAMPLES 180

FIGURE A3.9 AVERAGE RELATIVE ABUNDANCES OF 30 MOST ABUNDANT TAXA FROM 2 SHEEP USING 181

FIGURE A3.10 SIGNIFICANT DIFFERENCES IN RELATIVE ABUNDANCES OF TAXA OF FAECAL BACTERIA FROM 2 SHEEP SAMPLES..... 182

FIGURE A3.11 NMDS PLOT BASED ON THE BRAY-CURTIS DISTANCE MATRIX REPRESENTING THE FAECAL MICROBIAL COMPOSITION OF DIFFERENT SHEEP SAMPLES..... 183

FIGURE A3.12 CLUSTERING OF MICROBIOME BETWEEN THE SHEEP SAMPLES 184

FIGURE A3.13 DIVERSITY OF GUT MICROBIOTA OF SHEEP 185

FIGURE A4.1 COLLECTION OF FAECES FROM SHEEP AND CULTURE OF LARVAE IN THE WET LABORATORY.. 191

FIGURE A4.2 FILTRATION OF COLLECTED AND STORED *H. CONTORTUS* L3. 192

FIGURE A4.3 RAREFACTION CURVE BASED ON OTU RICHNESS VALUES. 194

FIGURE A4.4 DISTINCT CLUSTERING OF MICROBIOME BETWEEN HIGH AND LOW WORM BURDEN SHEEP.. 194

FIGURE A4.5 VENN DIAGRAM REPRESENTING THE CORE OTUS IN GROUP OF SHEEP WITH HIGH AND LOW BURDEN OF PARASITE. 195

FIGURE A4.6 RAREFACTION CURVE BASED ON OTU RICHNESS VALUES. 195

FIGURE A4.7 CLUSTERING OF MICROBIOME OF INFECTED AND UNINFECTED SHEEP. 196

FIGURE A4.8 HEATMAP+ OF THE RELATIVE ABUNDANCES OF THE IDENTIFIED PHYLA IN UNINFECTED AND INFECTED SHEEP. 197

FIGURE A4.9 HEATMAP+ OF THE RELATIVE ABUNDANCES OF THE 30 DOMINANT FAMILY IN UNINFECTED AND INFECTED SHEEP.....198

FIGURE A4.10 VENN DIAGRAM REPRESENTING THE SHARED AND UNIQUE OTUS PRESENT IN SHEEP BEFORE AND AFTER INFECTION. 199

FIGURE A4.11 VENN DIAGRAM REPRESENTING THE CORE OTUS PRESENT IN SHEEP BEFORE AND AFTER INFECTION. 200

FIGURE A5.1 DISTINCT CLUSTERING OF FAECAL AND ABOMASAL MICROBIOTA OF SHEEP 207

FIGURE A5.2 DISTINCT CLUSTERING OF FAECAL AND ABOMASAL MICROBIOTA OF SHEEP. 208

List of Tables

TABLE 1.1 IMPORTANT TAXA IN THE GUT MICROBIOTA OF SELECTED SPECIES OF ANIMALS. 15

TABLE 1.2 COMPARISON OF THE MOST COMMONLY USED COMMUNITY FINGERPRINTING TECHNIQUES. 22

TABLE 2.1 PRIMERS SELECTED FOR USE IN ARISA OPTIMIZATION STUDIES FOR THE ANALYSIS OF MICROBIAL COMPOSITION WITHIN SHEEP FAECES. 41

TABLE 2.2 EXPERIMENTAL PCR CONDITIONS FOR AMPLIFICATION OF THE ITS REGION. 43

TABLE 2.3 RANGE OF PEAK NUMBERS AND SIZES IN THE ARISA PROFILES OF TWO DIFFERENT MASTER MIXES AND PRIMERS WITH TWO DIFFERENT DNA EXTRACTION TECHNIQUES. 50

TABLE 2.4 RANGE OF PEAK NUMBERS AND SIZES IN THE ARISA PROFILES OF QIAGEN DNA EXTRACTION TECHNIQUES. 51

TABLE 2.5 PERMANOVA OF FAECAL MICROBIOTA SAMPLES BASED ON ARISA. 52

TABLE 2.6 TWO WAY ANOVA OF SHANNON DIVERSITY *(H)* INDEX OF FAECAL MICROBIOTA DETERMINED BY ARISA. 52

TABLE 3.1 EXPERIMENTAL SAMPLING PLAN TO INVESTIGATE THE COMMUNITY STRUCTURE, COMPOSITION AND STABILITY OF THE SHEEP GUT USING FAECAL SAMPLES. 63

TABLE 3.2 NUMBER OF TAXA IDENTIFIED FROM FAECAL SAMPLES OF SHEEP. 69

TABLE 3.3 ANOSIM ANALYSIS OF MICROBIAL COMPOSITION OF SHEEP ACROSS 2 WEEKS 74

TABLE 3.4 ANOSIM ANALYSIS OF MICROBIAL COMPOSITION OF SHEEP OVER A 6-MONTH PERIOD 82

TABLE 3.5 ANOSIM OF GUT MICROBIAL COMPOSITION FOR INDIVIDUAL SHEEP 90

TABLE 4.1 ANOSIM ANALYSIS OF MICROBIAL COMPOSITION OF SHEEP BETWEEN HIGH-BURDEN AND LOW-BURDEN SHEEP 111

TABLE 4.2 MICROBIAL DIVERSITY INDICES OF THE SHEEP FAECAL MICROBIOTA. 113

TABLE 4.3 IDENTIFIED GENERA WITH SIGNIFICANTLY DIFFERENT ABUNDANCES BETWEEN THE HIGH AND LOW BURDEN SHEEP 116

TABLE 4.4 ANOSIM OF MICROBIAL COMPOSITION OF SHEEP FOLLOWING INFECTION 124

TABLE 4.5 MICROBIAL DIVERSITY INDICES OF SHEEP FAECAL MICROBIOTA OF UNINFECTED AND INFECTED SHEEP. 126

TABLE 4.6 THE GENERA WITH SIGNIFICANTLY DIFFERENT ABUNDANCES BETWEEN THE UNINFECTED AND INFECTED SHEEP HIGH PARASITE BURDEN 130

TABLE 4.7 THE GENERA WITH SIGNIFICANTLY DIFFERENT ABUNDANCES BETWEEN THE UNINFECTED AND INFECTED SHEEP WITH LOW PARASITE BURDEN 131

TABLE A1.1 LIST OF MAMMALIAN SPECIES..... 170

TABLE A2.1 DNA YIELD OF SIX FAECAL SAMPLES OF SHEEP BY USING THREE DIFFERENT GENOMIC DNA EXTRACTION TECHNIQUES..... 172

TABLE A3.1 AVERAGE RELATIVE ABUNDANCES OF 50 MOST ABUNDANT FAMILY PRESENT IN MINIMUM 50% OF THE SAMPLES. 186

TABLE A3.2 AVERAGE RELATIVE ABUNDANCES OF 50 MOST ABUNDANT SPECIES PRESENT IN MINIMUM 50% OF THE SAMPLES IDENTIFIED 187

TABLE A3.3 ANOSIM PAIRWISE COMPARISON OF R VALUES OF 28 SHEEP SAMPLES 188

TABLE A4.1 THE 50 MOST ABUNDANT CORE OTUS WITH DIFFERENT ABUNDANCES BETWEEN THE HIGH AND LOW PARASITE BURDEN SHEEP..... 201

TABLE A4.2 SHARED AND UNIQUE TAXA PRESENT IN HIGH AND LOW PARASITE BURDEN SHEEP..... 202

TABLE A4.3 SHARED AND UNIQUE TAXA PRESENT IN UNINFECTED AND INFECTED GROUP OF SHEEP WITH HIGH AND LOW PARASITE BURDEN. 202

TABLE A4.4 THE 30 MOST ABUNDANT GENRERA OF UNIMFECTED AND INFECTED SHEEP WITH HIGH PARASITE BURDEN 203

TABLE A4.5 THE 30 MOST ABUNDANT OTUS OF UNIMFECTED AND INFECTED SHEEP WITH HIGH PARASITE BURDEN 204

TABLE A4.6 THE 30 MOST ABUNDANT GENERA OF UNIMFECTED AND INFECTED SHEEP WITH LOW PARASITE BURDEN 205

TABLE A4.7 THE 30 MOST ABUNDANT OTUS OF UNIMFECTED AND INFECTED SHEEP WITH LOW PARASITE BURDEN 206

TABLE A5.1 ANOSIM OF MICROBIAL COMPOSITION OF SAMPLES COLLECTED FROM THE ABOMASUM AND FAECES OF SHEEP. 209

TABLE A5.2 SHARED AND UNIQUE TAXA PRESENT IN ABOMASUM AND FAECAL SAMPLES OF SHEEP. 209

TABLE A5.3 SHARED AND UNIQUE CORE TAXA PRESENT IN ABOMASUM AND FAECAL SAMPLES OF SHEEP. 210

TABLE A5.4 AVERAGE RELATIVE ABUNDANCES OF IDENTIFIED PHYLA PRESENT IN MINIMUM 50% OF THE ABOMASAL AND FAECAL SAMPLES OF SHEEP 211

TABLE A5.5 AVERAGE RELATIVE ABUNDANCES OF 30 MOST ABUNDANT FAMILIES PRESENT IN MINIMUM 50% OF THE ABOMASAL AND FAECAL SAMPLES OF SHEEP. 212

TABLE A5.6 AVERAGE RELATIVE ABUNDANCES OF 30 MOST ABUNDANT GENERAPRESENT IN MINIMUM 50% OF THE ABOMASAL AND FAECAL SAMPLES OF SHEEP. 213

Chapter 1

Introduction and Review of the Literature

1.1 General Introduction

1.1.1 Global Food Security

By 2050, the global population is predicted to grow from the current 7.3 billion to approximately 9.7 billion people [1]. This rapid population growth brings with it two great food security challenges. First is the need to produce enough food for a global population set to increase by 33% in less than a quarter of a century. Secondly, there is a need to produce more with less. Urban expansion to house the growing population, environmental degradation of formerly arable land, and the potential for arable land to be used to meet renewable energy demands rather than food production *per se* means that increased agricultural outputs will likely be met using less farming land rather than more.

In addition to a growing global population, there are inadequacies and inequality in food supply and distribution globally at the current time. Care International estimates that 870 million people suffer chronic food insecurity; likely to increase to 1.4 billion people, if just and sustainable food systems are not implemented [2]. Moreover, 2.6 million children die each year due, at least in part, to malnutrition [3]. Clearly there is a need to increase agricultural productivity and efficiency; indeed it is estimated that agricultural productivity will need to increase by 70% to meet the increasing global demand by 2050 [4, 5]. While much of the increased production will need to be in cropping (e.g. cereals and vegetables), there is both a need and a demand for high-quality protein sources, including meat.

1.1.2 Increasing Demand for Meat and Animal Products

Demand for ruminant (i.e. cattle, sheep and goats) livestock products (e.g. meat, milk and fibre) has been growing steadily for many decades. Over the last 40 years of the 20th century per capita production of meat increased by approximately 70% (Figure 1.1). On average, animal protein makes up 37% of a person's total protein demand per year [6]; however, there is an imbalance in terms of per capita protein intake between people living in developed and developing worlds. Growing populations and incomes, particularly in developing countries, along with changing food preferences, are rapidly increasing demand for more livestock products.

Currently, around 40% of the total global agricultural production is animal products [7]. With a growing global population and increased per-capita consumption, the global demand for meat products is projected to more than double from the early 2000s where demand of 229 million tonnes will increase to 465 million tonnes in 2050 [8]. Due to the increasing demand, animal production systems, such as ruminant production systems, are of special interest [6]. Large-scale, high-density animal production facilities can have production efficiencies and offer convenience of handling for the administration of drugs such as anthelmintics. However, they are not without their environmental and 'one-health' (human-animal interaction) risks. Thus, if stocked at an appropriate density and well managed, grassland ruminant production may offer an efficient and sustainable method of high-quality animal protein production [9].

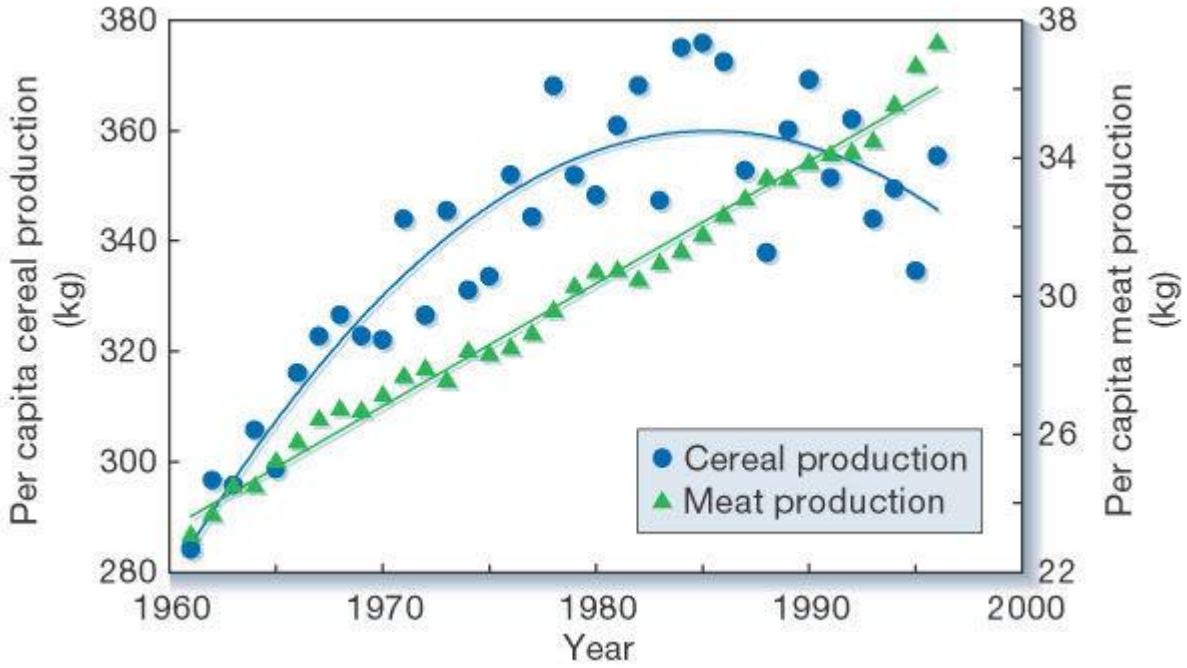


Figure 1.1 Increasing demand for meat, as demonstrated by the increasing meat production per capita in the later part of the 20th century. Taken from Tilman *et al.* [9].

1.1.3 Major Impediments to Agricultural Productivity

There remain numerous obstacles in place in regards to meeting the projected global demands for meat; and there is a clear need to develop efficient and sustainable approaches to animal production. Dumont and colleagues [10] proposed five principles for the design of sustainable animal production systems. Three of the principles were (i) adopting management practices aiming to improve animal health; (ii) decreasing the inputs needed for production and (iii) decreasing pollution by optimizing the metabolic functioning of farming systems. While these principles are overarching, there is a clear relationship between these principles and infectious diseases, in particular in terms of animal health and decreasing inputs.

Parasite infection remains one of the major impediments to increased productivity in the livestock industry. Indeed, in Australia, five of the eight most economically important diseases in the Australian sheep industry are caused by parasites [11]. In particular, nematode infection represents a major global health problem of both humans and other animals.

The three main species of nematodes causing production losses in small ruminants worldwide are *Haemonchus contortus*, *Teladorsagia circumcincta* and *Trichostrongylus* spp. [12]. Each species is prevalent in specific climatic conditions. *H. contortus* is mostly prevalent in sub-tropical regions, but can be present in places with seasonal activity of warm and cold weather, while temperate environments are suitable for *T. circumcincta* and *Trichostrongylus* spp.

Given its predisposition for sub-tropical regions, *H. contorus* is likely to be the biggest problem in countries where there is the greatest need to increase animal productivity, as a large proportion of the world's human population live in the sub-tropics and tropics. Hence, the following sections focuses on the parasite, *H. contortus*.

1.2 *Haemonchus contortus*

1.2.1 The Parasite, *H. contortus*

H. contortus, also called barber's pole worm, is one of the most common and harmful nematodes of the small ruminants including goats and sheep, worldwide [13]. It belongs to the Phylum Nematoda, Class Secernentea, Subclass Rhabditia and Family Trichostongylidae. The adult female is about 18–30 mm, with the adult male slightly

smaller at up to 20 mm long. Globally it has a wide distribution, having been detected in every continent except Antarctica. The geographical distribution is generally considered to be temperate and sub-tropical. The detection of *H. contortus* in Asia (Indonesia and India) suggests its range can extend to tropical areas.

1.2.2 Life Cycle of *H. contortus*

Like most other parasitic nematodes, *H. contortus* has a basic, direct life cycle without an intermediate host [14], as represented in Figure 1.2. Sheep become infected when they ingest infective L3 larvae from pasture. Larvae penetrate the mucosa of the abomasum and moult twice to become adults approximately 3 weeks after ingestion. The adult parasite can survive several months inside the host and the adult female lays 5,000 – 10,000 eggs per day, a fecundity up to 10 times higher than other two common genera of nematodes that infect ruminants, *Teladorsagia* (predominantly *T. circumcincta*) and *Trichostrongylus* spp. [12]. The eggs exit the host in faeces [15], where they can re-contaminate pasture. Eggs require sufficient moisture and optimal temperatures to hatch, but once hatched, larvae can survive for months on pasture. Even in extreme winter conditions, larvae can survive largely due to their ability to be metabolically inactive, a process termed hypobiosis [14]. Grazing sheep consume pasture with infective larvae to complete the life cycle.

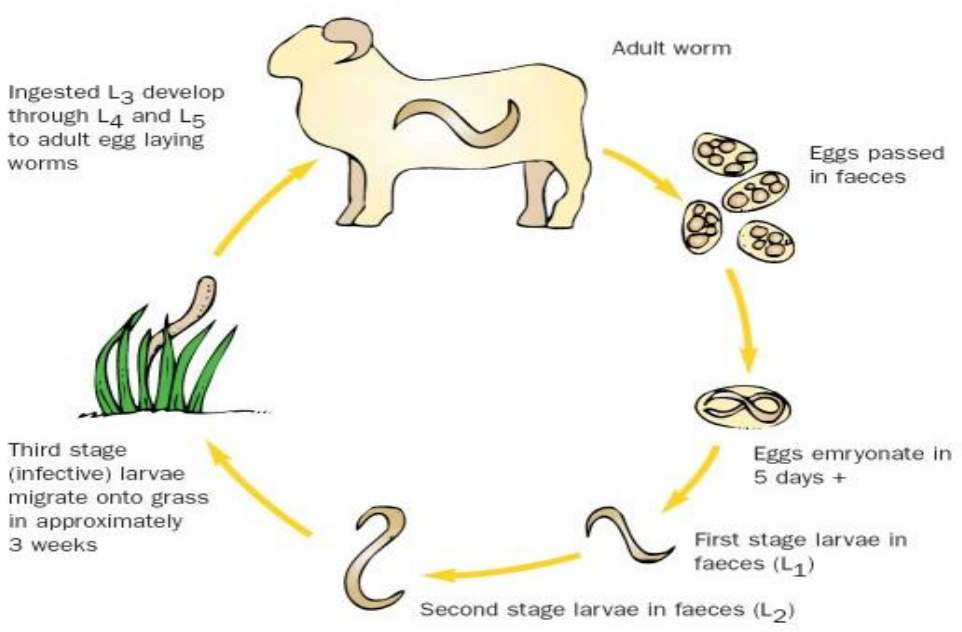


Figure 1.2 A diagrammatic representation of the *H. contortus* life cycle. Taken from [16].

1.2.3 Lost Productivity and Economic Importance of *H. contortus*

The pathology of *H. contortus* arises from the adult worm attaching to the abomasal mucosa and feeding on the blood of the host. Haemonchosis, the clinical disease caused by infection with *H. contortus*, is largely related to protein (blood) loss by the host from the feeding regime of the parasite. Infection can be fatal if untreated, especially in young sheep where immunity is less developed than in adults. Other outcomes of haemonchosis include anaemia and chronic ill thrift [17]. Animal fatalities have an obvious economic cost to the farmer; however, milder infections also reduce the overall income of sheep farmers due to concomitant reductions in milk, wool and meat production; as well as reduced reproductive performance [18, 19]. Reduced performance of animals is likely due to reduced food intake, poor nutrient utilisation and redistribution of protein within the body to enable damaged tissue to be repaired [20, 21].

Estimates of the cost of *H. contortus* and/or internal parasites are difficult to generate. A Meat and Livestock Australia report in 2008 estimated that haemonchosis cost the Australian sheep production industry around AUD 370 million each year, in terms of production loss, medications and sudden deaths of animals; equivalent to AUD 470 million in 2016 currency [19, 22]. Based on the estimates above, the cost of internal parasite infection equates to approximately 10% of the total value of the Australian sheep industry. Another more recent report on the economic impact of gastrointestinal nematodes estimated losses of £84 million in the United Kingdom [23]. The majority of these costs are due to lost productivity (see above); however, drug treatment, stocking rates and grazing management are some other avenues for lost income [24].

1.2.4 Optimising Production Traits of Small Ruminants

It is known that nematode parasite infection can significantly hamper production performances of small ruminants with wool growth, fat and meat deposition, and milk production all adversely affected [25, 26]. However, the degree of severity of production losses is complex and depends on the intensity of infection, host nutrition, host immunity and the level of mixed infections, to name just a few factors [26, 27]. Researchers have shown a negative correlation exists between body weight gain and faecal egg count during natural infections [26, 28-30]. However, the correlation between parasite resistance and growth of sheep differs between species of nematodes and the topographic regions [26, 30, 31]. The different genetic correlations in topographic zones could reflect differences in management conditions, breed or parasite species [26].

Most nematode parasite control, including *H. contortus*, involves the use of anthelminthic (drug) treatment, along with other facets of animal husbandry. At present, the worldwide annual expenditure on anthelmintics is over US$ 3 billion annually [13]. Anthelmintic treatment is expensive and requires repeated treatment [32, 33], but has been largely effective and thus is a mainstay of parasite control in the sheep and goat industries in industrialised countries. However, the cost and need for repeated treatment make anthelminthics poorly suited to animal production in low-income settings, where the burden of haemonchosis is greatest. In addition, it has been reported that anthelmintic treatment restores only part of the lost weight gain caused by the parasite infection, but not to the values seen in uninfected animals [26].

While anthelminthics have been an important tool in nematode control, there is increasing resistance to the available chemicals [34]. For example, 65-90% of sheep farms in Australia are estimated to have internal nematode parasites present that are resistant to the commonly used drugs, bezimidazole and levamisole [34]. High levels of nematode parasite resistance to anthelmintics, as well as consumer demand for chemical-free livestock products, has encouraged a research focus on chemical-free parasite control options [35].

Vaccines are one potential solution; however, despite several decades of research, relatively few commercial vaccines are available and are not suited for wide-scale use [36]. Grazing management has long been used as a way of managing infectious diseases of livestock, as well as providing animals with adequate nutrition, and continues to be an important aspect of animal husbandry [37, 38]. Breeding sheep for parasite resistance or using indigenous breeds has proven effective in reducing worm burdens, and is increasingly accepted by sheep breeders [39-41]. Other potential approaches include the

use of nematophagous fungi to reduce larval populations on pasture [42] and more recently, nutritional strategies [34, 35, 40].

Regardless of the genetic background and the sheep breeds production purpose (milk, meat, wool or combinations), nutritional management plays a crucial role in healthy and productive sheep. Nutritional practices that minimize the impact of nematode parasite infections and enhance the immune response could be potential strategies in the control of gastrointestinal infections in production animals. Given the negative impact *H. contortus* infection has on animal production due to factors such as food intake and poor nutrient utilisation, optimal nutritional intake could reduce the impact these changes have on overall nutrition and well-being of an infected animal.

Microorganisms living in the gut, commonly called the gut microbiota or gut microbiome, are an essential component of nutrition acquisition. As ruminants lack the enzymes necessary for optimal digestion of the plant material they ingest, they depend upon a symbiotic relationship with the microbial communities (bacterial, fungal, archaeal and protozoal) of their digestive tract [43]. The importance of the gut microbiota of ruminants has long been appreciated; but beyond their critical role in nutrition, it appears increasingly likely that the microbes in the gut of animals, and their community composition, has wide-reaching implications for host health in all higher animals [44]. The gut microbiota is likely to play a critical role in immunity to infectious diseases, including, potentially, parasite infections. The following sections give an overview of our current understanding of the gut microbiota in a healthy host.

1.3 Gut Microbiota

1.3.1 An Overview of Gut Microbiota and its Purported Importance

The animal gut is colonised by a wide range of microorganisms that have co-evolved with the host and play several important functions that affect host physiology and nutrition [43]. Microbes colonise the gut mostly via the oral route, with colonisation typically commencing during birth. Initially, the newborn gut is uncolonised prior to birth; and is subsequently colonised with bacteria from the mother (during and after birth), the infant's diet, and the surrounding environment (including interactions with other animals) [45, 46].

The vast majority of studies investigating the composition of the gut microbiota, and potential roles in health and disease, have been conducted in humans. The gastrointestinal tract is the home of the intestinal microbiome, defined as all the microbial inhabitants (the microbiota) and their collective genome. This definition of the microbiome holds true for humans and other animals. In humans, bacteria constitute the vast majority of the gut microbiota, but archaea, viruses, and protozoans are also present [43]. Recent data suggest that the human gut is inhabited by some 150–200 prevalent and up to 1,000 less common bacterial species [44-47]. This diversity of microorganisms in the gut microbiome of humans contains an estimated 150-fold more genes than there are human genes in the body. The collective genome of the bacterial population that inhabits mammals is estimated to contain 3 million genes [48], while the human genome contains approximately up to 25,000 genes [49]. In part, due to this complex community of microbes and associated genes, it is thought that the disruption of the gut microbiome could lead to adverse health effects. Dysbiosis of this commensal bacterial population has

been linked to various disease conditions including obesity, inflammatory bowel disease (IBD), irritable bowel syndrome, cancer and atopic disorders [44, 50, 51].

1.3.2 Ecological Principles in the Composition of the Gut Microbiota

Two types of microbial populations, autochthonous and allochthonous, have been described in mammals [52, 53]. Autochthonous bacteria are the resident stable microbes that have very closely co-evolved within the mammalian habitat. Allochthonous bacteria are non-resident microbes that are opportunistic colonisers of a habitat. They may be associated with disease states or other perturbations. Some taxa of microbes are consistently detected in most individuals of a given species sampled, and across various closely related mammalian species; thus, are predominantly or exclusively autochthonous. However, some taxa found might be autochthonous in one habitat and allochthonous in another.

It appears that succession of microbial populations continues until the establishment of as a climax community [54]. This climax community consists primarily of autochthonous taxa, with the population remaining largely stable within the individual host, and at the host population level. However, even after the climax community has been established, the microbial population is continuously changing in response to new microbes in the individual's environment, the disease and stress levels of the individual, and diet. Following these perturbations the microbial composition usually returns to the climax community after the cause of the disruption is removed [55].

Not only is there a succession of microbes during maturation of an animal, but there are also significant site predilections. Site predilections can be linked to the nutrients present

in a specific site, the availability of preferred tissue receptors that provide a means to colonise that site, such as pH, and what substrates are present (thus metabolic activities are needed in that site) [54].

Taken together, the data about the composition and function of the microbiome have led to a new view that the microbiome should be considered as a multicellular, complex organ that is important in the health and wellbeing of animals [47, 53, 56].

1.3.3 Composition of the Gut Microbiota in Animals

The vast majority of gut microbiota studies have been conducted in humans; however, there is increasing interest in microbial composition of the gastrointestinal tract of other animals. It has been reported that microbial communities at higher taxonomic levels are similar between birds and mammals. Studies show that Firmicutes and Bacteroidetes are the two most dominant phyla out of 75 known microbial phyla in mammals and birds [57, 58]. In addition to these two common phyla, Actinobacteria (Gram positive) are also reported as a dominating human gut microbiota [44]. The Firmicutes is a large bacterial phylum containing more than 200 genera, including *Lactobacillus, Mycoplasma, Bacillus and Clostridium*; whereas Bacteroidetes contains about 20 genera [59].

Comparing the composition of microbes across mammalian species is difficult due to the limited number of species sampled, varied conditions under which the studies were conducted, and different techniques used. However, more species are being sampled as the technology becomes more affordable. A summary of some key findings are provided in Table 1. Non-human studies have been conducted primarily in experimental models

and production animals, but some wildlife species have also been studied in more recent years.

Table 1.1 Important taxa in the gut microbiota of selected species of animals.

Host	Major Phyla	Dominant Phylum	Major Genus	References
Human	Firmicutes Bacteroidetes Actinobcteria	Firmicutes	*Lactobacillus, Mycoplasma, Bacillus, Clostridium*	[44, 59]
60 mammalian species (Appendix 1,Table A1.1 for the list)	Firmicutes, Bacteroidetes Proteobacteria, Actinobacteria Verrucomicrobia, Fusobacteria Spirochaetes, Fibrobacteres Cyanobacteria	Firmicutes	Unknown?	[60]
Pig	Firmicutes Bacteroidetes Proteobacteria, Actinobacteria Spirochaetes	Firmicutes	*Prevotella, Anaerobacter, Streptococcus, Lactobacillus, Coprococcus, Blautia,*	[58, 61, 62]
Chicken	Firmicutes Bacteroidetes Proteobacteria Actinobacteria	Firmicutes	*Lactobacillus, Clostridium, Ruminococcus, Enterococcus, Escherichia, Enterobacter, Bifidobacterium*	[63, 64]
Mouse	Firmicutes, Bacteriodetes, Proteobacteria, Actinobacteria, Tenericutes, TM7 Verrucomicrobia	Firmicutes	*Lactobacillus, Lactococcus, Helicobacter, Ruminococcu, Turicibacter etc*	[65]
Sheep	Firmicutes Bacteriodetes Verrucomicrobia Proteobacteria Actinobacteria	Firmicutes	*Treponema*	[66]
Cattle	Firmicutes Bacteriodetes Proteobacteria Acidobacteria	Firmicutes	*Prevotella* *Oscillibacter* *Turicibacter* *Roseburia* *Fecalibacterium*	[67]
Goat	Proteobacteria Bacteriodetes Firmicutes	Proteobacteria	*Prevotella*	[68]
Australian sea lion	Firmicutes Proteobacteria Actinobacteria	Firmicutes	*Englenozoa* *Lactobacillus* *Clostridia*	[69]

1.3.4 Factors Affecting the Stability of Gut Microbiota Composition

Several studies have documented that an individual's microbiota is more similar over time than to other individuals. However, in childhood, before the establishment of a stable and diverse microbiota (climax community) this may be different. The initial microbiota is characterized by low diversity and mainly facultative anaerobic bacteria belonging to Proteobacteria and Actinobacteria. The gut microbiota then becomes more diverse, and bacteria belonging to Firmicutes and Bacteroidetes are dominant [47, 48, 70].

It is now increasingly recognized that composition of gut microbiota may alter concomitantly with a change of body weight in animals. For example, Ley *et al*. [71] demonstrated that obese animals have reduced Bacteriodes and increased Firmicutes when compared to their non-obese counterparts. However, some human studies do not confirm this high Firmicutes/ low Bacteriodes hypothesis. Rather, they confirm a significant diet dependant reduction in Firmicutes and increased Bacteridetes in faecal samples from obese individuals on a low fat diet [44, 72, 73]. Furet *et al.* [74] demonstrated that the degree of changes among microbiota composition related with energy intake, which may provide a better explanation for the discrepancy between these studies.

Several positive and negative selection mechanisms involved in the development of the adult microbiota of the host gut have been suggested [75]. Positive selection mechanisms include host factors that let specific bacteria attach on to the gut surface, like glycolipids on the epithelium or specific glycans in the host mucus. On the other hand, negative selection mechanisms by the host immune system may alter the gut microbial community by killing specific bacterial groups. For example, mice deficient in toll-like receptors or nucleotide-binding oligomerization domain receptors, which recognize conserved

microbial signatures or downstream signalling molecules, have altered gut microbial composition [76-78]. Furthermore, epithelial expressed effector molecules of the innate immune system also affect the gut microbial composition and may act protectively in patients with IBD [79]. Interestingly, several studies have now demonstrated that the gut microbiota of IBD patients has reduced microbial diversity, and animal models have provided evidence that the gut microbiota may be involved in the development of IBD [80].

Microbial composition changes with an increase in host age. In pigs the proportion of the phylum Firmicutes increased with increase of age, while the proportion of bacteria in the phylum Bacteroidetes decreased at the same time period. Pigs the same age were more similar to each other compared to pigs of different ages [58, 61]. Studies in humans have also revealed differences to gut microbioal composition among different age groups [81-84].

1.3.5 Microbiota in Nutrition and Metabolism

The intestinal microbiota has a pivotal role in converting nutrients into energy [44]. This role, although widely appreciated, has been shown through experiments with germ-free mice; which, despite increased food intake, have reduced capacity to harvest energy from the diet relative to wild-type mice [85]. Furthermore, the gut microbiota is an important detoxifier of xenobiotic compounds that are ingested, thus further contributing to health and nutritional status [86].

In many mammals, microbes aid in the host's ability to utilize plant polysaccharides as energy sources through producing different enzymes [74, 87, 88]. Particularly, vertebrate

herbivores cannot digest plant fibre auto-enzymatically, but rather rely on gut microflora for this purpose [89]. These enzymes are critical to host nutrition because most animals lack the genes for lycoside hydrolase, polysaccharide lyase, and carbohydrate esterase enzymes that are necessary to facilitate this process [90].

During the catabolism of dietary polysaccharides, bacteria in the gastrointestinal tract produce short chain fatty acids (SCFAs) [91, 92]. The composition and proportions of these SCFAs vary depending on microbial composition, which is to some degree adaptable, and modulated by the composition and structure of the fibre component of the diet [92]. Acetate is the primary SCFA produced in most gastrointestinal tract environments, followed by propionate and butyrate [91]. Other SCFAs such as valerate, isobutyrate, and isovalerate are also produced in trace amounts [91]. These SCFAs are also of critical importance to host energetics and hydration. SCFAs stimulate fluid and electrolyte uptake and are absorbed trans-epithelially as a source of energy that contributes between 10% (humans) and up to 70% (ruminants) of the host's daily energy requirements [92, 93].

The gastrointestinal microbiome also contributes to nitrogen metabolism, both the catabolism of proteins and the anabolism of nitrogenous compounds such as amino acids [94]. The catabolism of proteins by bacteria in the digestive system leads to the liberation of essential amino acids that can be utilised further along the digestive tract by other gut bacteria. In turn, some of these bacteria may produce amino acids and other nitrogenous compounds that are required by the host. While the gut microbiota appears to play an important role in the cycling of biologically active nitrogen compounds, there is no strong evidence to suggest that the gut microbiota can positively contribute to the overall amount of biologically active nitrogen independent of that derived from the diet. Nitrogen fixing

bacteria have been isolated from humans, guinea pigs and pigs [95], but a recent study was unable to demonstrate significant nitrogen fixing activity in humans with a low nitrogen intake [96]. Nonetheless, the gut microbiota is likely play an important role in host nutrition through metabolism of nitrogenous compounds in the digestive tract.

The above studies highlight the central role of microbiota in host nutrition and homeostasis. Our ability to understand the relationship between gut microbiota and host homeostasis, or in altered disease states, is dependent on accurately discerning the microbial community of the host. The proceeding section summarises different methods to investigate microbiota currently available and potential benefits and limitations of such approaches.

1.4 Methods for the Investigation of Gut Microbial Communities

1.4.1 Detection of Microbes in Complex Samples

Microorganisms are a highly disparate group of organisms and constitute about 60% of the earth's biomass [97], contributing to every known natural ecosystem. However, until relatively recently we have had a limited understanding of the true diversity of microbes present. With the advent of culture-independent detection methods, specifically genetic amplification detection methods, we are gaining a better knowledge of the diversity of microbes present in many ecosystems.

One ecosystem that has long been of interest to science is the gut microbiota, particularly in higher animals such as mammals and birds. Initially, efforts to investigate the microbial community diversity of the mammalian and avian gut depended on selective,

culture-based techniques to identify and characterize microbes of interest; and mostly focused on detecting potentially harmful microbes [57, 98]. These culture based techniques are not ideal, especially when studying mutualistic microbial diversity, as up to 99% of microbial species cannot be cultured under laboratory conditions [99].

The advent and widespread application of molecular approaches has greatly increased our potential to understand complex microbial communities, through the detection and characterisation of molecules such as nucleic acids, lipids and proteins [100]. Culture independent nucleic acid approaches to microbial community studies include 16S ribosomal ribonucleic acid (rRNA) sequencing and analysis of whole genomes. In particular, the recent development in advanced molecular techniques such as genomics and sequence technologies have initiated a new era of microbial ecology, leading to studies that investigate the entire microbial community, often termed metagenomics. Implementation of these recently adopted technologies has led to the detection of many phyla, genera and species that were previously undetected [101, 102].

Currently, there are several approaches to microbial community analyses, and the methods used depend on various factors including the ecological question being asked, the type of specimen being sampled, and budgetary constraints [102, 103]. Several relevant community analysis methods well suited to application in gut microbiota studies are presented below.

1.4.2 Community Fingerprinting

Community or deoxyribonucleic acid (DNA) fingerprinting techniques are applied to gain an understanding of the microbial population structure from environmental samples. Commonly used techniques include denaturing gradient gel electrophoresis (DGGE) [104], terminal restriction fragment length polymorphism (T-RFLP) [105], and automated ribosomal intergenic spacer analysis (ARISA) [106]. In their most recent incarnations, all three methods use polymerase chain reaction (PCR) for the amplification of ribosomal genes, followed by a technique to identify the variety present within the amplified fragments. The main differences between these commonly used techniques are shown in Table 1.2. The variety observed, and used to differentiate organisms, is due to differences in the length of polymorphisms of certain rRNA genes, or in the non-coding regions of the genome (between genes) of prokaryote cells. These techniques offer fast, relatively inexpensive and reproducible evaluations of microbial community composition based on DNA fragment profiles; however, taxonomic identification is difficult.

Table 1.2 Comparison of the most commonly used community fingerprinting techniques.

	DGGE	T-RFLP	ARISA
Mechanism	Different portions of 16S rRNA have different melting points	Length of polymorphisms differ in the small subunit ribosomal RNA genes	Length of polymorphisms differ from species to species in the highly variable regions between 16S rRNA and 23S rRNA.
Digestion	No digestion is needed	Digestion used to cut the DNA fragments into smaller pieces	No digestion is needed to amplify
Resolution	Provides the highest resolution, can even detect a single base-pair variation in the DNA fragments	TRFLP offers lowest resolution out of these three methods	ARISA offers medium-level resolution, approximately equating to species level

1.4.3 ARISA

ARISA was first introduced as rRNA Intergenic Spacer Analysis (RISA) [107]. In 1999, Fisher and Triplett [106] automated this technique, thus developing automated RISA (ARISA), to analyse freshwater bacterial community diversity. ARISA utilises the inherent length heterogeneity of the intergenic transcribed spacer (ITS) region between prokaryotic 16S and 23S rRNA genes. PCR-amplification across this region, and subsequent capillary sequencing, provides a characterisation of the taxa present in the sample.

The general procedure of ARISA is as follows:

1. DNA is extracted from the sample.
2. The intergenic space between the 16S and 23S small subunit ribosomal RNA genes is amplified by PCR with a fluorescent-labelled primer.

3. The amplified products are run through capillary electrophoresis.
4. The laser-induced fluorescence detector records the fluorescence intensity at different time points and generates electropherograms.
5. Electropherograms are analysed by software (e.g. GeneMapper © or Peak Scanner™ software) to enable the size of the amplified product.

Many studies have described the effectiveness of ARISA in characterising and understanding microbial communities in a wide range of environments [106, 108-111]. However, like any experimental technique, ARISA is not without its limitations. An overlap in the size of the ITS region between species can occur, though this is uncommon [112]. On the other hand, some bacteria may have multiple rRNA operons which may result in multiple peaks from a single 'species' [106, 113]. ARISA is unable to detect those organisms which are present in low numbers in the microbial community and are unable to detect those bacteria which lack the ITS region due to absence of linked 16S-23S rRNA operons (such as the phylum of aquatic bacteria, *Planctomycetes* [114]. Moreover, ARISA does not work when the intergenic space between the 16S and 23S genes are more than 1200 base pairs apart. For example, the species *Thermoplasma volcanium* has an intergenic distance of 155,293 base pairs between the two genes.

ARISA has some limitations, but most fingerprinting techniques are vulnerable to methodological artefacts. Importantly, ARISA offers robust and repeatable profiles, allows for a high throughput of data, facilitates spatial and temporal analysis of microbial communities and allows simultaneous analysis of many samples [102]. Relative to other commonly used fingerprinting methods (Section 1.4.2) ARISA has found favour in microbial community analysis because it offers sufficient resolution to differentiate at

approximately the species level, is sensitive, is suitable for cross-laboratory comparison, is highly reproducible, and is cost effective [106, 115].

1.4.4 Next-Generation Sequencing

The challenge of metagenomics is to differentiate the sequence of individual bacteria in a complex microbial community, without the need to isolate and culture individual organisms [116]. Initially, most community analysis work focused on sequencing 16S rRNA genes, making data analysis more manageable than analysing entire bacterial genomes. In this way, only a small component of the total metagenome need to be sequenced. The 16S rRNA gene is like a barcode for each species, and sequence of this gene can enable identification of which species are present. The 16S rRNA gene is about 1,500 base pairs. Although highly conserved amongst bacterial genera due to the important role of ribosomes in cells, it has nine variable regions. These variable regions contribute most of the information that enables species to be differentiated [116]. Publicly available databases, which house many thousands of 16S sequences, allow for the rapid and potentially accurate identification of taxa. Accuracy of identification depends on various factors, including the accuracy of the initial bacterial identification when entered into the database, other factors pertaining to the curation of databases, and the length of sequence obtained during PCR. As with other genetic detection and sequencing methods, 16S rRNA sequencing is effective in that it is phylogenetically valid. However, it can be difficult to differentiate organisms at lower taxonomic ranks such as genus and species.

Sequencing techniques that are not constrained to certain parts of the genome are becoming more popular as the costs of sequencing decreases. Additionally, affordable

software and computing systems for large-scale analysis are becoming increasingly available and more accessible to biologists. Like 16S rRNA sequencing, high-throughput metagenomic sequencing allow scientists to detect and quantify organisms that are difficult to culture, leading to an improved understanding of prokaryotic community composition. There are some limitations in high-throughput methods, such as the accurate differentiation between closely related species [115, 117] and the cost and technical difficulty associated with these sequencing methods. Nonetheless, it is now possible to attain more detailed sequence samples and the detail of the community structure of a given sample is enhanced. Large scale sequencing technology currently used in 'metagenomic' studies now allow us to investigate the microbial communities with improved accuracy. As the scientific community moves towards identifying microbes, community composition and functional diversity will allow less biased ways of analysis than has previously been possible [100, 118]. However, for the moment metagenomic analysis remains a relatively costly and time-consuming approach.

The preceding sections have highlighted various approaches used to advance our understanding of microbial community structures in animals. The application of these technologies has allowed researchers to ascertaine the critical role microbial communities play in nutrition and metabolism homeostasis. How infectious disease can alter or influence the microbiota, and visa-versa, is an area of intense research. Key studies of microbial communities that are altered or otherwise by gastrointestinal infectious diseases are discussed below.

1.5 Role of Gut Microbiota in Immunity and Resistance to Infection

1.5.1 Gut Microbial Composition and Infectious Diseases

The main benefit of hosting microbes in the mammalian digestive tract was thought to be in nutrient extraction from the diet and normal gut function. However, it is now recognised that microbes in the digestive tract play an important role in many aspects of an animal's physiology, including proper development of intestinal morphology and immune function [119, 120]. Host nutrition directly links with innate immunity and nutritional supplementation can lower the level of infection in mammals [121-125].

Gastro-intestinal commensal bacterial communities are thought to limit pathogenic infection predominantly by one of the two key mechanisms: **(1)** competing for space and resources; and **(2)** stimulating intestinal epithelial and immune cells [50]. The autochthonous gut microbiota play a significant role in host defence by colonizing the intestinal lumen and competing with potentially pathogenic organisms for the environmental niche [50, 120, 126]. Dysbiosis of these microbial communities can lead to improved infectivity by pathogenic bacteria. Kamada *et al*. [101] observed that commensal bacteria reduce the extent and duration of colonization of *Citrobacter rodentium* relative to germ-free mice (no microbiota on or within these mice), despite similar immune responses (recruitment of neutrophils, inflammatory macrophages and $CD3^+$ T cells) in the germ-free and specific pathogen free mice (those with a gut microbiota). It is likely that maintenance of a diverse composition of commensal bacteria in the intestine, to some degree at least, limits the potential for bacterial pathogens to establish infections within intestinal microenvironment [50].

In humans, a healthy gut microbiota may prevent colonisation of the gastrointestinal pathogen, *Clostridium difficile.* This premise is based on the act that C. difficile infection is a leading cause of nosocomial infection, with onset commonly after prolonged courses of antibiotics. Unfortunately, to date most studies have been retrospective, though are still indicative of changes to the microbiota. Rea *et al*. observed that patients with active *C. difficile* infection harboured a less diverse gut microbiota compared with their healthy counterparts. Increases in Lactobacillaceae and Enterobacteriaceae, but decreases in Enterococcaceae, were observed in patients positive for *C. difficile* [127]. Using nonsequencing methods, Hopkins and colleagues observed that elderly patients with *C. difficile* infection had higher counts of Enterobacteriaceae (Proteobacteria), *Enterococcus*, and *Lactobacillus* (both Firmicutes), whereas healthy elderly patients harbored more diverse *Bacteroides* strains (Bacteroidetes) [128]. Healthy adults were also more likely to have more *Bifidobacteria* and *Bacteroides* compared with either elderly population.

Intestinal microbes are known to greatly influence the development and effectiveness of mucosal and systemic immune responses in mammalian systems [129]. Germ-free mammals overall have depressed immune functions (despite the aforementioned study [130] suggesting immune function was similar in germ-free and specific pathogen free mice). In general, germ-free animals have decreased cytokine production, systemic immunoglobulin levels, intraepithelial lymphocyte counts, and relative amounts of gut-associated lymphoid tissue. As a result, these animals are more susceptible to infection [131]**.**

It is difficult to ascertain the importance of the gut microbiota in immune function through the use of germ-free mice, which are a biologically artificial model. However, in studies where 'normally' colonised animals are used, the microbiome produces SCFAs,

polysaccharide A, alpha-galactosylceramide, and tryptophan metabolites. These metabolites can induce immune responses such as IL-22, Reg3γ, IgA and IL-17 responses [132].

In a series of studies by Hooper and colleagues, commensal bacteria-derived signals were found to be critical for expression of RegIIIγ, an antimicrobial lectin that is secreted into the intestinal lumen by epithelial cells and kills Gram positive bacteria by binding to peptidoglycans exposed on the surface of the bacteria [50, 133-135]. Loss of commensal bacteria induced RegIIIγ expression, which led to increased bacterial dissemination and susceptibility to bacterial pathogens such as *Salmonella Typhimurium* [135]. Thus, commensal bacterial stimulation of immune and non-immune cells along the gut results in the mutually beneficial consequence of limiting expansion of invasive bacteria [50].

It is not yet well established whether gut microbiota have any direct or indirect effect on gastrointestinal invasion of parasites. Commensal bacteria can augment the ongoing immune response to intestinal parasitic infections. Mice depleted of commensal bacterial communities via oral antibiotic treatment exhibited a diminished $CD4^+$ T cell response and increased parasite burden following infection with the intestinal protozoan, *Encephalitozoon cunniculi* [136]. Metabolites produced by commensal bacteria may have an adjuvant like effect on the immune response and aid in the formation of protective immunity to protozoans, thereby accelerating pathogen clearance [50, 136]. Hayes *et al*. [137] documented that the parasitic nematode, *Trichuris muris,* fails to establish an infection following antibiotic depletion of commensal bacteria. The hatching of *T. muris* embryonated eggs is partially dependent on type I fimbriae from commensal bacteria binding to proteins on the surface of the egg [137]. This parasite utilises commensal bacteria-derived signals to gain a competitive advantage over the host immune response.

This mechanism, along with mechanisms by which the gut microbiota appear to limit infection, is illustrated in Figure 1.3. Another recent study suggested that helminth-colonised humans had higher microbial species richness [138]. These studies demonstrate there are likely to be many examples of integral host-microbiome-parasite interactions, which are currently unknown due to our poor understanding and lack of current research.

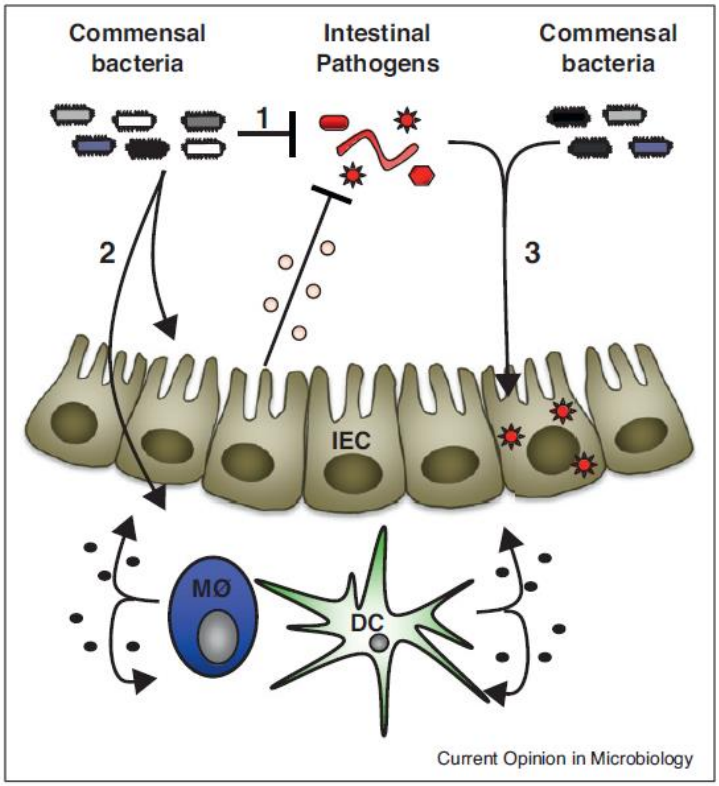


Figure 1.3 Dynamic host–commensal bacteria–pathogen interactions in the intestinal microenvironment (Taken from [50]). Intestinal commensal bacteria communities limit intestinal infection by (1) competing for space and resources or (2) stimulating intestinal epithelial and immune cells. Epithelial cells can produce antimicrobial peptides, such as RegIIIγ, that inhibit colonization of invasive bacteria. Resident innate immune cells can secrete cytokines that activate host-defence mechanisms and shape the quality and magnitude of the adaptive immune response to infection. Conversely, (3) some intestinal pathogens have evolved to utilize commensal bacterial derived signals to improve infectivity.

1.5.2 Studies in Ruminants

1.5.2.1 Microbial communities of the digestive tract of ruminants

A general predominance of Firmicutes over Bacteroidetes occurs in cattle and sheep, with Ruminococcaceae and Lachnospiraceae being the most representative microbial families of the Firmicutues [66, 67, 139]. However, predominance is site specific within the gastrointestinal tract. Bacteroidetes was identified as the most abundant phylum in the sheep rumen [140]. In goats there appears to be a dominance of Proteobacteria, followed by Bacteroidetes and Firmicutes, with *Prevotella* as the dominant genus [68]. In brief, there is a general consensus towards shared dominant taxa of the digestive tract of ruminants, with variation among species at lower taxonomic ranks. Moreover, the microbial community composition of the rumen is different to the community composition of the lower gastrointestinal tract and faeces.

1.5.2.2 Parasite infection and gut microbiota

To date there have been relatively few studies investigating the interactions of gastrointestinal parasite infection in ruminants. However, there are some data that suggest infection with helminth parasites can induce a significant change in the structure and function of the host gut microbiome in livestock. For example, infection of pigs with the whipworm *Trichuris suis* altered the microbiome (approximately 13% of genera in the proximal colon microbiome and ~26% of metabolic pathways), with the effect on the porcine proximal colon microbiome appeared to be long-lasting and independent of worm burden [141].

In ruminants, helminth infections in goats and cattle have been studied [68]. In goats, no significant change of gut microbial diversity was recorded following *H. contortus* infection, but increased abundance of the genus *Prevotella* was observed. The mean abundance of *Prevotella* in the abomasal microbiome of the uninfected goats was 16.65% but increased to 25.35%; $P < 0.05$) in the infected goats. In cattle *Ostertagia ostertagi* increased abomasal pH and hyper-gastrinaemia [142]. Infections by adult parasites rapidly altered abomasal secretory activities. Elevated abomasal pH values are closely associated with increased anaerobic bacterial densities in abomasum luminal contents [142].

1.5.2.3 Ruminant immunity and parasite infection

Ruminant animals, such as sheep, have an intricate relationship with their microbiome, as the microbiome provides the enzymes necessary for optimal digestion of the plant material. In brief, ruminants depend upon the symbiotic relationship with the gut microbiota for nutrient acquisition. This symbiotic relationship likely impacts on immunity too, as there is a well-established link between nutrition and immune responses.

Much work has been conducted on the immune response of ruminants to *H. contortus* infection, with a primary focus being on the development of a vaccine [36]. However, we also know that it is immune-mediated pathology (clinical manifestations such as reduced appetite, weight loss, and diarrhea), rather than direct effects of the nematode parasite itself, that may be responsible for much of the parasite induced disease [35]. There are various other immune-related observations that are not fully understood in relation to gastrointestinal parasites and immunity. For example, different sheep breeds are known to

have different susceptibilities to nematode infections [41]. The driving factors behind these differences are unknown. Although there are many factors that can contribute to resistance and susceptibility to infectious diseases, one aspect that has not yet been sufficiently investigated is interactions and potential role of the gut microbiota in decreasing the severity or risk of gastrointestinal nematode infection in ruminants.

1.6 Project Aims

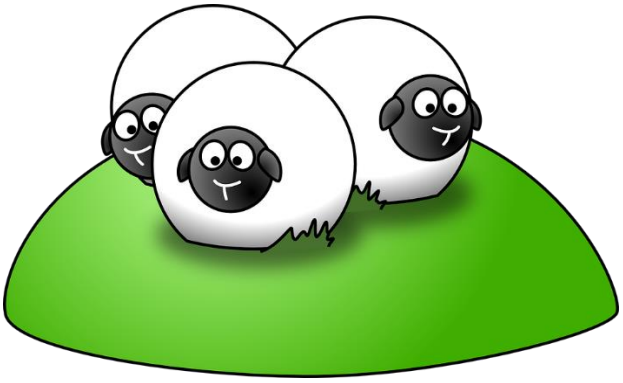
Little is currently known about the microbial populations colonizing the sheep gastrointestinal tract, despite their expected key role in host metabolism, physiology and immunity. Even less is known about the impact that nematode infections have on the gut microbiota and no studies have yet investigated if there is any link between the gut microbiota and susceptibility or resistance to parasite infection in sheep. Such a limited understanding of any potential role the gut microbiota might play in improving animal production, or in prevention of nematode infection in sheep, suggested the need to first gain a basic understanding of the sheep gut microbiota. This also included the need to establish microbiota stability over time through a longitudinal study and determine whether parasite infection would modulate the microbial population. As such, the aims of this study were:

1. To determine that ARISA is robust and suitable method for the characterisation of gut microbial composition of sheep
2. To determine the composition and the temporal stability of the sheep gut microbiota by using ARISA and 16S rRNA sequencing

3. To investigate the interactions between gut microbiota of sheep and *H. contortus* to:
    i. investigate the link between gut microbiota and *H. contortus* burden in sheep; and
    ii. determine the impact of *H. contortus* infection on the gut microbiota of sheep.

Chapter 2

Optimisation of ARISA for the Analysis of Microbial Composition within Sheep Faeces



2.1 Introduction

Community or DNA fingerprinting techniques have been adopted to gain an understanding of the microbial population structure from environmental samples. Commonly used techniques include DGGE [104], T-RFLP [143] and ARISA [106] (refer to Section 1.4.2 and 1.4.3). For community analysis these methods use PCR for the amplification of ribosomal sequences, followed by a technique to identify the variety present within the amplified fragments. These techniques offer fast, relatively inexpensive and reproducible evaluations of microbial community composition in the way of DNA fragment profiles.

In 1999, Fisher and Triplett automated the ribosomal intergenic spacer analysis technique to analyse freshwater bacterial community diversity, giving rise to ARISA [106]. ARISA utilises the inherent length heterogeneity of the intergenic transcribed spacer (ITS) region between prokaryotic 16S and 23S rRNA genes (Figure 2.1). PCR-amplification across this region allows for the characterisation of the microbial community structure in a sample. Many studies have described the effectiveness of ARISA providing an insight into microbial community composition in a variety of sample types [106, 108-110, 144].

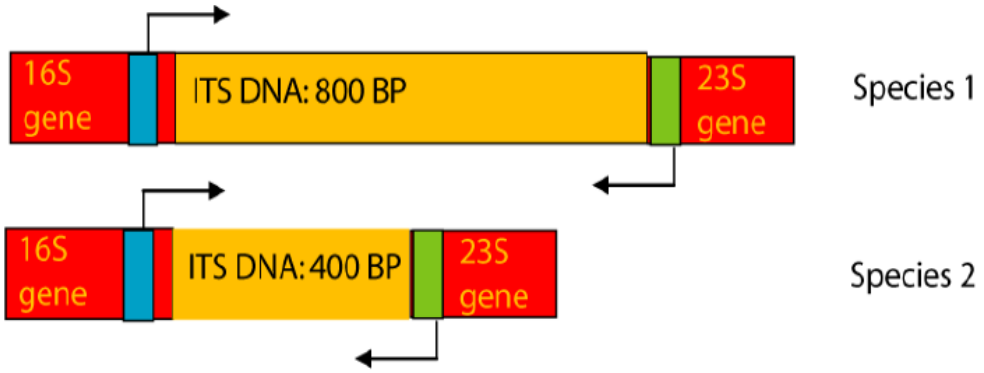


Figure 2.1 Intergenic region between 16S and 23S genes of the bacterial genome. Different species usually have different intergenic lengths. The blue and green boxes are the primer binding site on the 16S and 23S genes for PCR amplification. Adapted from Shimizu *et al*. [145].

As with all techniques, there are limitations of the ARISA method including the potential overlap in the size of the ITS region between species. An ARISA fragment of a specific size (represented as a peak on an electropherogram) might represent multiple species of different sequences, thus underestimating the diversity in the sample [106, 146]. Conversely, some bacteria may have multiple rRNA operons which may result in multiple fragments (electropherogram peaks) from a single 'species' [106, 147]. Another potential limitation of ARISA is that it is unable to detect those bacteria which lack the ITS region due to absence of linked 16S-23S rRNA operons, such as the phylum of aquatic bacteria, *Planctomycetes* [148]. Again, there is a converse situation, in which ARISA does not detect species with a large ITS region. The largest size standard used in the sequencing is 1200 nucleotides, so when the intergenic space between the 16S and 23S genes is more than 1200 base pairs products cannot be accurately sized and differentiated from other large fragments. Also, there are PCR related difficulties in

amplifying larger DNA fragments. For example, the species *Thermoplasma volcanium* has an intergenic distance of 155,293 base pairs between the two genes [145]; a product that is too large to resolve and detect through ARISA. Finally, ARISA may be unable to detect those organisms that are present in low numbers in the microbial community; although this is a problem in many community-profiling approaches that are dependent on DNA amplification.

Although ARISA has some limitations as discussed, the method still allows for a high throughput of data, and can be readily applied to spatial and temporal analyses of microbial communities [149]. Given that most fingerprinting techniques are vulnerable to methodological artefacts, ARISA offers robust and repeatable profiles, and allows simultaneous analysis of many samples. In microbial community analysis, ARISA is often preferred above other fragment-based fingerprinting methods such as DGGE and T-RFLP; as it offers sufficient resolution to differentiate at approximately the species level (though the actual species identity cannot be determined), is sensitive, is suitable for cross-laboratory comparison, is highly reproducible, and is cost effective [106, 150].

ARISA has been applied to many sample types, including faecal samples from human [151, 152], calf [153], foal [154] and mice [155], but to date ARISA has not been applied to sheep faeces for the purpose of analysing microbial populations in the ovine digestive tract. Therefore, there was a need to establish ARISA in our laboratory and optimise ARISA for application to sheep faeces; with the expectation that the technique could subsequently be applied to a large-scale study of the sheep faecal microbiota.

2.2 Materials and Methods

2.2.1 Animal Handling and Sampling

The experimental procedures used in this study were approved by the Monash University Animal Ethics Committee (MARP2012 040). Australian Merino wethers (n= 3), 2 years of age, were housed at the Monash Animal Research Platform, Gippsland Field Station. The animals had free access to water, and were provided with the same feed *ad libitum.* The sheep had no outdoor access for grazing. Faecal samples were collected directly from the rectum by hand with sterile gloves. Samples were collected twice daily at 9.30 am and 3.30 pm, every alternate day, for 5 days (i.e. three sample collection days yielding six samples per sheep over a 5 day period) (Figure 2.2). Immediately after collection, samples were taken to the laboratory in an ice box ('esky'), labelled and stored at -80° C.

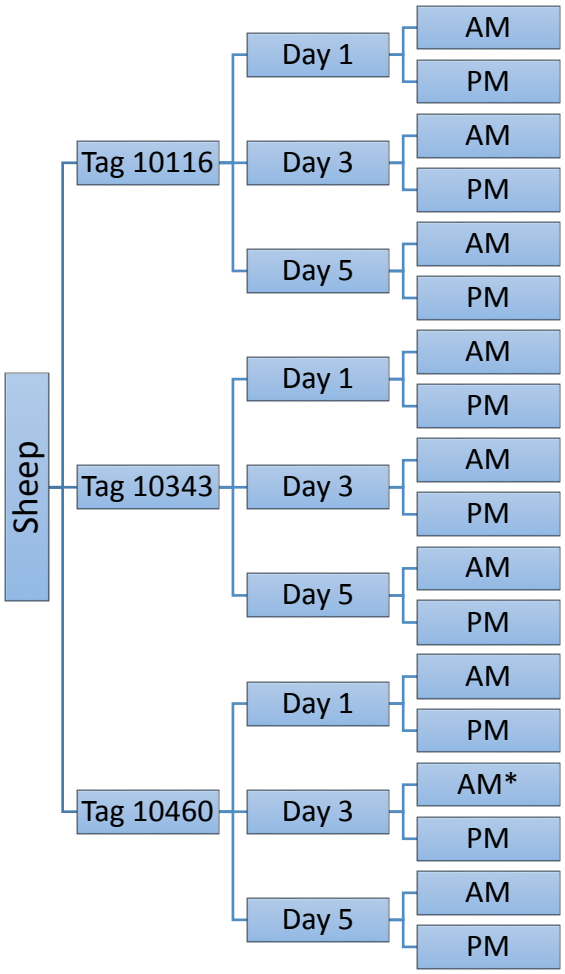


Figure 2.2 Experimental sampling design. Three individual sheep (tag numbers 10116, 10343 and 10460) were sampled six times over a 5 day period. ***** No sample collected due to lack of faecal material in sheep at the time of sample collection.

2.2.2 Primer Selection

In order to select an appropriate primer set for the application of ARISA to analyse sheep faecal microbiota, a thorough review of relevant published literature was conducted, focused primarily on the application of ARISA to comparable samples. Some primers were selected that had been used on environmental and food samples also. The primer pairs deemed highly relevant are listed in Table 2.1.

Table 2.1 Primers selected for use in ARISA optimization studies for the analysis of microbial composition within sheep faeces.

No.	Forward primer	Reverse primer	Previous sample/s analysed	References
1	1046F-GYACACACCGCCCGT	23SR-GGGTTBCCCCATTCRG	Sheep rumen, human stool, mouse and calf faeces, water, soil, milk	[106, 151-153, 155-160]
2	ITSMF-GTCGTAACAAGGTAGCCGTA	ITSMR-GCCAAGGCATCCAAC	Cow rumen	[161-164]
3	ITSCF-GTCGTAACAAGGTAGCCGTA	ITSCR-GCCAAGGCATCCACC	Soil, milk, farmyard manure, culture media, mouse caecum	[156, 165-167]
4	1552F-TCGGGCTGGATGACCTCCTT	132R-CCGGGTTTCCCCATTCGG	Foal feces, soil, milk, farmyard manure, culture media, mouse caecum	[154, 156]

2.2.3 Master Mix Selection

Trials were conducted with two commercially available pre-mixed master mixes: GoTaq Green® (Promega, NSW, Australia) (https://au.promega.com/resources/protocols/product-information-sheets/g/gotaq-green-master-mix-m712-protocol/) and HotStarTaq® Plus (Qiagen Pty Ltd, Vic, Australia). These two master mixes were selected as commonly used in similar studies and are readily available. GoTaq Green® master mix is a premixed ready-to-use solution containing Taq DNA polymerase, dNTPs, $MgCl_2$ and reaction buffers at concentrations that enable the efficient amplification of DNA templates by PCR. HotStarTaq® Plus master mix contains Taq DNA polymerase that has been chemically modified to be inactive at ambient temperatures (until it is heat activated), PCR buffer, $MgCl_2$ and dNTPs.

2.2.4 Genomic DNA Extraction, Quantification and Homogenization

Genomic DNA was extracted from faecal samples using three commercially available DNA extraction kits: i) QIAamp DNA Stool Mini Kit (Qiagen Pty Ltd., Vic, Australia) ii) QIAamp DNA Stool Kit reagents with modification by using EconoSpin columns (Epoch Life Science, Inc, Missouri City, USA) and iii) PowerSoil® DNA isolation kit (MO BIO Laboratories, Inc., Carlsbad , CA, US. Extracted DNA was quantified using Qubit® Fluorometer (Life Technologies Australia Pty Ltd. Vic, Australia) according to the manufacturer's instructions. Quantified DNA was then diluted to 20ng/µL using RNase free water (Qiagen Pty Ltd., Vic, Australia) for use as template for PCR [161, 168].

2.2.5 PCR

PCR amplification of the ITS region was performed using combinations of the primer sets listed in Table 2.1 with the fluorescent dye HEX (6-carboxy-1,4 dichloro-20,40,50,70-tetra-chlorofluorescein) labelled on either the forward or reverse primer (GeneWorks Pty Ltd, SA, Australia). Each reaction contained 10µl of one selected mastermix, 0.5µl of each of the forward and reverse primer (0.25µM), 1µl of bovine serum albumin (2µg/µl) (Genesearch Pty Ltd, QLD, Australia) and 6µl of RNase-Free water. PCR conditions differed for the two master mixes (Table 2.2). PCR was conducted using a Veriti® thermal cycler (Life Technologies Australia Pty Ltd., Vic, Australia).

Table 2.2 Experimental PCR conditions for amplification of the ITS region.

	GoTaq Green®			**HotStarTaq® Plus**		
	Temp (° C)	**Time**	**Cycle**	**Temp (° C)**	**Time**	**Cycle**
Initial denaturation	95	4 mins		95	5 mins	
Denaturation	96	15 s		94	1 min	
Annealing	45	35 s	10 cycles	55	1 min	30 cycles
Extension	72	1 min		72	1 min	
Denaturation	96	15 s				
Annealing	55	35 s	30 cycles			
Extension	72	1 min				
Final extension	72	7 mins		72	10 min	

2.2.6 Fragment Separation and ARISA Analysis

Fragment separation was carried out at the Australian Genome Research Facilities (AGRF) (Melbourne, Vic, Australia) using an Applied Biosystems 3730 DNA analyser (Applied Biosystems, CA, USA) with a GS1200 LIZ ® internal size standard (Life Technologies Australia Pty Ltd. Vic, Australia).

For each DNA sample, PCR was done in triplicate, and each product was sent for capillary electrophoresis. Peak size, height and area data were extracted to Microsoft Excel after performing accurate size calling by using GeneMapper software Version 4.0 (Applied Biosystems, CA, USA) for further analysis. The software converted fluorescence data into electropherograms; peaks represented fragments of different sizes, and the peak's areas represented the relative proportion of the fragments. All peaks with fluorescent intensity of ≤50 relative fluorescence units (RFU) were excluded as they might be the part of instrumental noise (sometimes referred to as background peaks) [106, 157, 165, 168, 169]. Given the approximate minimal known lengths of the ITS region

(143bp) [106] included in the primer sets ITSC and 1552/132, fragment lengths below 229bp and 300bp, respectively, were eliminated from analysis. Data comprising the true peak sizes and peak areas were converted to abundance per binned operational taxonomic units (OTUs) using the custom binning script interactive binner [168] in the R software package [170], with a relative fluorescence intensity cut-off of 0.09%, a window size (WS) of two and a shift size of 0.1 [168]. To determine the best binning strategy for a dataset without a priori knowing the ideal WS value, the script automatic binner [168] in R was used which allows for an automatic calculation of a series of WS values (e.g. 0.5, 1, 2, 3, 4, and 5 bp) for a given shift value (e.g. 0.1 bp). A compromise between high resolution (low WS) and high similarity among samples (high WS) was made based on the output of the script. Triplicates were compared as if they were individual samples. Within the triplicate, if two were similar, the outlier was discarded and analysis was conducted on duplicate samples only. If all triplicates were discordant, all three were discarded, the PCR was re-run, and the new product had capillary electrophoresis conducted in triplicate. Average of the duplicates/triplicates were used for statistical analysis.

2.2.7 Statistical Analysis

Abundance data obtained from interactive binner were used to generate a resemblance matrix using the Bray-Curtis similarity algorithm [171]. Similarities between sample groups were visualised using non-metric multi-dimensional scaling (nMDS) [172]. The result of nMDS ordination is a map where the position of each sample is determined by its distance from all other points in the analysis. To test for differences in composition of the faecal microbiota between sheep, sampling date and time of day (am & pm), a three

factor permutational multivariate analysis of variance (PERMANOVA) [173] was carried out based on the similarity of ARISA profiles. Each test was done using 999 permutations under Type III sum of squares (SS) to generate a permutated F statistic (*F*) and *p-value* (*P*). Results were considered significant where *p-value* ≤ 0.05. Homogeneous dispersion of samples within each factor was assessed by Permutational multivariate dispersion (commonly referred to as PERMDISP) [174]. Compared to Analysis of similarity (ANOSIM), PERMANOVA assumes homogenous dispersion of data. Shannon diversity index (*H*) were calculated using the equation: $H = -\sum P_i \ln(P_i)$ where P_i is the proportion of individuals belonging to species *i*.

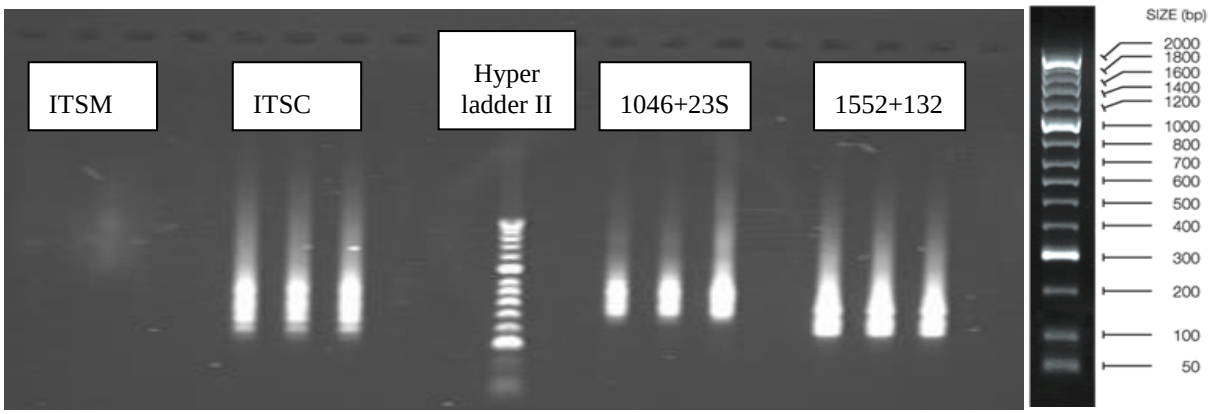
All statistical tests were performed using the software PRIMER-E v7 [175]. Two-way analysis of variance (ANOVA) was performed using GraphPad Prism version 6 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

2.3 Results

2.3.1 PCR Profiles of Different Primer Sets

In order to compare the efficiencies of the four different primer sets selected for use in this preliminary experiment, trials were conducted using two different master mixes. Success of PCR amplification was adjudicated based on visual characteristics of the amplicon when visualized in 0.8% agarose gels. Amplicon size, thickness, and brightness; and the reproducibility of replicate amplifications were used as criteria for success (Figure 2.3). Of the four primer sets 1552/132 and the ITSC appeared to work best, and therefore, selected for further downstream analysis (capillary sequencing).

(A)



(B)

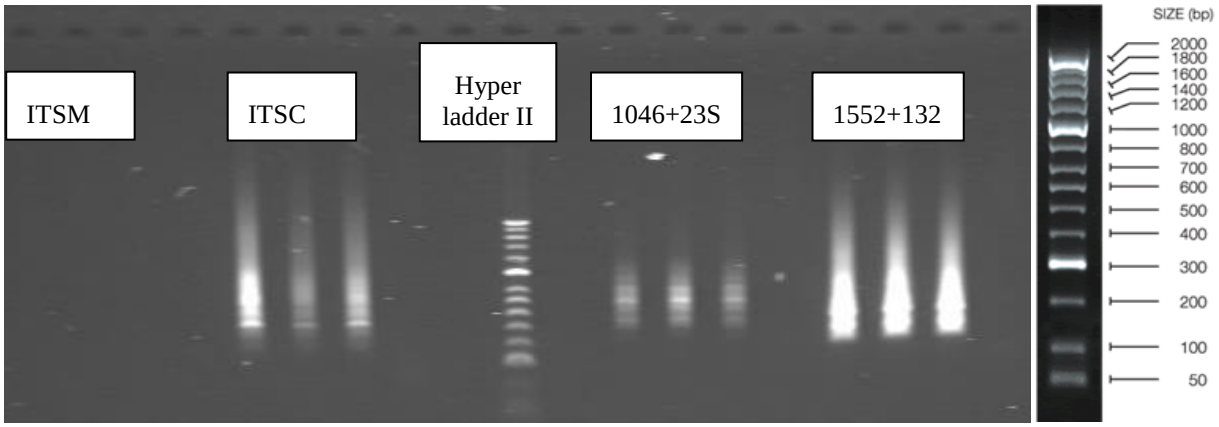


Figure 2.3 PCR amplification of four primer sets with HotstarTaq Plus (A) and GoTaq green (B) master mix visible in 0.8% agarose gel. For every primer set, PCR was conducted in triplicate from the same DNA extraction as shown.

2.3.2 ARISA is Reproducible and Sensitive

PCR products using primer sets ITSC and 1552/132 (triplicate strong banding profiles) were sent for capillary sequencing. ARISA profiles were highly reproducible within the same faecal sample for an individual sheep: triplicate samples from the same DNA

extraction from a single faecal sample yielded reproducible electropherograms (Figure 2.4). Visual analysis of ARISA electropherograms also enabled the differentiation of community faecal composition between individual sheep (Figure 2.5).

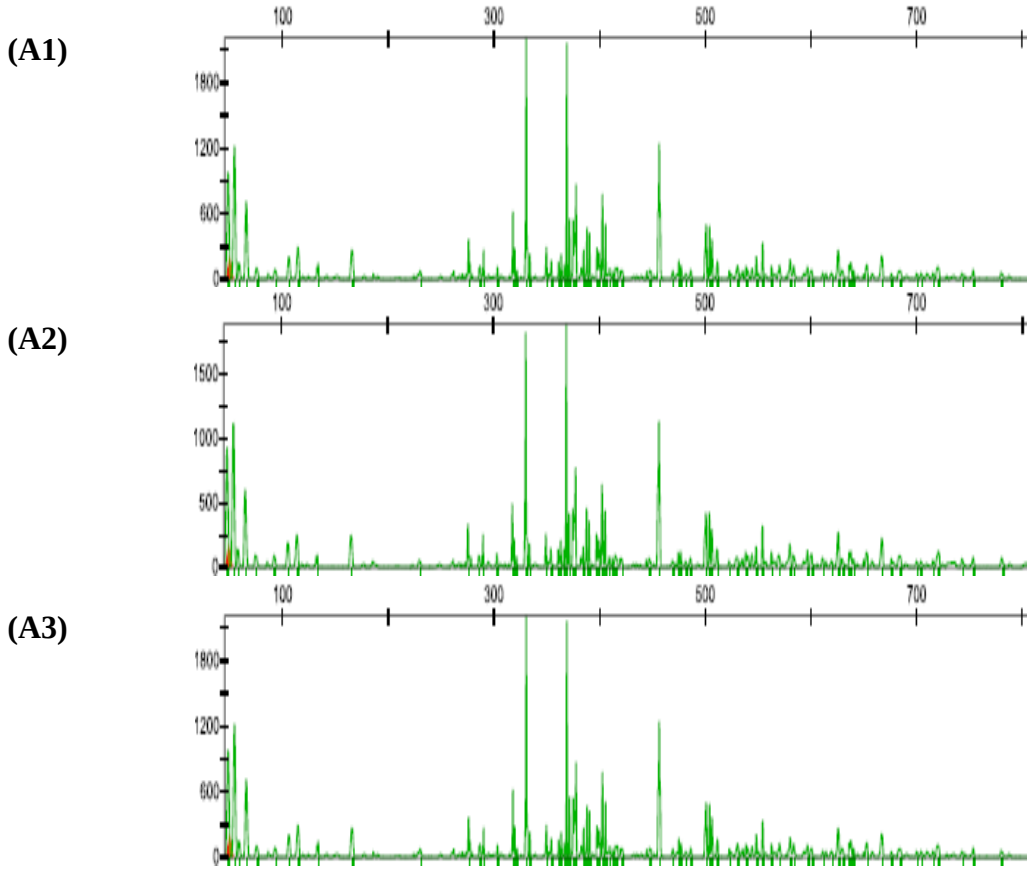


Figure 2.4 Electropherograms of the triplicate samples of ARISA from the same DNA extraction (A1, A2 & A3) demonstrates reproducibility. PCR was conducted using HotstarTaq Plus master mix with ITSC primer set. The presence and size of peaks is highly similar amongst the three repeats. Each peak is considered to represent a bacterial OTU.

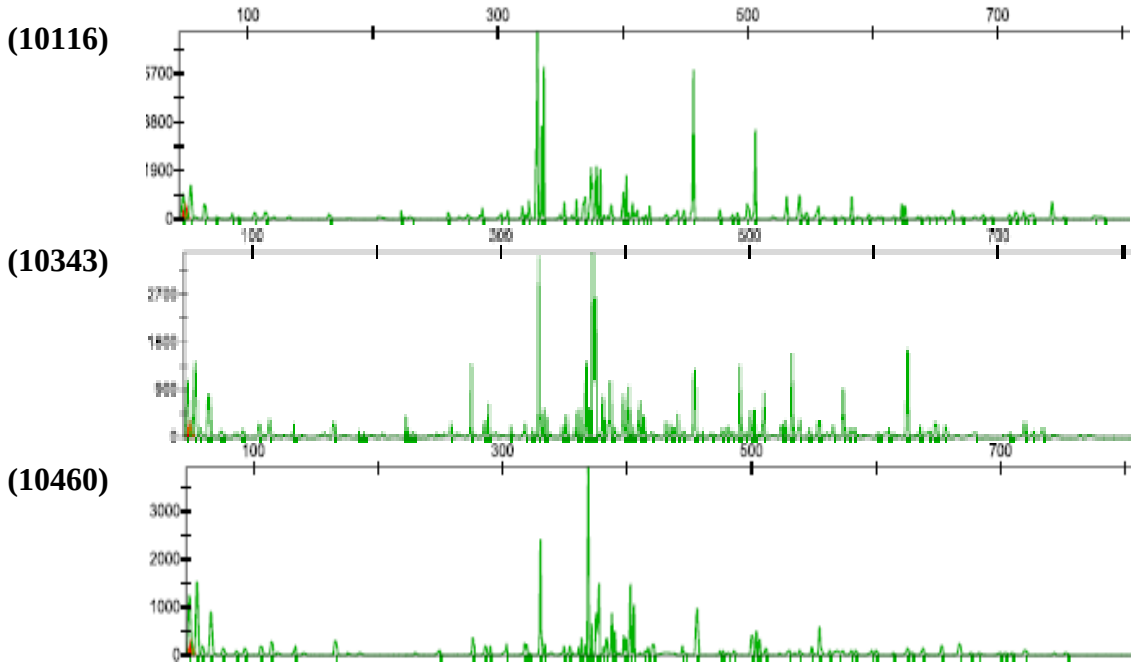


Figure 2.5 ARISA can differentiate faecal microbial communities in different individual sheep. Each electropherogram (Tag 10116, 10343 & 10460) represents a different sheep. Differences in peak location and size are indicative of differences in bacterial community structure.

2.3.3 Selection of the Best Combination of Master Mix, Primers and DNA Extraction

The number of peaks generated by the capillary sequencing, the range of the peak sizes, and the reproducibility of the triplicates were used to determine the best combination of DNA extraction technique, master mix and primers (Table 2.3). Using HotStarTaq® or GoTaq® with ITSC primers generated comparable size and total number of peaks; in contrast, the 1552 primers gave a reduced number of peaks. Hence, ITSC primers were chosen for subsequent PCR reactions. Sporadic contamination issues (band in no template control) were detected with GoTaq Green master mix and, therefore, it was decided to exclude GoTaq Green master mix from further use in this study.

Table 2.3 Range of peak numbers and sizes in the ARISA profiles of two different master mixes and primers with two different DNA extraction techniques.

Master mix	Primer	DNA extraction tool	No of replicates	No. of peaks (mean±SD)	Range of peak size (bp)	DNA extraction cost/sample (Aus $)
HotStar Taq	ITSC	Qiagen	6	50.8±3.5	265-827	10.00
		PowerSoil	3	50	260-826	8.35
	1552	Qiagen	6	31±3.8	317-737	
		PowerSoil	3	47	354-1015	
GoTaq Green	ITSC	Qiagen	6	52.5±4.5	230-770	
		PowerSoil	3	54.3±2.5	230-684	
	1552	Qiagen	6	43.1±2.1	318-816	
		PowerSoil	3	49.3±0.6	318-1016	

SD: standard deviation

A further consideration of using Qiagen extraction kits with HotStarTaq® was the cost of DNA extraction/sample ($10) as it was envisaged a large number of samples would be processed. Using a modified method by incorporating lower cost extraction columns (Econospin) in the extraction method lowered the cost ($10 to $3.60 per extraction sample). The combination of ITSC primers with HotStarTaq® master mix using template DNA extracted with Qiagen reagents and the EconoSpin column produced >60 peaks over a broad range of sizes (Table 2.4). Moreover, using the ITSC-HotStarTaq®-Qiagen/EconoSpin combination yielded highly consistent triplicates. This combination also detected an acceptable range of peak sizes per sample. Hence, the Qiagen and the Qiagen with Econospin extraction techniques were considered comparable. Future studies in this thesis, therefore, used the combination of ITSC primers with HotStarTaq® master mix using template DNA extracted with Qiagen reagents and the EconoSpin column.

Table 2.4 Range of peak numbers and sizes in the ARISA profiles of Qiagen DNA extraction techniques.

Master mix	Primer	DNA extraction tool	No of replicates	No. of peaks (mean±SD)	Range of peak size (bp)	DNA extraction cost/sample (Aus $)
HotStar Taq	ITSC	Qiagen	6	50.8±3.5	265-827	10.00
		Qiagen with EconoSpin	6	63±1	270-975	3.60

SD: standard deviation

2.3.4 Preliminary ARISA Trial to Determine Reproducibility and Differential Capacity

Faecal microbial composition was compared in three sheep, using samples collected morning and afternoon (see Figure 2.2). Using PERMANOVA, there was no evidence to suggest that the gut microbial composition differed between morning and afternoon samples within individual sheep (F= 1.3033, P(perm) = 0.318; Table 2.5); nor was there evidence of changing faecal microbiota in individual sheep across the 5 days (F= 1.7618, P(perm) = 0.165; Table 2.6). PERMANOVA also revealed that the gut microbial composition of individual sheep were significantly different (F= 8.5574, P(perm) = 0.002; table 2.4), which was illustrated using a non-metric multi-dimensional scaling (nMDS) plot (Figure 2.6). This finding was further confirmed by two way ANOVA analysis (p= 0.004) of Shannon diversity *(H)* index, with no significant differences observed across days (Table 2.5).

Table 2.5 PERMANOVA of faecal microbiota samples based on ARISA.

Source of Variation	DF	SS	Pseudo-F	P(perm)	Unique perms
Tag	2	15313	8.5574	**0.002****	998
Day	2	3152.7	1.7618	0.165	999
Time	1	1166.2	1.3033	0.318	999

PERMANOVA of faecal microbial composition data to generate a permutated F statistic (*F*) and permutated p-value (*P*) with calculated degrees of freedom (DF) and sums of squares (SS) noted. **P ≤ 0.01. Samples were collected from three sheep, twice a day (am and pm) on three sample days (days 1, 3, 5).

Table 2.6 Two way ANOVA of Shannon diversity *(H)* index of faecal microbiota determined by ARISA.

Source of variation	DF	SS	F (DFn, DFd)	P value
Tag	2	0.2193	F (2, 8) = 11.72	**0.004****
Day	2	0.00738	F (2, 8) = 0.3946	0.686

Two way ANOVA of Shannon diversity *(H)* data to generate a p-value (*P*) with calculated degrees of freedom (DF) and sums of squares (SS) noted. **P ≤ 0.01. Samples were collected from three sheep, twice a day (am and pm) on three sample days (days 1, 3, 5).

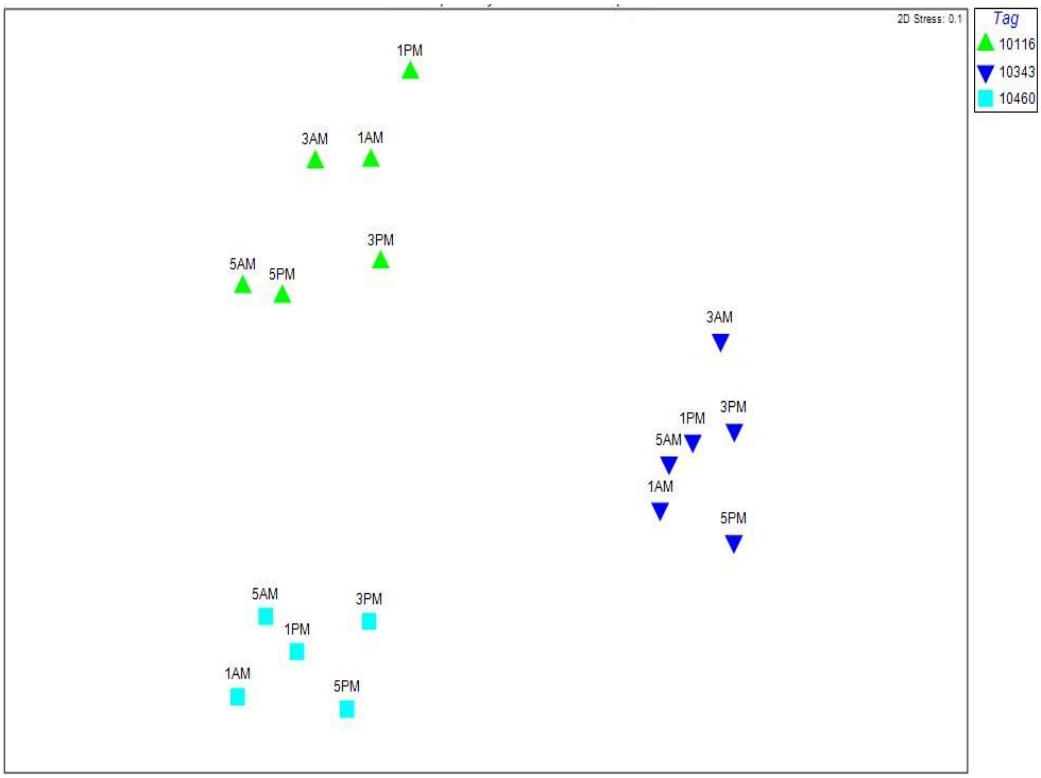


Figure 2.6 An nMDS plot representing the faecal microbioal composition of three individual sheep (tag numbers 10116, 10343, 10460) as determined by ARISA. The number indicates the day of sample collection, while am and pm indicate morning & afternoon sample collection time, respectively. Each sample is represented by a single point. Sheep (tag) 10460 morning sample for day three was not processed as it was not collected.

2.4 Discussion

The advent and widespread application of molecular approaches has greatly increased our potential to understand complex microbial communities [176]. In particular, the recent developments in advanced molecular techniques such as genomics and sequence technologies have initiated a new era of microbial ecology, leading to studies that investigate the entire microbial community. Despite these developments, there is still a need to analyse samples quickly and cheaply. This preliminary study attempted to demonstrate the applicability of ARISA in the analysis of community composition of faecal bacteria in sheep.

DNA of sufficient yield and quality is the crucial starting material for ARISA, as the more DNA that is recovered, the more likely rare species will be represented and thus detected. Faecal microbiota is highly diverse and not all DNA extraction methods work equally well for different microbial groups. Several studies have shown that the DNA extraction method used has an impact on the microbial community representation in samples from different habitats [177-181]. Several factors should be taken into consideration with regards to DNA extraction methods such as: the quality required for downstream analysis, overall variability of the method (between different researchers, and by the same researcher from one sample to the next); the availability of equipment, and reagents; contamination by PCR inhibitors; and primers to be used [177, 180]. In this study, it was noted that the Qiagen with EconoSpin column kit, which was the most economical method, yielded less genomic DNA compared to the other two techniques (Appendix 2, Table A2.1). However, a higher number of peaks was obtained in the ARISA electropherograms by using the DNA extracted using Qiagen reagents with the EconoSpin column compared to the PowerSoil and Qiagen extraction kits. This trend was

observed irrespective of the primer set and master mix being used. This suggested the use of Qiagen reagent with EconoSpin columns was satisfactory; whether the lower yield reflected better quality DNA, perhaps by having less PCR inhibitors or un-fragmented DNA, was not determined.

An important step in standardizing and optimizing PCR-based methods such as ARISA for the study of faecal microbial populations is to choose an appropriate primer set [156]. The selection of primers for PCR can significantly influence the data generated from ARISA. For example, Maggi and Breitschwerdt outlined how primer selection affects the accuracy in detecting *Bartonella sp* using ARISA [182]. Another study demonstrated that using two different primer sets results in differences in bacterial profiles [169]. This study initially investigated the use of four sets of primers, and based on PCR amplification narrowed our selection to two pairs of primers. Neither of these two primer sets previously had been used for ARISA on faecal samples of sheep; although both had been used in the analysis of microbial populations in faeces from other animals. A consistently greater number of peaks was observed in the ARISA electropherograms when using the ITSC primers compared to the 1552/132 primers, irrespective of the master mix used. This result was in agreement with the findings of Cardinale *et al.* [156] who compared three different primer sets to analyse six different environmental samples. Higher number of peaks in the ARISA electropherograms of environmental samples was achieved by using the ITSC primer set, thus suggesting a more informative power (better representation of the OTUs) of this primer set than the others [156]. The DNA extracted from the PowerSoil kit, using the 1552/132 primer set resulted in greater peak sizes with larger spacer size irrespective of the master mix used. These findings are in contrast with the results of Cardinale *et al.* [156] who obtained peaks with larger spacer size using the ITSC primer set rather than the 1552/132 primer set when analysing different

environmental samples. It is hypothesized that these differences were due to the preferential amplification of the shorter templates, as suggested by Fisher and Triplett [106] and Cardinale *et al.* [156], rather than the biological absence of species possessing longer spacers.

Premixed master mixes were chosen in this study as they provide all components in a single solution (other than template and primers). The use of premixed master mix also increases throughput, and is likely to improve reproducibility through decreasing pipetting errors. It also reduces pipetting steps and the potential risk of contamination. In molecular analysis of bacterial communities, contamination with any bacterial DNA would negatively impact on the veracity of the results, thus contamination must be avoided. Though caution was exercised, sporadic contamination was seen when GoTaq Green master mix was used. Although both master mixes resulted in similar ARISA electropherograms for a given primer set (suggesting a limited influence of the master mix or different Taq DNA polymerases in ARISA efficiency), the sporadic contamination seen with GoTaq Green master mix excluded its use in subsequent studies.

Statistical analysis of the ARISA results demonstrated that ARISA was able to detect differences in the faecal microbial composition of the three individual sheep used in this trial. Encouragingly, for repeat samples from the same individual sheep, there was no significant difference in the microbial composition of samples. This was true for samples collected on the same day (morning and afternoon samples) and over a 5 day period. These results are consistent with the previous ARISA findings of large sample sized studies in ruminants [164, 183]. A study on cow rumen samples revealed high similarity (93-95%) in rumen bacterial community within an individual cow across different sampling times and lower similarity of 85% between the different cows sampled on a

controlled diet [183]. Another study showed that the ruminal bacterial communities were more similar within the same cows across the feeding cycle than between the different cows sampled [164]. Therefore, this study suggested that ARISA from faecal samples of sheep will be useful as means of identifying groups of animals that differ substantially in microbial community composition or in grouping animals having similar community profiles from more refined studies.

In conclusion, this preliminary study has enabled the optimisation of ARISA in the laboratory, through the determination of a suitable combination of DNA extraction, primers and polymerase. This preliminary study has also demonstrated the applicability of ARISA in the analysis of community composition of faecal bacteria in sheep through the generation of reproducible results, while also differentiating the gut microbial community composition of individual sheep. ARISA may be applicable in industrial and agricultural settings, as it is cost-effective and has lesser requirements for advanced bioinformatic analysis than next-generation sequencing based approaches.

Chapter 3

The Composition and Stability of the Faecal Microbiota of Sheep

3.1 Introduction

The digestive tract of an animal is colonised by a wide range of microorganisms that have co-evolved with their host. These microorganisms, often referred to as the gut microbiota, play an integral role in host nutrition and health. In recent times, the knowledge and understanding of gut microbial community composition has increased tremendously; particularly in humans, but to a lesser degree in other animals of economic importance.

In humans and other mammals it is generally considered that the gut microbiota develops through infancy [45, 46], with a succession of microbial populations occurring until the establishment of a climax community [54]. The time it takes for the intestinal microbiota to reach its climax state appears to vary with animals [154, 184, 185]. Even after the climax community has been established, microbial community composition within an individual can and do change, due to the influence of certain factors including antimicrobial agents, exposure to other organisms, diet [186], age [61, 187] and host factors (host species and breed) [188, 189].

Although general ecological trends are observed in the development and maintenance of microbial communities; within the population of a given animal species, gut microbial community composition and structure varies considerably across individuals. Nonetheless, there are typical microbial taxa that are present among all, or a large proportion, of individuals. These taxa are defined as the core microbiome (or core microbiota), even though their abundance across individuals of a given host species varies remarkably [161].

Unsurprisingly, there are differences in microbial composition of the gut of different animals (e.g. horse, rabbit, pig) [61, 190, 191]. The composition of the gastrointestinal

microbiota is likely to contribute to overall production performance and/or health of economically important animals through feed and drug metabolism, immune system development and pathogen colonization resistance [89, 192-195]; thus an understanding of the gut microbial composition amongst these economically important species is required. Of particular interest are ruminants, which depend on their gut microbiota to produce enzymes for the digestion of cellulose and other plant polysaccharides and the synthesis of certain vitamins [7, 89]. Although there are key bacterial taxa that we commonly find associated with ruminants, there are differences in overall community composition. Studies have revealed differences in rumen microbial diversity of different breeds of cows [188] and goats [196]. Moreover, recent studies suggest that cows fed the same diets can have notable differences in rumen bacterial community composition; whereas an individual's microbiota is more similar over time than it is to other individuals [164, 183]. These findings are in keeping with the 'core microbiome' and the theory of a climax community described in humans and experimental animals.

Currently, research on gut microbiota of sheep is limited, with only one study conducted to date on sheep faecal samples using next generation sequencing techniques [66]. Therefore, a detailed study on faecal samples of the "normal" gut microbiota of Merino sheep was carried out to further our understanding of the sheep gut microbiota. Specifically, 1) to determine the predominant bacterial species present in the sheep faeces; and, 2) to determine the similarity and stability of the faecal microbiota, both within individuals and across a small cohort of sheep, over short (2 – 4 weeks) and longer-term (~6 month) intervals.

3.2 Methods

3.2.1 Animal Handling and Sampling

This study was conducted on 2 year old Merino wethers. Details of animal handing and sampling are addressed in Section 2.2.1. To investigate the stability of the gut microbiota over time, both ARISA and 16S rRNA sequencing was conducted. Initially, two sheep were selected to enable the long-term stability to be investigated. In these two sheep, 16S rRNA sequencing was conducted on faecal samples collected at the commencement of the study (week 1) and 2 weeks later (week 3). Approximately 5 months later, the main component of the study commenced: a detailed investigation to monitor stability over a relatively short period (4 weeks). All 28 sheep had samples collected once a week (every Monday) over four consecutive weeks (weeks 22 – 25) and analysis was conducted by ARISA. For a subset of sheep (n=11) 16S rRNA sequencing was conducted over two consecutive weeks (weeks 24 and 25). Refer to Table 3.1 for sampling details.

Following collection, faecal samples were aseptically bagged, labelled and stored at -80°C, as described in Section 2.2.1.

Table 3.1 Experimental sampling plan to investigate the community structure, composition and stability of the sheep gut using faecal samples.

Technique	No of sheep	Week 1	Week 3	Week 22	Week 23	Week 24	Week 25	Number of samples analysed
Long term stability of gut microbial composition in selected sheep (6 months)								
16S sequencing	2*	✓	✓	21 weeks		✓	✓	8
Short term stability of gut microbial composition in selected sheep (4 weeks)								
ARISA	28			✓	✓	✓	✓	112
16S sequencing	11					✓	✓	18

***4** samples for week 24 and 25 (a sample from each of the 2 sheep for each time point) was included in the 16S-sequencing for the short term stability study. Thus, 22 samples were analysed as part of the 16S short term stability study.

3.2.2 DNA Extraction, Quantification and Homogenization

Genomic DNA was extracted from frozen faecal samples using the commercially available QIAamp DNA Stool Kit reagents with the EconoSpin column (Epoch Life Science, Inc, Missouri City, USA) following the QIAamp DNA Stool Kit protocol (see Section 2.2.4 for details).

3.2.3 ARISA

For each DNA sample, PCR was conducted in duplicate. PCR amplification of the ITS region was performed using the previously described primer set ITSF/ITSReub [156, 165-167] with HotStarTaq® Plus master mix. The primer set ITSF (GTCGTAACAAGGTAGCCGTA) and ITSReub (GCCAAGGCATCCACC) are

complementary to positions 1423 and 1443 of the 16S rDNA and 38 and 23 of the 23S rDNA of *Escherichia coli*, respectively. Refer to Section 2.2.5 for details of the PCR conditions.

Fragment separation and ARISA analysis were conducted as previously outlined (Section 2.2.6).

3.2.4 16S rRNA Sequencing

PCR targeting the V1_V3 region of the 16S rRNA gene was conducted using primers 27F [AGAGTTTGATCMTGGCTCAG] and 519R [GWATTACCGCGGCKGCTG]. These primers were selected based on the available literature [197, 198]. Important selection criteria in this study were that the primers used had adequate resolution to analyse to low taxa (genus/species) by sequencing 600bp of the selected variable region, while obtaining consistent, highly quality data. Amplicon sequencing was performed at AGRF on the MiSeq platform utilising Illumina's paired end chemistry. All 26 samples that had 16S sequencing conducted were included in the same sequencing run.

3.2.5 Bioinformatics Methods

Paired-end reads were assembled by aligning the forward and reverse reads using PEAR (version 0.9.5) [199]. Primers were trimmed using Seqtk (version 1.0) then sequences were quality filtered with a maximum expected error threshold of 0.5, full length duplicate sequences were removed, and sequences sorted by abundance using USEARCH

[200, 201]. Singletons or unique reads in the data set were discarded. Sequences were clustered and subsequently chimera filtered using both de novo and reference based methods ("rdp_gold" database). To obtain the number of reads of each OTU, reads were mapped back to OTUs with a minimum identity of 97%. Taxonomy was assigned using Greengenes database [202] (Version 13_8, Aug 2013) by QIIME [203]. A rarefied (10,000 sequences per sample) biom table was imported to Calypso [204] for further downstream analysis. For 16S sequencing data, all statistical analyses and graphs were produced in Calypso.

3.2.6 Ecological and Statistical Analyses

ARISA abundance data obtained from interactive binner, and square root transformed abundance data from 16S sequencing, were used to generate Bray-Curtis, and weighted and unweighted UniFrac distance matrix. Similarities between sample groups were visualised using nMDS, as described in Section 2.2.7 and principal coordinates analysis (PCoA) plot. To test for differences in composition of the faecal microbiota between sheep and over time, ANOSIM was performed. ANOSIM produces a statistic, *R* (0 to +1), which indicates the magnitude of difference among groups: an *R* of 1 indicates that the communities completely differ among defined groups, and an *R* of 0 indicates no separation among groups. The statistical significance of *R* was also tested. All statistical tests for ARISA data were performed using the software PRIMER-E v7 [175] whereas Calypso was used for 16S rRNA sequencing data. For ARISA data, species accumulation plots and different diversity indices - namely total species/OTUs (*S*), species richness (Margalef's index, *d),* Shannon diversity *(H)* and evenness *(J)* - were calculated in PRIMER-E v7. All diversity indices for 16S sequencing data were computed in Calypso.

Significant differences in microbial diversity and abundances were tested using two way ANOVA followed by Tukey's multiple comparisons test or paired sample t-test with graphs in GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California USA; www.graphpad.com. All other graphs were generated in Microsoft Excel and Calypso.

3.3 Results

3.3.1 Community Structure and Composition of the Sheep Gut Microbiota

3.3.1.1 Community structure determined by ARISA

Using ARISA, 289 OTUs were identified from 28 sheep sampled weekly over 4 weeks. On average 43.5 ± 11.8 (mean ± SD) OTUs were detected per individual faecal sample, ranging from 24 to 68 OTUs per sample.

To investigate the distribution of the different OTUs across individual sheep samples over 4 weeks, and to determine whether any OTUs were present in most samples (core microbiota), the OTUs were binned according to their percentage of occurrence across sheep. Most of the OTUs (59.5%; 172 OTUs) were present in ≤10% of all samples in this study (n=112). A small proportion of the total OTUs (4.8%) were present in over 60% of the 112 samples: these 14 OTUs were expected to be part of the 'core' microbiota of sheep. The average relative abundances of these core microbes across the sheep samples over 4 weeks was determined (Figure 3.1), showing a variable pattern of abundances. Collectively, these 14 OTUs compromise 41.8% of the total detected gut microbial population; however, of these 14 OTUs only two OTUs (329.8 and 455.8) made up more than 20% of total bacteria present.

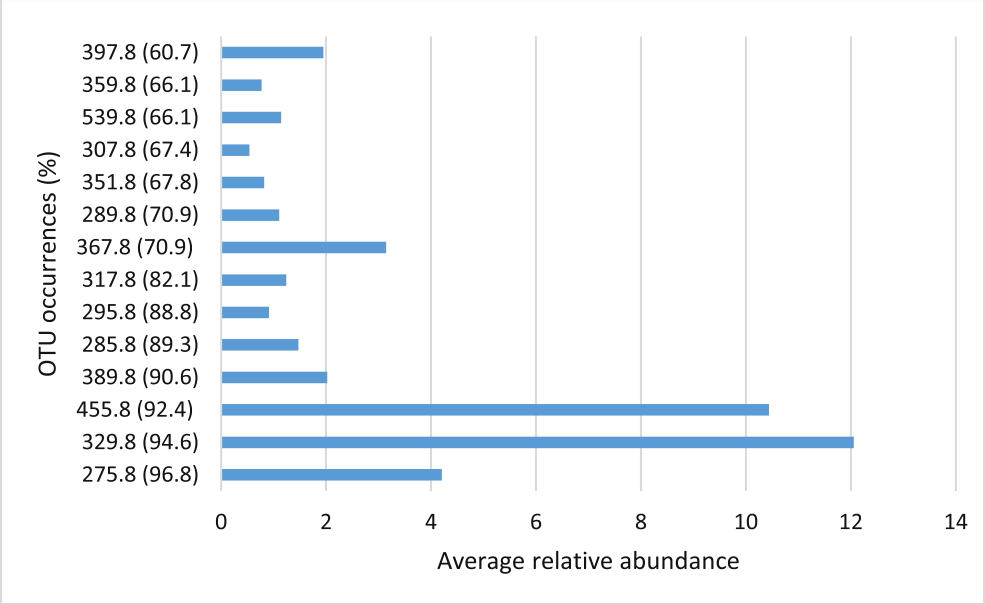


Figure 3.1 Average relative abundance of the 14 most prevalent OTUs (those detected in >60% of samples) detected by ARISA over 4 weeks of sampling. The X-axis shows the average relative abundance (%).The Y axis represents the OTUs with their frequency of occurrence (%).

Accumulation plots were used to assess whether the coverage depth of the ARISA OTUs was sufficient (Appendix 3, Figure A3.1). An asymptotic curve is indicative that sufficient sampling size has been reached, thus most of the OTUs are likely to be detected. As can be seen in Figure A3.1 (Appendix 3), 90% of all OTUs were detectable when 14 sheep were analysed (210 of 234 different OTUs). This suggests that this experiment on 28 sheep was of a sufficient sample size to detect an acceptable proportion of all OTUs detectable by ARISA.

3.3.1.2 Community composition determined by 16S rRNA gene sequencing

A total of 2,646,581 (120,299 ± 29,379; mean±SD) sequences were obtained from the hyper variable V1-V3 region of the bacterial 16S rRNA gene. After quality control (QC) and chimera removal, samples contained a total 1,989,361 sequences; an average of 90,426 ± 21,762 (mean±SD) sequences per sample. This resulted in a total of 8,839 OTUs at ≥97% sequence similarity. Rarefaction analysis was conducted based on the OTU richness values (Appendix 3, Figure A3.3), suggesting that sequencing depth for this experiment was adequate. Table 3.2 shows the number of detected taxa, and Figure A3.2 (Appendix 3) shows the dominant taxa with corresponding proportion of OTUs in each group.

Table 3.2 Number of taxa identified from faecal samples of sheep (n=11) using 16S rRNA gene sequencing.

Taxa	Number
Phylum	27
Class	47
Order	82
Family	136
Genus	206
Species	215
OTU	8859

Among the 27 phyla detected, the community was dominated by two phyla (Figure 3.2): Firmicutes (53.55±4.32% of the total abundance) and Bacteroidetes (27.43±2.77% of the total abundance).

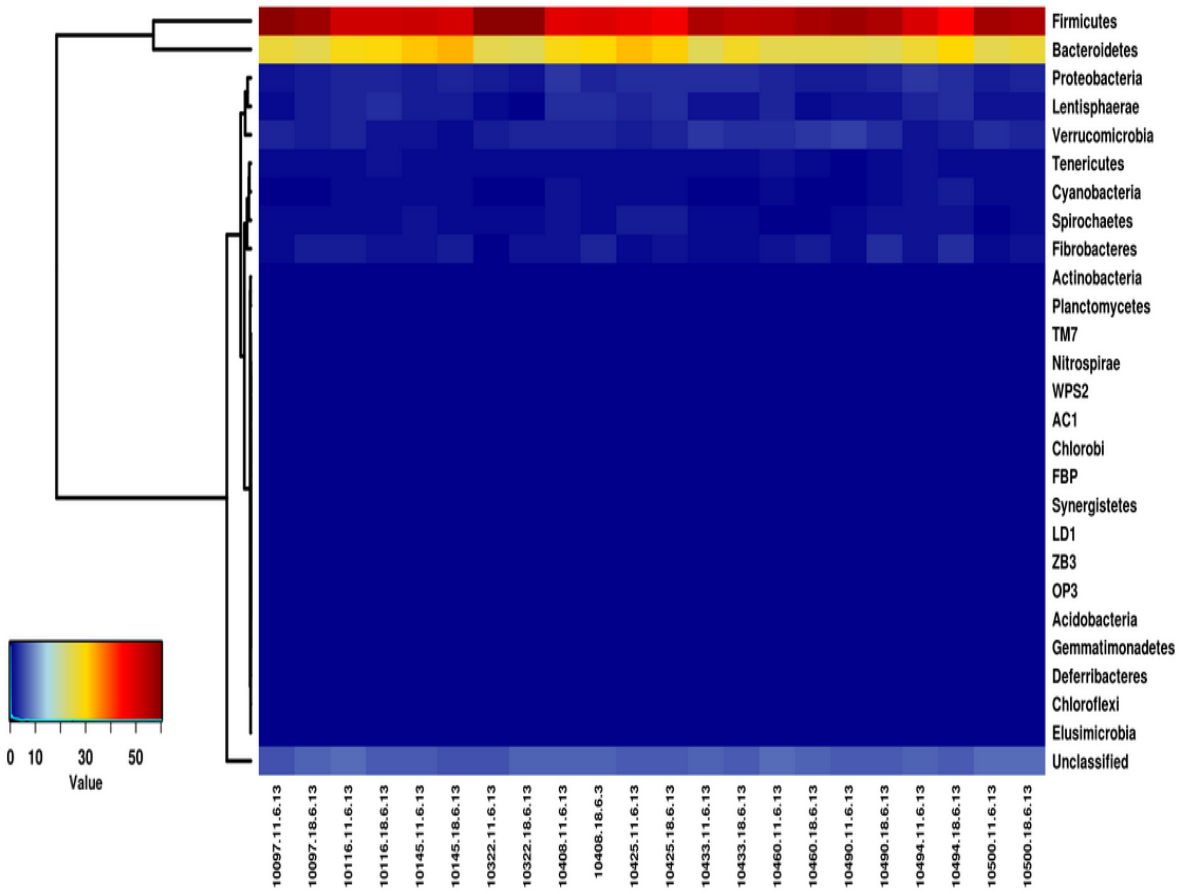


Figure 3.2 HeatMap+ illustrating the average relative abundances (%) of identified phyla using 16S rRNA sequencing.

3.3.2 Similarity and Stability of Faecal Microbiota Over Time

3.3.2.1 Community structure over 4 week period determined by ARISA

Temporal similarity of faecal microbial composition was investigated by ARISA over a period of 4 weeks for 28 sheep. ANOSIM analysis based on the Bray-Curtis similarity matrix was conducted on square root transformed data. Results showed very little separation (ANOSIM: R=0.074, P = 0.01) in microbial composition between the four weekly samples. This finding was supported by two way ANOVA analysis with Tukey's multiple comparison test of different diversity indices: total species/OTUs (*S*); species richness (Margalef's index, *d),* Shannon diversity *(H)* and evenness *(J)*; with no significant differences observed between the sampling weeks (Figure 3.3). Analysis of the ten most abundant OTUs revealed four OTUs to have significantly different average relative abundances across the different weekly samples (Figure 3.4); the other six highly abundant OTUs showed no difference in relative abundance over the 4 week sampling period.

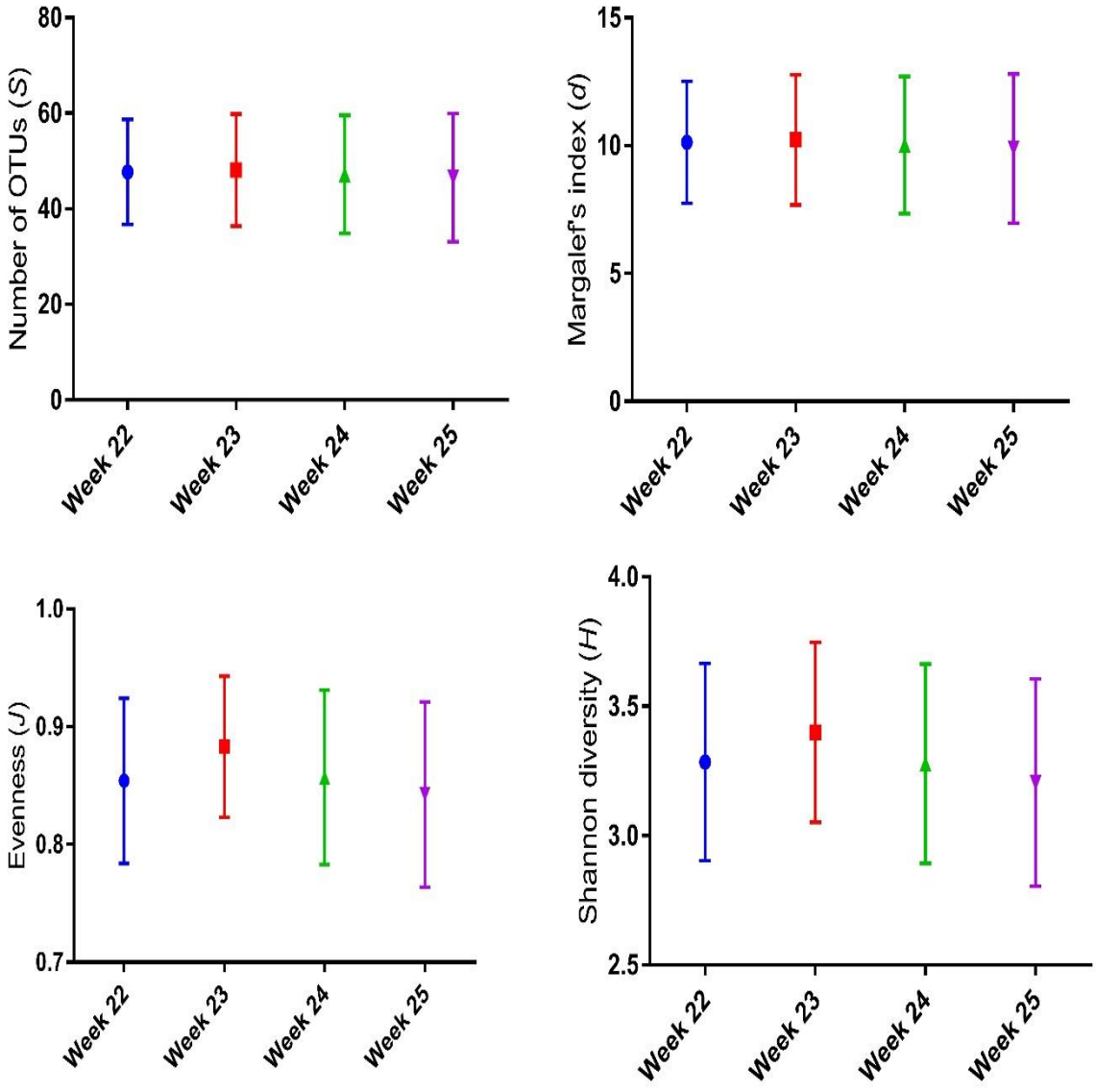


Figure 3.3 Diversity of faecal microbiota of sheep across 4 weeks of sampling by ARISA. Total OTUs (*S*), Margalef's index (*d*) for species richness, evenness (*J*) and Shannon Diversity (*H*) of week samples was calculated. Two way ANOVA with Tukey's multiple comparison test was conducted to test the significance between the weeks. No significance was recorded. Error bars represent SD.

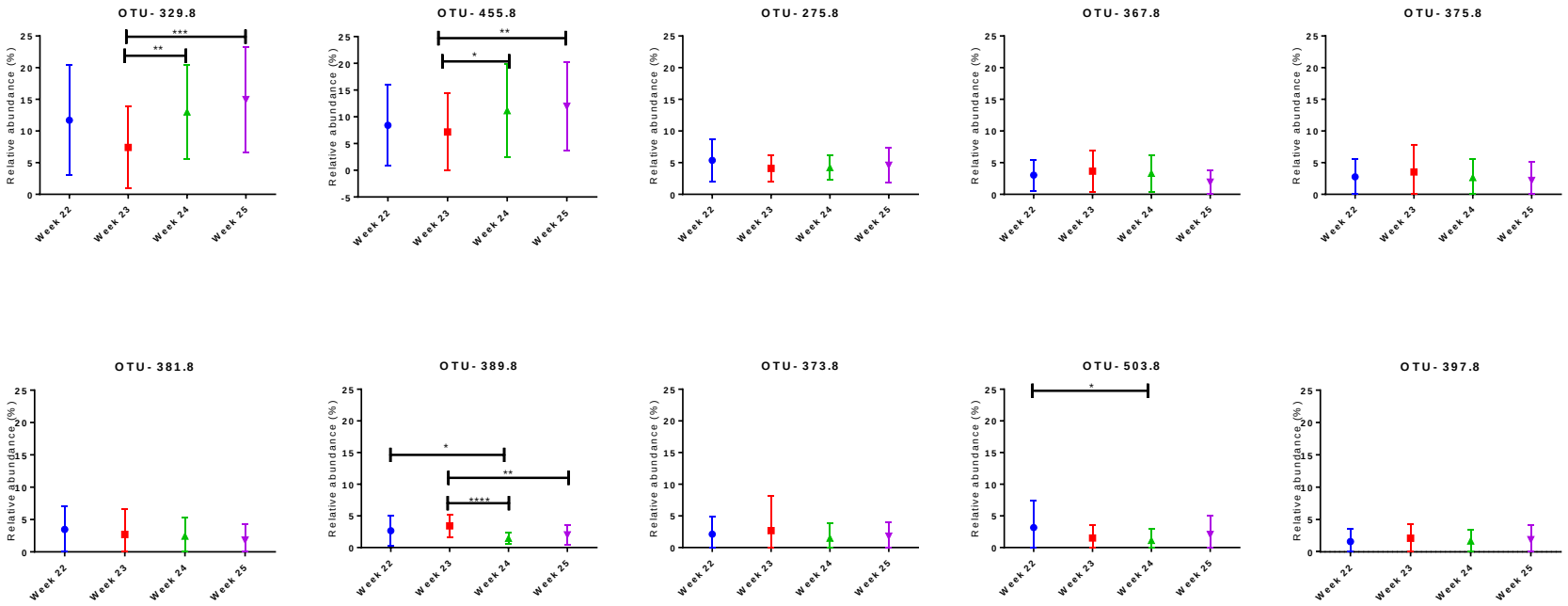


Figure 3.4 Relative abundances of dominant OTUs as detected by ARISA. Average relative abundances of top 10 abundant OTUs was calculated. Two way ANOVA with Tukey's multiple comparison test was conducted to test the significance between the weeks. Error bars represent SD. **P ≤ 0.05; **P ≤ 0.01.

3.3.2.2 Community composition over 2 week period determined by 16S rRNA sequencing

16S rRNA sequencing analysis was conducted on 11 samples (selected based on ARISA outcomes) collected over a 2 week period. Multivariate ANOSIM based on Bray-Curtis, weighted UniFrac and unweighted UniFrac distances revealed no separation of gut microbiota over the 2-week sampling period (Table 3.3). These results were further supported by the nMDS (Figure 3.5) and a PCoA plot (Appendix 3, Figure A3.4), which do not indicate distinct clustering based on the week samples (week 24 compared to week 25). Common microbial diversity indices such as Shannon, Simpson, Chao 1, richness, evenness and Fisher's alpha were also determined (Figure 3.6). With the exception of Chao1 index, no significant changes in diversity occurred between the week 24 and week 25 samples.

Table 3.3 ANOSIM analysis of microbial composition of sheep across 2 weeks using 16S rRNA sequencing. No statistical significance was observed.

Parameter	**R-value**	**P-value**
Bray-Curtis	-0.006	0.439
Weighted UniFrac	-0.07	0.92
Unweighted UniFrac	-0.079	0.967

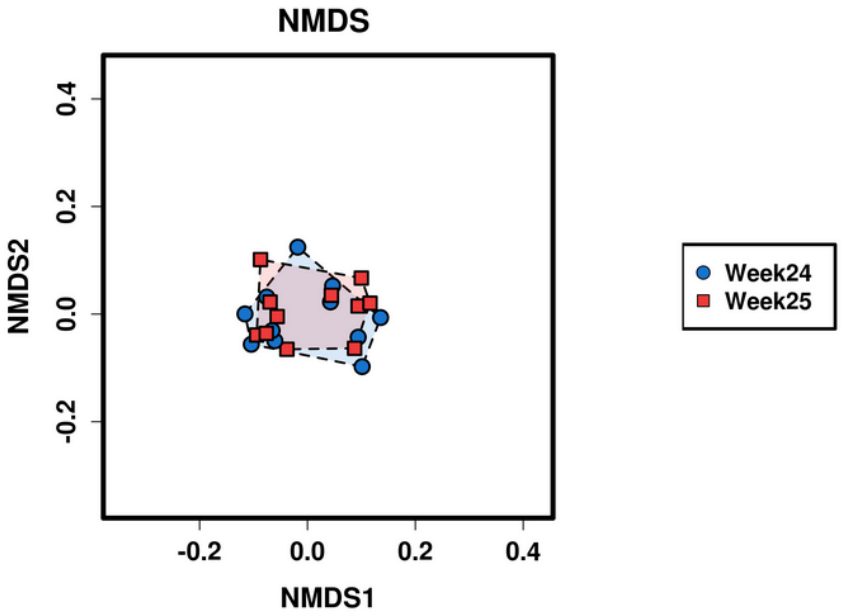


Figure 3.5 Clustering of faecal microbiota of week 24 and week 25 sheep samples. The nMDS plot is based on Bray-Curtis distance of faecal microbial composition using 16S rRNA analysis. Each symbol represents an individual sheep sample.

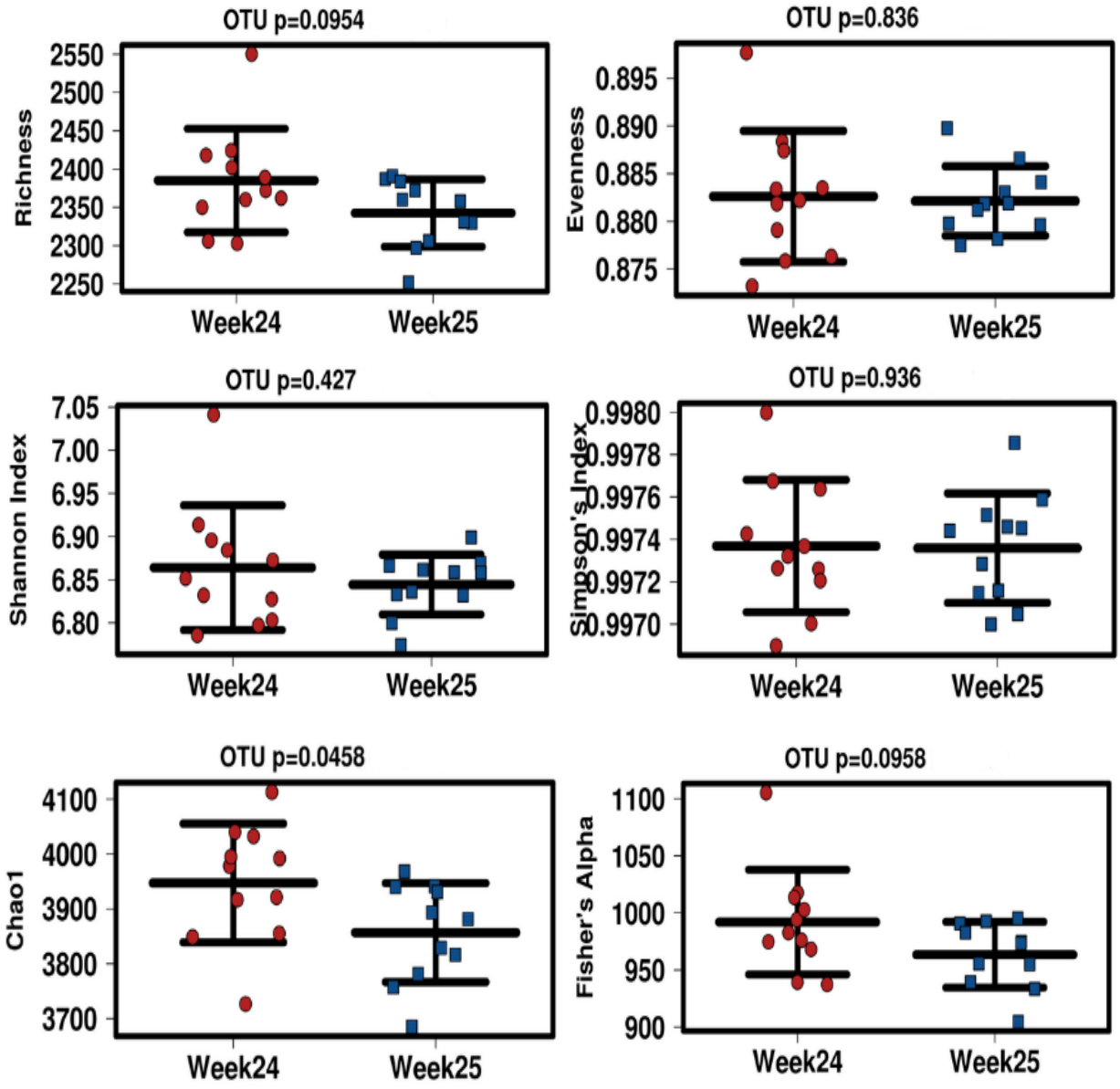


Figure 3.6 Diversity of gut microbiota of sheep across 2 weeks (n=11). OTU richness, Evenness, Chao1, Shannon, Simpson and Fisher's alpha diversity were compared for weeks 24 and 25. Unpaired t test was conducted to test for significant differences in diversity over the two sampling weeks, but no significant differences were detected.

The proportion of taxa present in both weeks 24 and 25 was determined. When comparing the 'core microbiota', that being the taxa present in at least 50% of samples, there was little change in the taxa present in week 24 relative to week 25. This observation was true at the higher taxonomic level of family and at the species level; with approximately 90% of core taxa (be it family or species) present in both weeks 24 and 25 (Figure 3.7). At the OTU level 66% core OTUs were present in both weeks 24 and 25 (Appendix 3, Figure A3.5). Refer to the Appendix 3 (Table A3.1 and Table A3.2) for the relative abundances of the 50 abundant core microbial taxa detected over these 2 weeks of sampling.

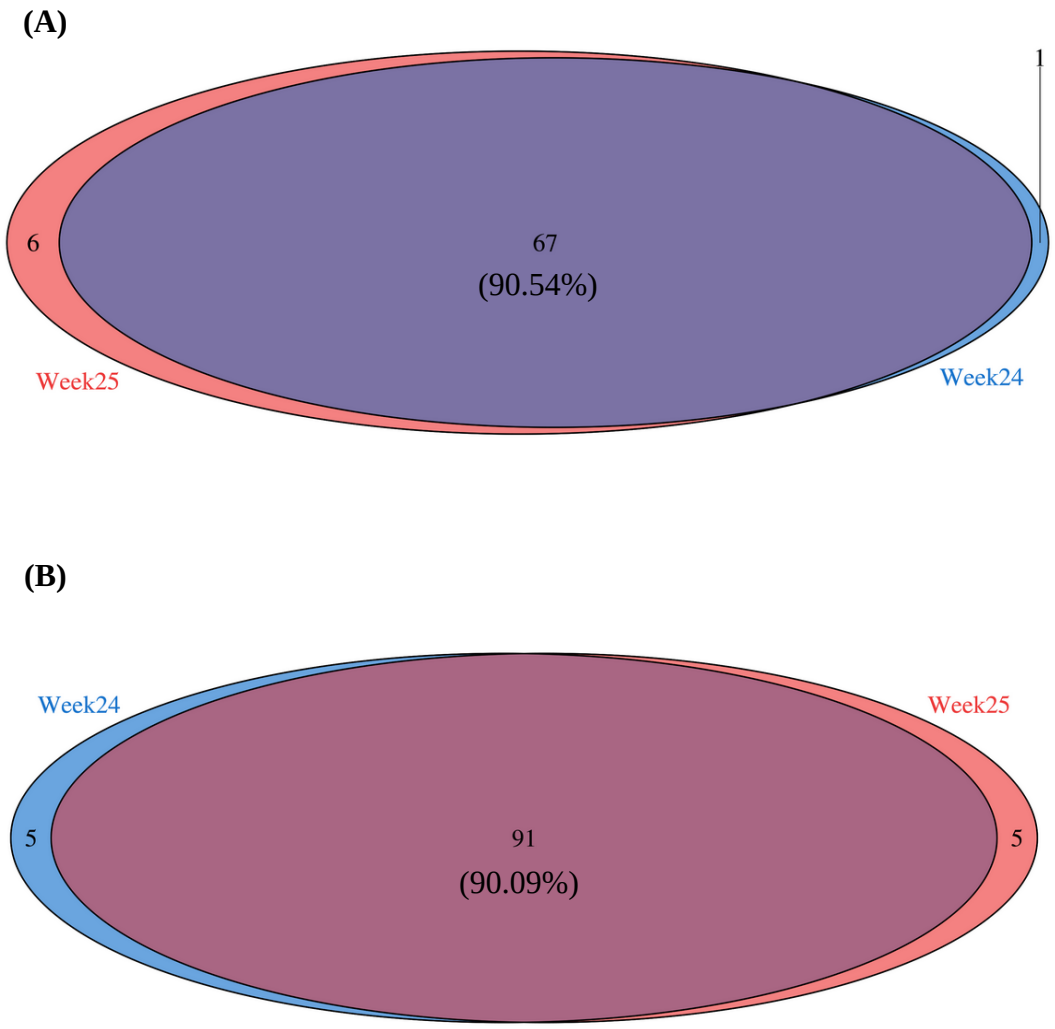


Figure 3.7 The stability of the core microbiota of sheep over a 2-week period. Venn diagrams representing the shared and unique taxa at (A) family and (B) species level in weeks 24 and 25 samples from 11 sheep. Approximately 90% of all core taxa are present in both weeks. A bacterial group was marked as present in a sample group if it was detected in at least 50% of the samples within the group.

Relative abundances of genera (Figure 3.8), families and phyla (Appendix 3, Figure A3.6) were also determined. It can be seen in Figure 3.8, where the week 24 and week 25 samples are paired for each sheep, that there was little difference in the relative abundance of the most commonly detected genera. The same trend exists at the family and phylum level (Appendix 3, Figure A3.6). Analysis was conducted to determine whether differences in relative abundance (from week 24 to week 25) were statistically significant. Of the 206 genera detected, four genera had significantly different relative abundances across the 2 weeks (Figure 3.9). Two genera had a relative abundance of over 1% on at least one week of sampling; however, the other two genera were non-dominant organisms, constituting less than 0.5% of the overall faecal microbiota. Similar outcomes were observed for phylum and family level analyses, with three of 27 phyla and four of 136 families differing in relative abundance over the consecutive weeks (Appendix 3, Figure A3.7).

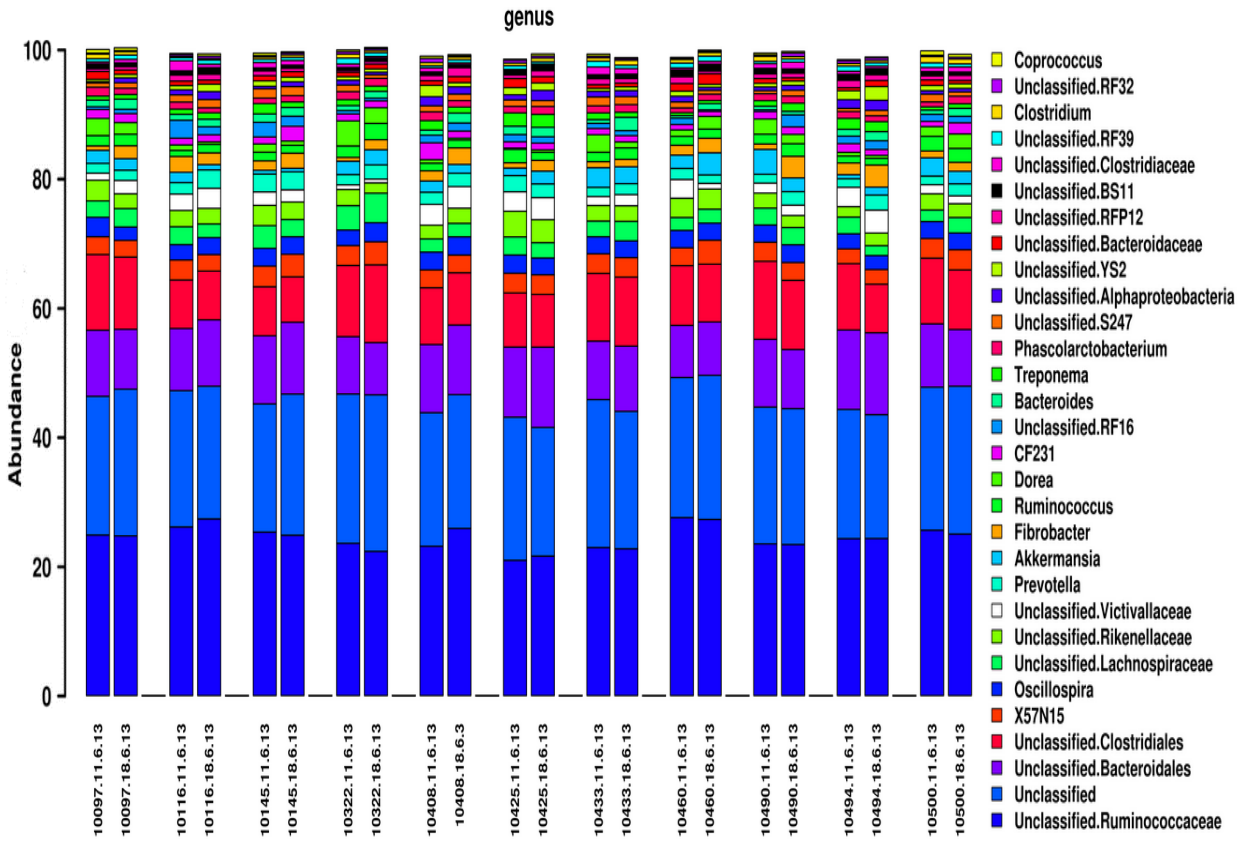


Figure 3.8 Average relative abundances (%) of the 30 most abundant genera from 11 sheep using 16S rRNA sequencing. The Y-axis shows the average relative abundances (%); X-axis shows sample identification (five digit identification code followed by the date of sample collection). Each pair of columns represents the same sheep in weeks 24 (left column of the pair) and week 25 (right column).

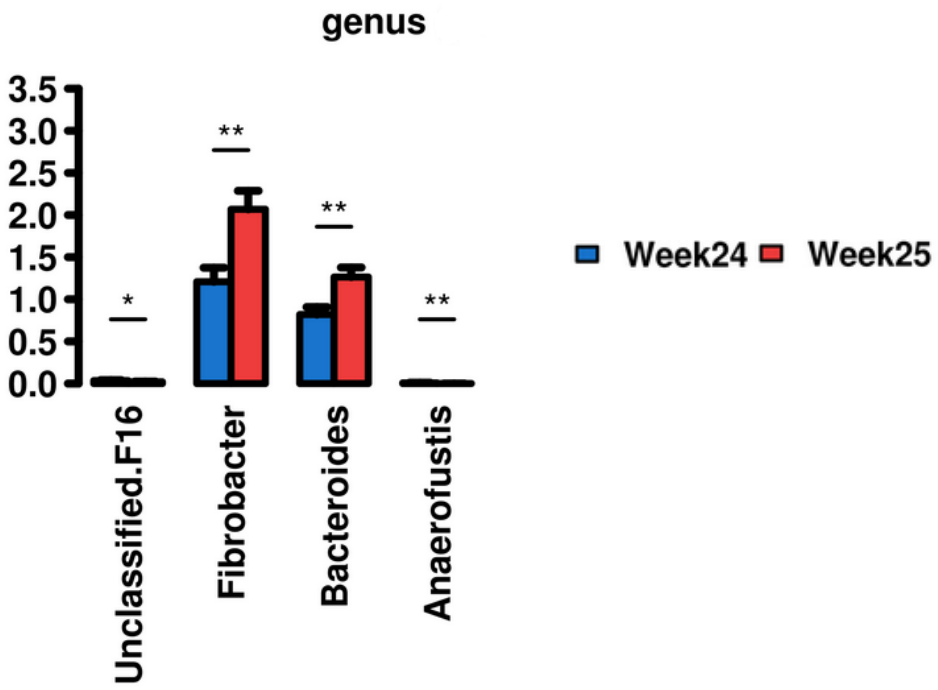


Figure 3.9 Significant differences in relative abundances of genera of faecal bacteria from 11 sheep samples collected in weeks 24 and 25 and analysed by 16S rRNA sequencing. The Y-axis shows the average relative abundances (%). Pair-wise comparisons are done by unpaired t-test; *$p \leq 0.05$, **$p \leq 0.01$.

3.3.2.3 Community composition over 6 month period determined by 16S rRNA sequencing

In order to investigate the long-term stability of gut microbiota, samples taken from two sheep in weeks 1 and 3 were analysed and compared the data generated from the same two sheep from samples taken in weeks 24 and 25. This enabled comparison of the faecal microbiota over approximately 6 months using 16S rRNA sequencing. To do so, analyses were conducted by grouping early samples (weeks 1 and 3) and late samples (weeks 24 and 25) for each sheep.

Although a difference in gut microbial composition between the two sheep was detected (Table 3.4), ANOSIM suggested no significant shift of gut microbial community

composition over the 6-month period. An nMDS plot based on Bray-Curtis distance matrix showed at the community level there was some change to microbial community composition between the time points (Figure 3.10 and Appendix 3, Figure A3.8), yet still overlap of community composition. The same nMDS plot presented for individual sheep across two sample periods (Appendix 3, Figure A3.8) rather than the population of sheep presented at two time points (Figure 3.10), reveals some small shift in faecal microbial composition for each individual sheep.

Table 3.4 ANOSIM analysis of microbial composition of sheep over a 6-month period using 16S rRNA sequencing. *$p \leq 0.05$.

Parameter	**R-value**		
	Bray-Curtis	**Weighted UniFrac**	**Unweighted UniFrac**
Weeks 1-3 vs weeks 24-25	0.062	0.198	0.115
Sheep (pair)	0.365*	0.115	0.417*

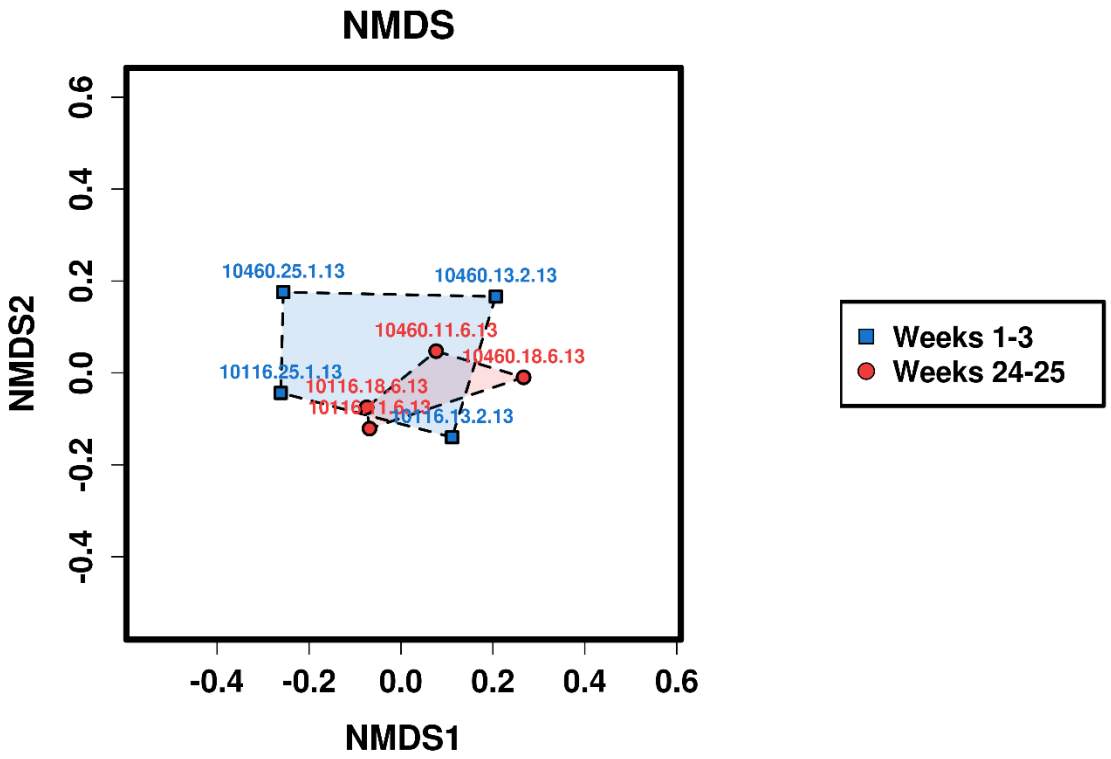


Figure 3.10 nMDS plot based on the Bray-Curtis distance matrix representing the faecal microbial composition (determined by 16S sequencing) of two sheep sampled on two consecutive weeks approximately 6 months apart. Each sample is represented by a single point. First five digits indicate the sheep tag; next digits indicate the date of sample collection.

Analysis of the core microbiota (taxa present in at least 50% of samples) revealed moderate stability at the family and species level, with more 70% core taxa were shared between week 1-3 and week 24-25 samples (Figure 3.11).

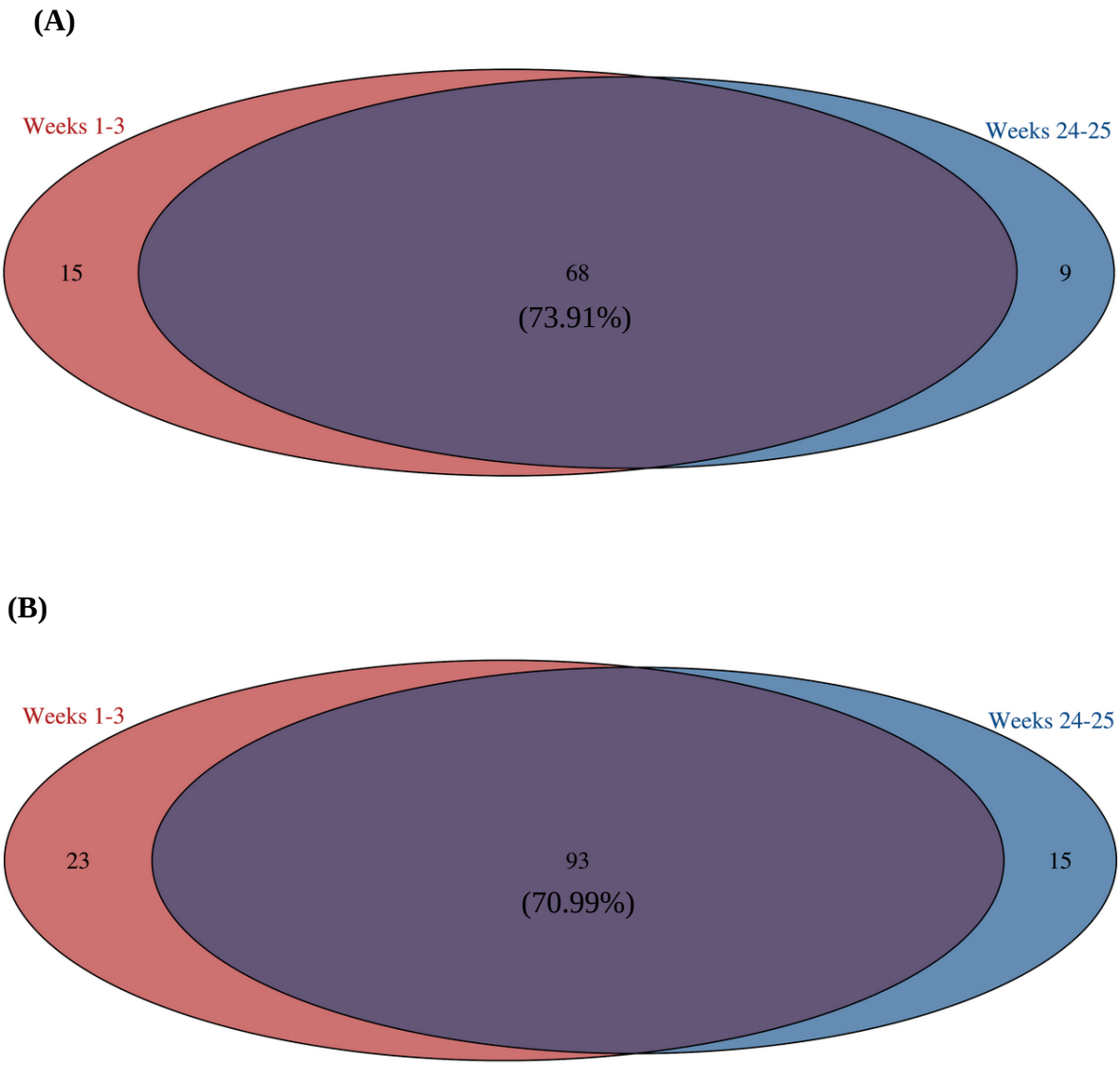


Figure 3.11 The stability of the core microbiota of sheep over a 6 months period. Venn diagrams representing the shared and unique taxa at (A) family (B) species level in weeks 1-3 and weeks 24-25 samples from 2 sheep. Approximately 70% of all core taxa are present in both sampling times. A bacterial group was marked as present in a sample group if it was detected in at least 50% of the samples within the group.

Relative abundances of genera (Figure 3.12), families and phyla (Appendix 3, Figure A3.9) were determined. For these analyses, each of the four time points for the two sheep were analysed separately (rather than pooled as early and late, as was done for

community composition analyses by ANOSIM and nMDS). The relative abundance varied across the four time points; including in the most abundant genus (unclassified Ruminococcaceae) and the third most abundant genus (unclassified Bacteriodales). It is not possible to visually ascertain differences in the less dominant taxa; however, statistical analysis revealed a significant difference in three phyla (out of 24), 10 families (out of 123) (Appendix 3, Figure A3.10) and 16 genera (out of 179), including the two abundant genera mentioned above (Figure 3.13).

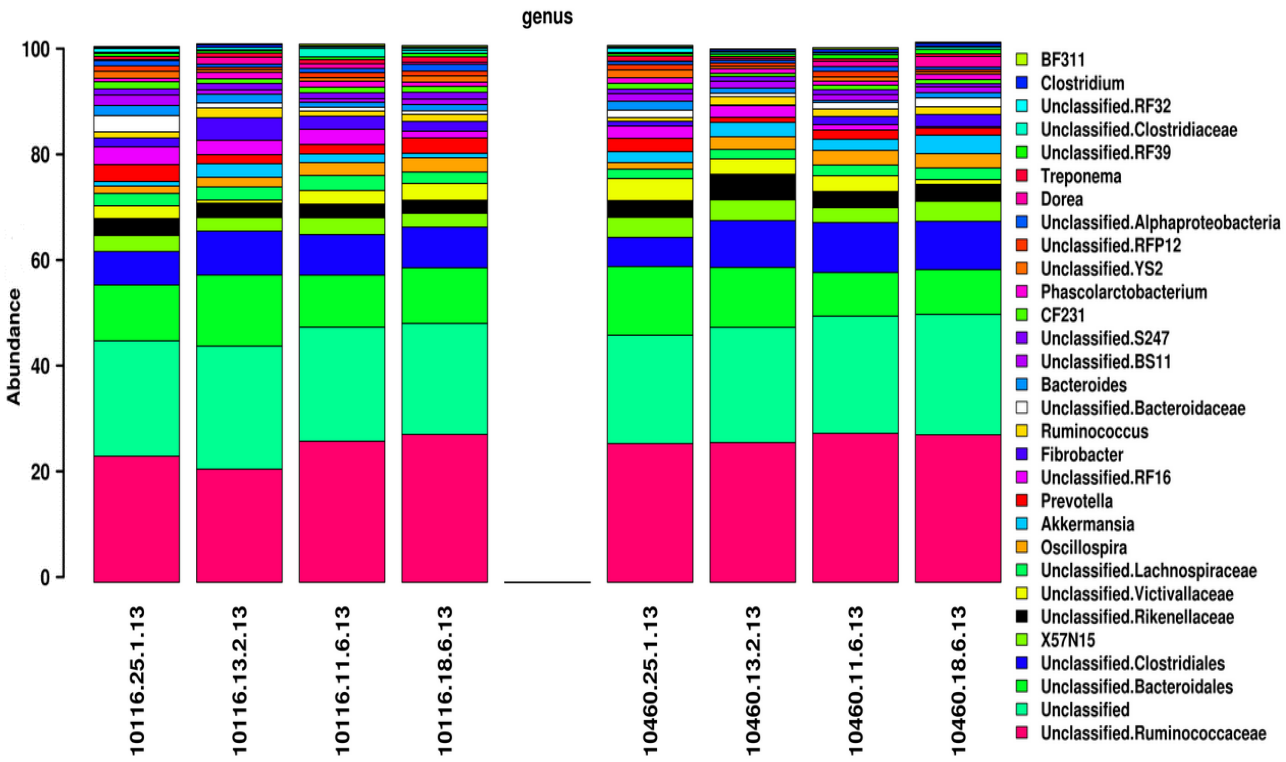


Figure 3.12 Average relative abundances (%) of 30 dominant genera from two sheep using 16S rRNA sequencing over a 6 months period. The Y-axis shows the average relative abundances (%); X-axis shows sample identification (five digit identification code followed by the date of sample collection). The first four columns represent one of the two sheep (tag 10116) in weeks 1, 3, 24 and 25 (left to right); and the second four columns represent the other sheep (tag 10460) over the same sample collection points.

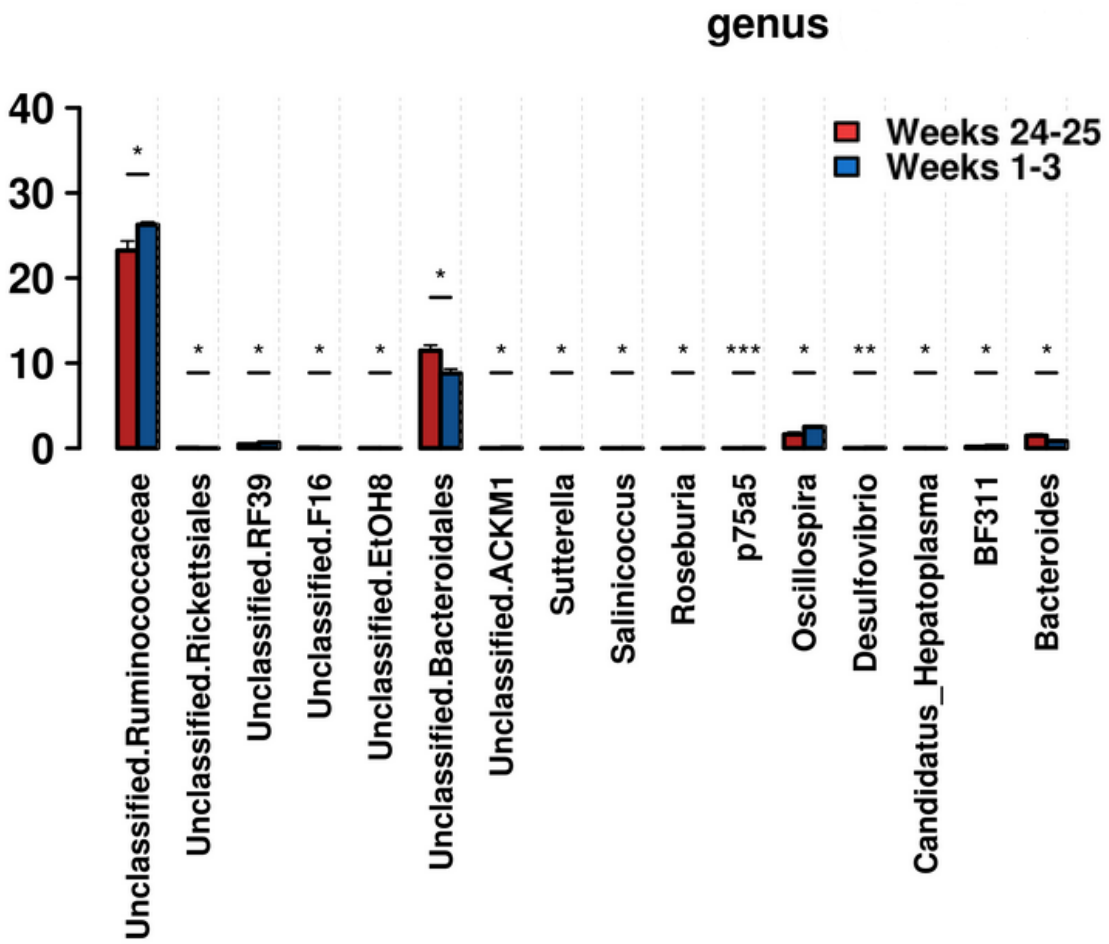


Figure 3.13 Significant differences in relative abundances of genera of faecal bacteria from two sheep samples collected in weeks 1-3 and weeks 24-25, analysed by 16S rRNA sequencing. The Y-axis shows the average relative abundances (%). Pair-wise comparisons are done by unpaired t-test; *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

3.3.3 Comparison of Faecal Microbiota of Individual Sheep

3.3.3.1 ARISA demonstrates that the sheep faecal microbiome is not homogenous

The analyses presented thus far in Chapter 3 were conducted at the community level, and sought to investigate the stability of the faecal microbiota over time. ANOSIM based on the Bray-Curtis similarity matrix was conducted by pooling 4 weeks of ARISA data for each sheep. Separation (ANOSIM: R=0.584, P = 0.001) of microbial composition between the sheep samples was observed. Pairwise ANOSIM for ARISA data was also conducted (Appendix 3, Table A3.3), demonstrating that when paired with every other sheep individually, the vast majority of sheep had significantly different faecal compositions. An nMDS plot based on the Bray-Curtis similarity matrix illustrated the separation of microbial composition of individual sheep (Figure 3.14).

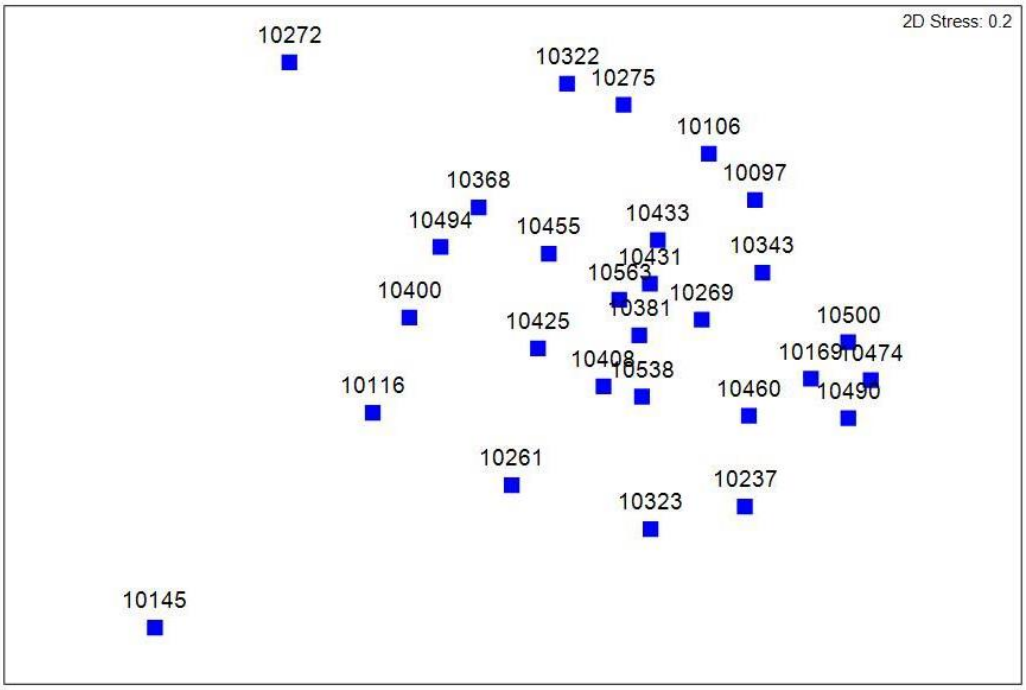


Figure 3.14 An nMDS plot based on the Bray-Curtis similarity matrix representing the gut microbial composition of different sheep samples (n=28). Each sheep is represented by a single point that was created by taking the average OTU abundance over 4 weeks of sampling. Numbers on the graph represent the sheep identification tag.

3.3.3.2 Diversity, relative abundance and bacterial species of individual sheep by 16S rRNA sequencing

Multivariate ANOSIM (using week 24 and 25 data) based on Bray-Curtis, weighted UniFrac and unweighted UniFrac distance revealed a difference in gut microbiota composition between the individual sheep samples (Table 3.5). These results are supported by the nMDS and PCoA plots (Appendix 3, Figure A3.11 and A3.12 respectively), which indicate that sheep have their own community composition.

Table 3.5 ANOSIM of gut microbial composition for individual sheep using 16S rRNA sequencing data (n=11). ***p≤0.001.

Parameter	R-value	P-value
Bray-Curtis	1	0.001***
Weighted UniFrac	0.974	0.001***
Unweighted UniFrac	0.924	0.001***

3.4 Discussion

It is commonly proposed that there are complex interactions between the gut community structure/composition and health. Given that microbiota differ in various species [66, 67, 139, 188], the first step in exploring such interactions is a sound knowledge of the composition of the gut microbiota of a given animal species, and factors which could impact on the gut microbiota. This study has added to the limited current knowledge available in the scientific literature regarding the composition of the sheep gut (faecal) microbiota.

A recent study by Tanca *et al*. [66] is the first published description of the sheep faecal microbiome. Studying five lactating Sarda sheep at a single time point, the most abundant phyla were Firmicutes and Bacteroidetes. This is consistent with this study's finding, where the same two phyla were the most abundant in the 11 Merino sheep analysed by 16S sequencing (Appendix 3, Figure A3.6). In both studies the combined contribution to overall abundance by these two phyla was in the vicinity of 80%; this suggests common dominant phyla across breeds within a common host species. At the family level, consistencies in this study and the findings of Tanca and colleagues [66] were also evident. The most abundant family detected in sheep faeces in both studies was Ruminococcaceae; with Lachnospiraceae, and families from the phylum Clostridiales also commonly detected and present in high numbers. Many of the study variables differed in the two studies conducted on sheep to date, such as the breed of sheep used, sex (and lactation status) of sheep, and feeding regime. Nonetheless, at the higher taxonomic levels similar microbial taxa are present in sheep.

Other studies investigating the microbial community composition in the digestive tract of sheep have been conducted [140, 205]. However, these studies did not set out to

determine the ‘normal’ gut microbial composition of sheep, and sampling protocols differed considerable from this study. Nonetheless, in studies investigating the rumen contents of sheep, Kittelmann *et al*. [140] and Morgavi *et al*. [205] commonly detected phyla such as Bacteriodetes, Firmicutes and Proteobacteria; in keeping with findings from this study and that of Tanca *et al*. [66] where samples from the lower digestive tract (faecal samples) were used.

Studies have been conducted in cattle, though many focus on rumen microbiota. In brief, there is consistency on the key higher taxa present in sheep faeces with that of faecal and gastrointestinal tract samples of other ruminants such as cattle. Existing studies reporting a general predominance of Firmicutes, Bacteroidetes and Proteobacteria in cattle and goat [67, 139] [68].

The concept of a core microbiota is sound, though unfortunately there is little consensus on what actually constitutes the core microbiota. In this study, the core microbiota has been determined to be the taxa present in at least 50% of samples, as determined by 16S sequencing. The core microbiota of sheep in this study consisted of 67 families of bacteria. This compares to 45 core families (of a total 76 families) detected by 16S rRNA sequencing in the study conducted by Tanca *et al.* [66]. Interestingly, using the more discriminatory shotgun sequence approach, the core microbiota consisted of 168 (of a total 385) families [66]. In this study, the core microbiota consisted of 67 of 136 detected families. We also conducted core microbiota analysis at the species level, and detected 91 of 215species to compromise the core microbiota. Thus at the braod ecological level, there is similarity in the proportion of taxa that constitute the core microbiota in sheep.

Many studies have sought to determine a 'normal' gut microbiota in various animals, but have used small samples sizes and single time points to do so. Here, I have used a combination of 16S sequencing and ARISA to investigate numerous sheep (11 and 28, respectively) over a time-course of up to 6 months. This study provides valuable data on temporal community stability/variability. The results clearly demonstrate stability of the faecal microbiota over 2 – 4 weeks, as demonstrated by both ARISA and 16S sequence analysis. The level of stability of the faecal microbiota over a 6 month duration is more difficult to ascertain. ANOSIM did not reveal significant differences in the gut microbiota over the ~6 month period, and when observing general trends in genus composition (Figure 3.12), there are no obvious or directional shifts in community composition in either of the two sheep samples in this experiment. Statistical analyses revealed that most of the changes in relative abundance occurred in non-dominant genera. Thus while the community composition did differ over the 6 month duration, the most abundant genera present (with the exception of members of the Ruminococcaceae and Bacteroidales families) remained mostly stable. Our preliminary analysis over a reasonable long-term (6 month) reveals moderate stability. Further studies, using a larger sample size for sequencing analysis, may be warranted to further our understanding of the sheep faecal microbial stability.

At the host population level, within the cohort of the 28 sheep in this study, similarities and consistencies in the faecal microbial composition were seen. However, this does not then suggest a completely homogenous microbial composition among sheep. In this study, all sheep were a similar age, the same sex, housed under the same conditions, had access to the same feed, and were, at this stage of the study, in good health. Nonetheless, this study reveals that composition of the gut microbiota differs among the individual sheep. A study on cow rumen microbial populations found that cows fed the same diets

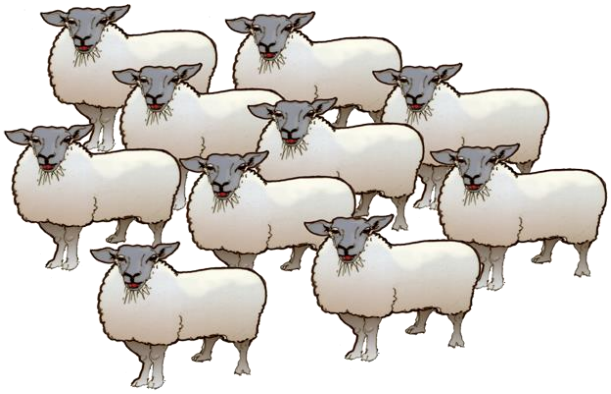
had substantial differences in bacterial community composition [164]. The findings of this study in sheep also reveals a level of uniqueness of the faecal microbiota for individual sheep. While no cause or effect in this study was investigated, other studies have speculated that various host factors may play a role in the development of a similar, but unique faecal/gut microbiota among individuals [161]. For example, glycolipids on the epithelium or specific glycans in the host mucus favour some bacteria over others to effectively attach to the gut surface [75, 206]. Similarly, the host immune system may alter the gut microbial community by killing specific bacterial groups. The relationship between gut microbiota and the immune system is complex and not fully understood; the immune system may impact on the development of the gut microbiota or visa-versa [207, 208]. For example, experimentally, mice deficient in toll-like receptors or nucleotide-binding oligomerization receptors, which recognize conserved microbial signatures, have altered gut microbial composition [77, 209, 210]. It is perhaps not surprising that deficits of the immune system would impact on the development of a microbial community; the significance of this finding in relation to healthy, non-genetically modified production animals is difficult to ascertain.

Community fingerprinting techniques such as ARISA are useful as a means of rapidly obtaining broad-scale profiles of bacterial community composition and for gaining an insight into the diversity of the bacterial population. ARISA may be particularly useful as a means of identifying groups of animals that differ substantially in microbial composition or in grouping animals having similar microbial community. Thus, this would subsequently allow more specific phylogenetic information on community members through methods like 16S amplicon sequencing analysis or whole genome shotgun sequencing to be achieved. This observation is in keeping the conclusions of de la Fuente *et al*. [211], who found that an alternative profiling method, T-RFLP, remained

a valuable tool for the study of rumen microbial communities. However, de la Fuente and colleagues did conclude that next generation sequencing techniques are becoming more cost effective and are generally more informative. While true, the use of ARISA in combination with sequence based methods has enabled a 28 –sheep, multiple time point cohort study to be conducted. In the absence of ARISA analysis it may have been unfeasible to conduct the study over multiple time points.

Chapter 4

Interactions between Sheep Gut Microbiota and the Parasite *Haemonchus contortus*



4.1 Introduction

Haemonchosis, the clinical disease caused by infection with *Haemonchus* spp., infects many breeds of goats, sheep and cattle in tropical and sub-tropical regions globally [41]. A burden of 1000 parasites can cause acute anaemia in small ruminants, which can be fatal if untreated, especially in young sheep where immunity is less developed than in adult sheep [212]. Infections reduce the overall income of sheep farmers due to concomitant reductions in milk, wool and meat production, reduced reproductive performance, sudden death of animals and cost of on-going drug treatment [213, 214].

Infection with enteric parasites, such as *H. contortus*, affects the gut microbial population in different host animals, including ruminants [68, 142, 197, 215-222]. Infection with *H. contortus* activates numerous biological pathways, including immune-mediated pathways in the mucosa of the pyloric abomasum [68] which are likely to interact and modulate the resident microbiota; although this is a poorly defined area of research. In addition, infection with *H. contortus* is likely to have an impact on the gut microbiota as the parasite causes serious physiologic changes (e.g. pH) within the digestive tract of sheep [68].

The importance of microbes in the digestive tract of ruminants has long been appreciated. Over the past 10-15 years there has been an explosive growth in metagenomic analysis of microbial populations; and there is a hope that research in this field can translate to improved health. To date much of the work conducted has been in humans [60, 71, 223], though there have been some studies that have investigated the microbial composition of the digestive tract of other animals, including production animals [66, 67, 139]. Previously, it was believed that the main benefit of hosting these microbes was to be able to utilize various food sources, including difficult to digest substrates such as cellulose.

However, recent research has revealed that microbes in the digestive tract play a large role in many aspects of an animal's physiology, including proper development of intestinal morphology and digestive function, as well as immune function [43, 193-195]. Moreover, intestinal microbes are thought to greatly influence the development and effectiveness of mucosal and systemic immune responses in mammalian systems [224]. Indeed, immune-mediated pathology rather than direct effects (blood loss) of the parasite itself may be responsible for some of the clinical manifestations, such as reduced appetite, weight loss, and diarrhoea [35]. Thus, the microbiota are likely to be intimately involved in moderating such clinical manifestations either directly, through increased nutrition acquisition (well defined in ruminant research), or indirectly through immune modulation (poorly defined in ruminant research). Due to the paucity of current research, there is a need to a) better understand the interactions between gastrointestinal nematode infection and gut microbial composition; and b) explore relationships between gut microbiota and severity of gastrointestinal nematode infection.

4.2 Materials and Methods

4.2.1 Experimental Animals

Adult Merino wethers aged 2 years were used in this experiment. The 28 sheep (the same sheep studied in the previous chapters), derived from the Sheep CRC Information Nucleus Flock (http://www.sheepcrc.org.au/), were considered to have a wide spectrum of parasite resistance, as measured by their Australian Sheep Breeding Value. The sheep were progeny derived from a 7 year breeding program which commenced in 2006, from commercial sires used for sheep breeding in the Australian sheep industry. The sires were selected to represent a range of traits typical for production systems across Australia. Progeny were tested for a range of production traits such as growth, carcass, meat, wool, reproduction and nematode parasite resistance.

4.2.2 Experimental Infection with *H. contortus* Parasites

Experimental sheep were transported to the Monash Animal Facility, Gippsland, from Hamilton, Victoria. After arrival animals were treated with anthelmintic (Cydectin®) and kept indoors on raised flooring and fed *ad libitum*. Each animal was confirmed as uninfected by faecal egg count (FEC) prior to experimental infection. Each animal was infected with two doses of 7,000 *H. contortus* L3 larvae given 3 days apart (14,000 larvae in total). Refer to Appendix 4 for details of the larvae culture, cleaning and filtering of L3, and experimental infection procedure.

4.2.3 Parasitological Examination

At 21 days post-infection, FEC were performed twice a week for 3 weeks using the modified McMaster method (Appendix 4) to determine the burden of infection. After six counts, the cumulative FEC (cFEC) was taken to determine the status of infection. Faecal samples from the five highest-burden (with highest egg count) and five lowest-burden (with lowest egg count) sheep were archived, and subsequently were used for bacterial community profiling based on this information.

4.2.4 Experimental Design and Sampling for ARISA and 16S Amplicon Sequencing

Faecal samples from 10 selected Merino wethers (five high-burden and five low-burden) were analysed by ARISA (samples collected for 8 of the 9 weeks of the study; 4 weeks before and 4 weeks after infection; total 80 samples) and 16S amplicon sequencing (samples collected in 4 of the 9 weeks; 2 weeks before and 2 weeks after infection; total 40 samples) (Figure 4.1). Samples were collected every Monday morning. Details of animal handling and sampling were discussed in Section 2.2.1.

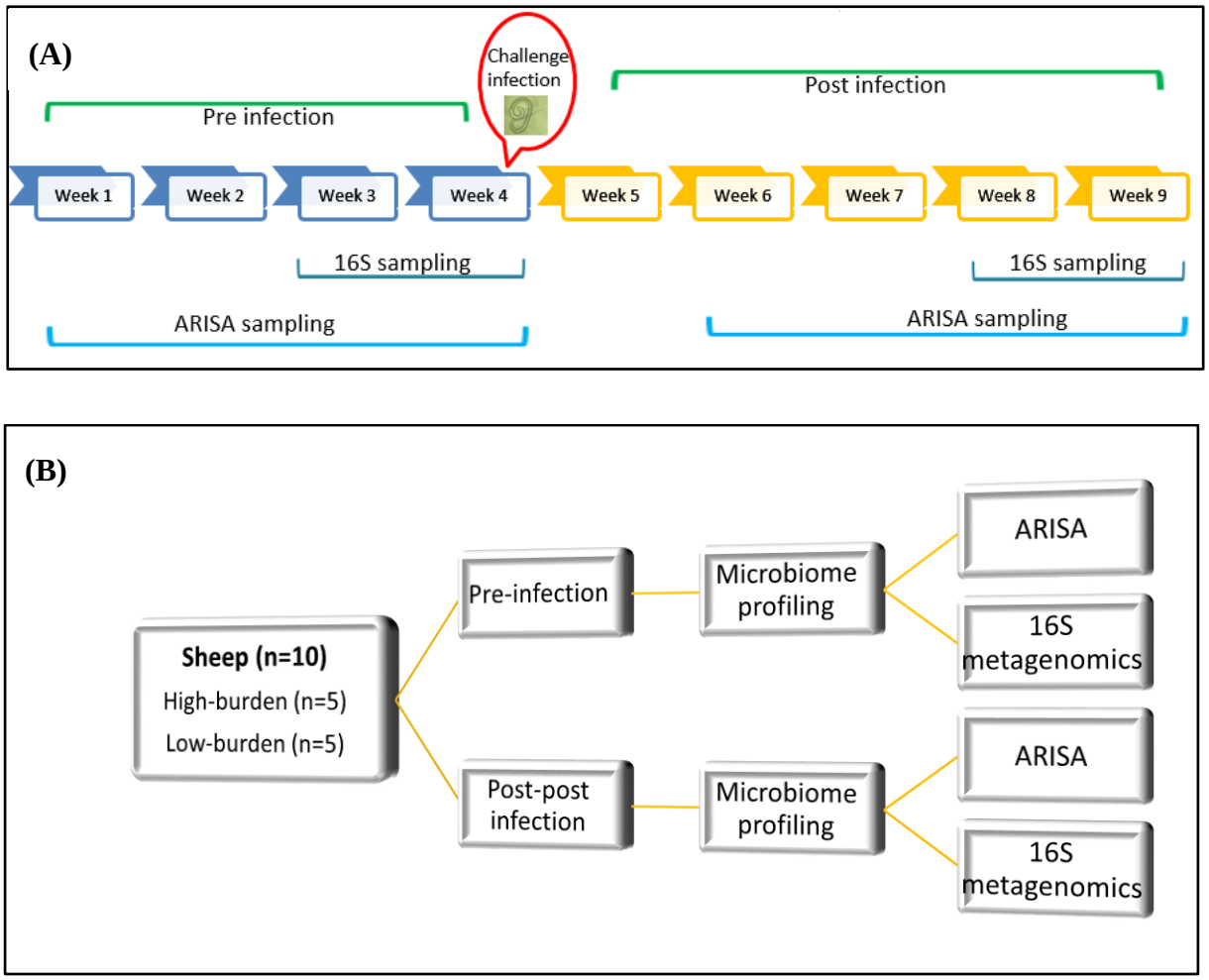


Figure 4.1 Diagrammatic representation of experimental design. (A): Time course of sampling and experimental infection; (B): Analytical procedure for sheep before and after experimental infection with *H. contortus*.

4.2.5 DNA Extraction, Quantification and Homogenization

DNA extraction, quantification and homogenization were conducted as described previously (Section 2.2.4).

4.2.6 ARISA

For each DNA sample, PCR was conducted in duplicate. PCR amplification of the ITS region was performed using the previously described primer set ITSF/ITSReub [156, 165-167] with HotStarTaq® Plus master mix. The primer set ITSF (GTCGTAACAAGGTAGCCGTA) and ITSReub (GCCAAGGCATCCACC) are complementary to positions 1423 and 1443 of the 16S rDNA and 38 and 23 of the 23S rDNA of *Escherichia coli*, respectively. Refer to Section 2.2.5 for details of the PCR conditions.

Fragment separation and ARISA analysis was conducted as previously outlined (Section 2.2.6).

4.2.7 16S rRNA Gene Illumina MiSeq Sequencing

16S rRNA sequencing was conducted by AGRF, as described in Section 3.2.4.

4.2.8 Bioinformatic Analysis

The bioinformatic pipeline used in this study was described in Section 3.2.5.

4.2.9 Ecological and Statistical Analysis

Statistical analysis of the 16S amplicon sequence data and graphs were produced in Calypso as previously described in Section 3.2.5.

Ecological analyses were conducted as described previously (Section 3.2.6). All ARISA data were analysed using GraphPad Prism software, as described in Section 3.2.6.

4.3 Results

4.3.1 Faecal Egg Count of infected sheep

To determine the burden of infection following experimental infection with *H. contortus* larvae (L3), FECs were conducted twice weekly between days 21 – 38 post infection. (Figure 4.2) In comparing the five sheep with the highest cFEC to the five sheep with the lowest cFEC, there was a significant difference between the two groups (Figure 4.3). These 10 sheep were selected for further analyses of the gut microbiota, and are referred to as high burden (n=5) and low burden (n=5) sheep throughout the remainder of the thesis.

Microbial community profiling using community fingerprinting tools ARISA and partial 16S rRNA gene sequencing were used to determine whether differences in bacterial community structure existed between the two groups.

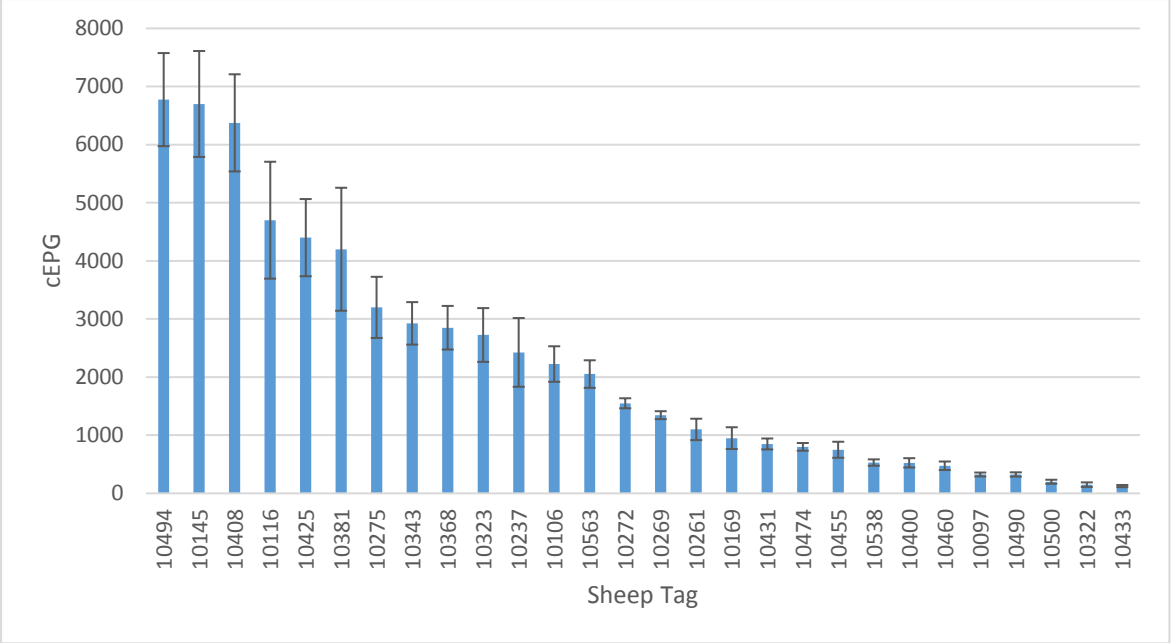


Figure 4.2 Cumulative eggs per gram (cEPG) for each of the 28 sheep. Variation between individual sheep after infection with same number of larvae of *H. contortus* was observed. Error bars symbolise SD of different faecal egg counts for an individual sheep.

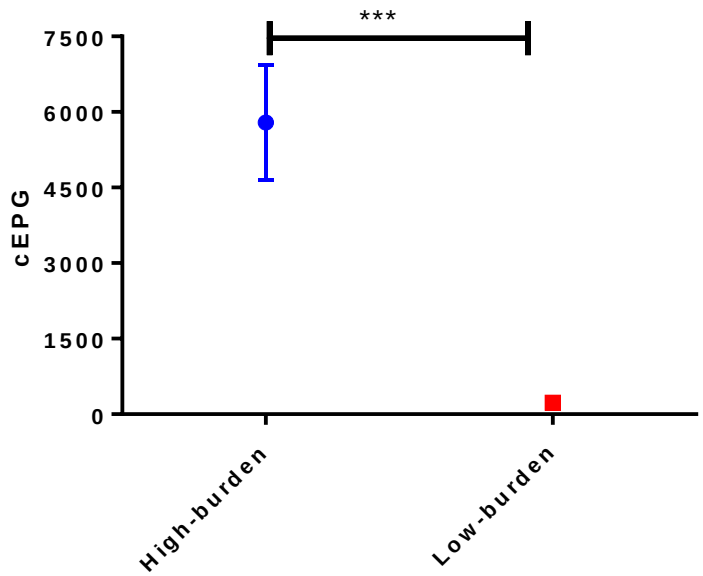


Figure 4.3 Comparison of cEPG of high (n=5) and low (n=5) parasite burden sheep. Unpaired t-test. ***p≤0.001. Error bars symbolise SD.

4.3.2 The Community Structure of Faecal Microbiota of High- and Low-Burden Sheep Prior to Infection Determined by ARISA

Three way ANOSIM revealed a separation (R=0.243, p= 0.008) of microbial composition between high and low parasite burden sheep. Differences in the microbial composition were visualised by nMDS plot, which showed distinct clustering of faecal microbiota in sheep with high parasite burden and a separate cluster for low parasite burden sheep (Figure 4.4). Most OTUs were shared among high and low parasite burden sheep groups (Figure 4.5), but abundances of dominant OTUs differed (Figure 4.6).

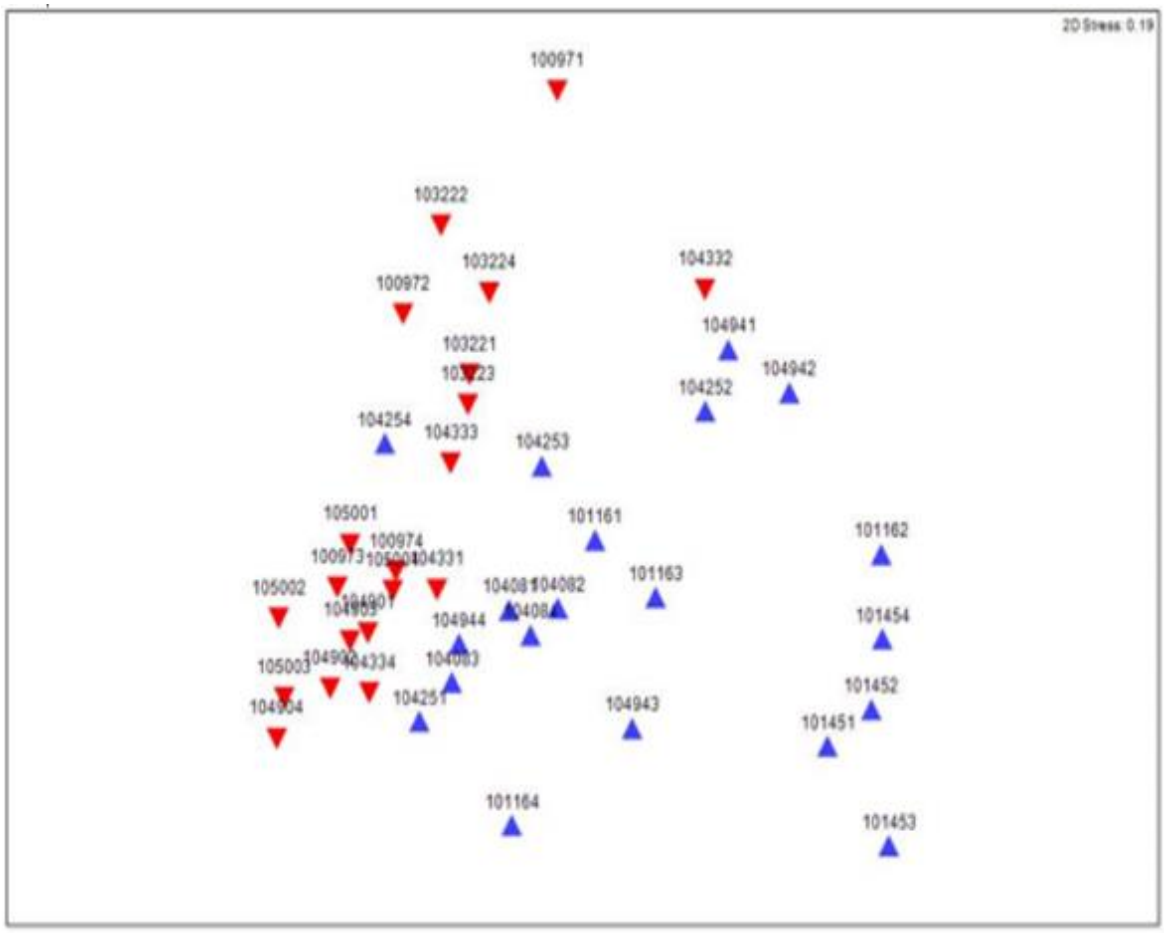


Figure 4.4 Distinct clustering of microbiome between high and low parasite burden sheep. nMDS plot of sheep faecal microbiota based on Bray-Curtis similarity matrix of faecal microbial composition determined by ARISA. Blue indicates high-burden (five sheep, four sampling events) and red represents low-burden sheep (also five sheep, four sampling events). Each symbol represents an individual sheep sample at a given time.

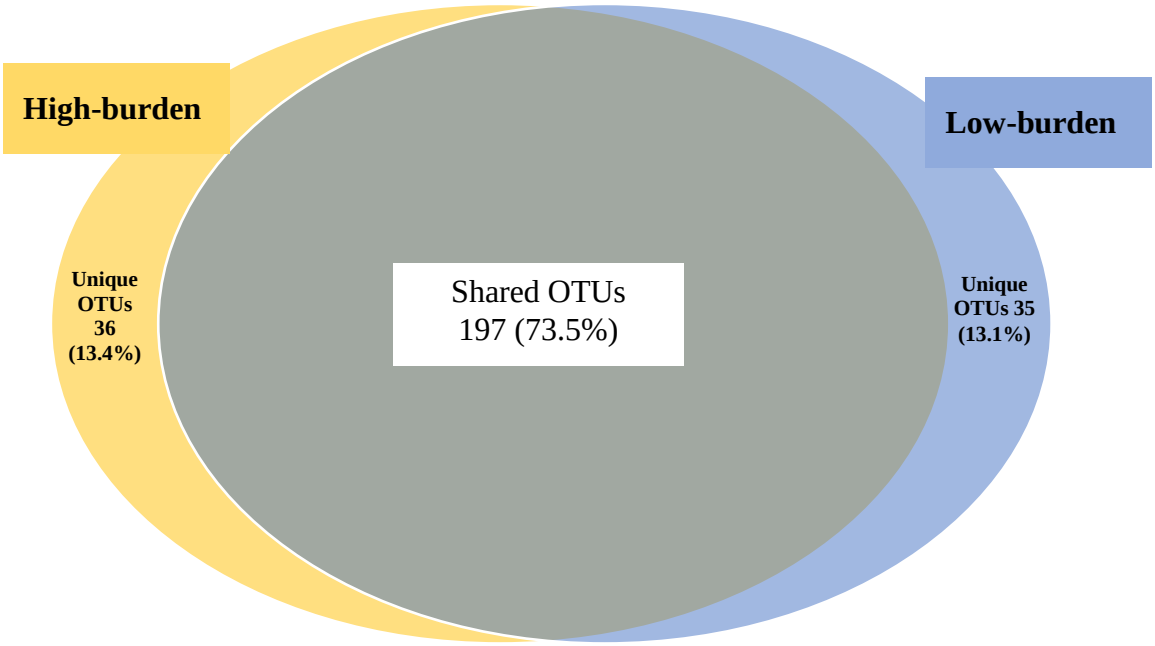


Figure 4.5 Venn diagram representing the shared and unique OTUs in high and low parasite burden sheep as determined by ARISA sampled over 4 weeks. An OTU was considered to be present in a sample group if it was identified in at least one of the samples within the group.

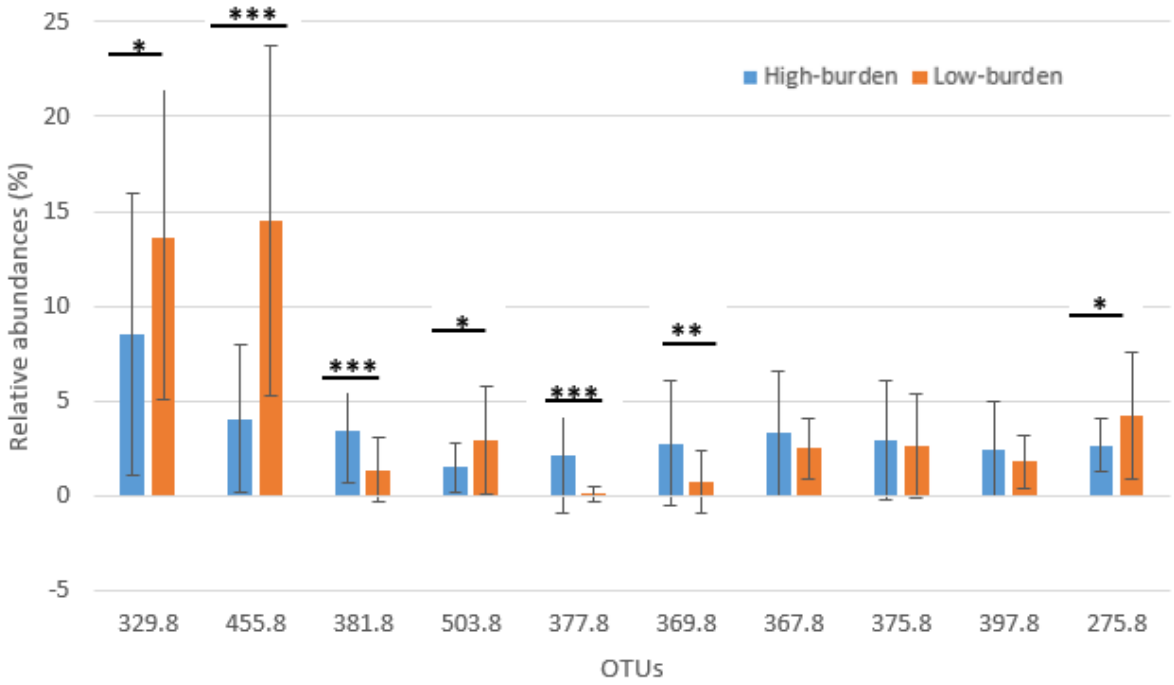


Figure 4.6 Average relative abundances of 10 most dominant OTUs of high-burden and low-burden sheep, as determined by ARISA. The Y-axis shows the average relative abundances (%). X-axis represents OTUs. Unpaired t- test; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

4.3.3 The Community Structure and Composition of Faecal Microbiota of High- and Low-Burden Sheep Prior to Infection Determined by 16S rRNA Sequencing

4.3.3.1 Rarefaction

16S amplicon sequencing analysis was conducted on the samples collected from high- and low-burden sheep for 2 weeks immediately prior to experimental infection with *H. contortus*. Rarefaction analysis was conducted based on the OTU richness values (Appendix 4, Figure A4.3), and it suggested that sequencing depth for this study was adequate.

4.3.3.2 Cluster analysis of faecal microbiota in high and low burden sheep prior to infection

Multivariate ANOSIM analysis based on Bray-Curtis, and weighted and unweighted UniFrac distance, revealed a separation of microbial composition between the sheep with high and low parasite burden (Table 4.1). nMDS (Figure 4.7) and PCoA (Appendix 4, Figure A4.4) plots showed two distinct clusters of faecal microbiota which aligned with sheep with high and low worm burdens.

Table 4.1 ANOSIM analysis of microbial composition of sheep between high-burden and low-burden sheep. **$p \leq 0.01$.

Parameter	**Bray-Curtis**	**Weighted UniFrac**	**Unweighted UniFrac**
R Value	0.878	0.88	0.435
p value	0.001**	0.001**	0.001**

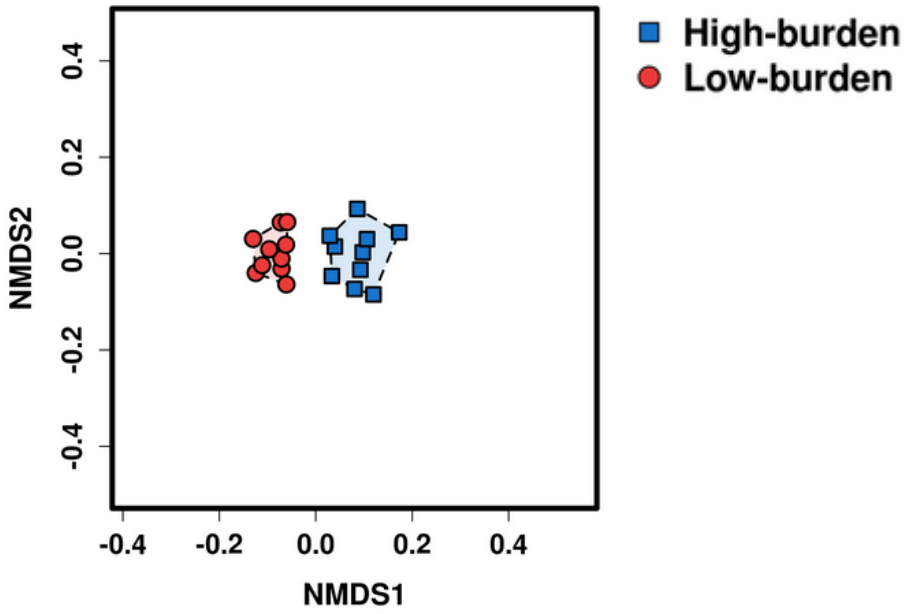


Figure 4.7 Distinct clustering of faecal microbiota between high and low parasite burden sheep. nMDS plot of sheep based on Bray-Curtis distance matrix of faecal microbial composition. Each symbol represents an individual sheep sample at a given time.

4.3.3.3 Diversity of microbial community prior to infection

Microbial diversity indices were evaluated (Table 4.2). Species richness and Fisher's alpha indices were significantly ($p \leq 0.05$) different between high-burden and low-burden sheep; other indices did not show a significant difference.

Table 4.2 Microbial diversity indices of the sheep faecal microbiota. Nonparametric unpaired t-test (Mann Whitney test); *p ≤ 0.05.

Diversity index	**High-burden** (mean±SD)	**Low-burden** (mean±SD)	**p value**
Species richness	2335.5 ± 42.33	2383.4 ±30.10	0.03*
Species evenness	0.88±0.005	0.88±0.004	0.22
Chao1	3849.39±101.84	3926.63±86.53	0.14
Fisher's alpha	956.86±26.76	984.46±26.06	0.03*
Shannon	6.83±0.04	6.86±0.03	0.13
Simpson	0.997±0.0003	0.997±0.0002	0.31

4.3.3.4 Composition of microbial community prior to infection

The gut microbiota of sheep was dominated by 24 phyla; with eight phyla having significantly (P≤ 0.05) different relative abundances in high-burden sheep relative to low-burden sheep (Figure 4.8). Notably, there were significantly more Firmicutes and less Bacteroidetes in low-burden sheep relative to the high-burden sheep. Out of 133 families identified, a total of 25 families had significantly different abundances in the two aforementioned groups of sheep (Figure 4.9). Moreover, 36 out of 200 genera identified had significantly different (p≤0.05) abundances in the two groups of sheep (Table 4.3).

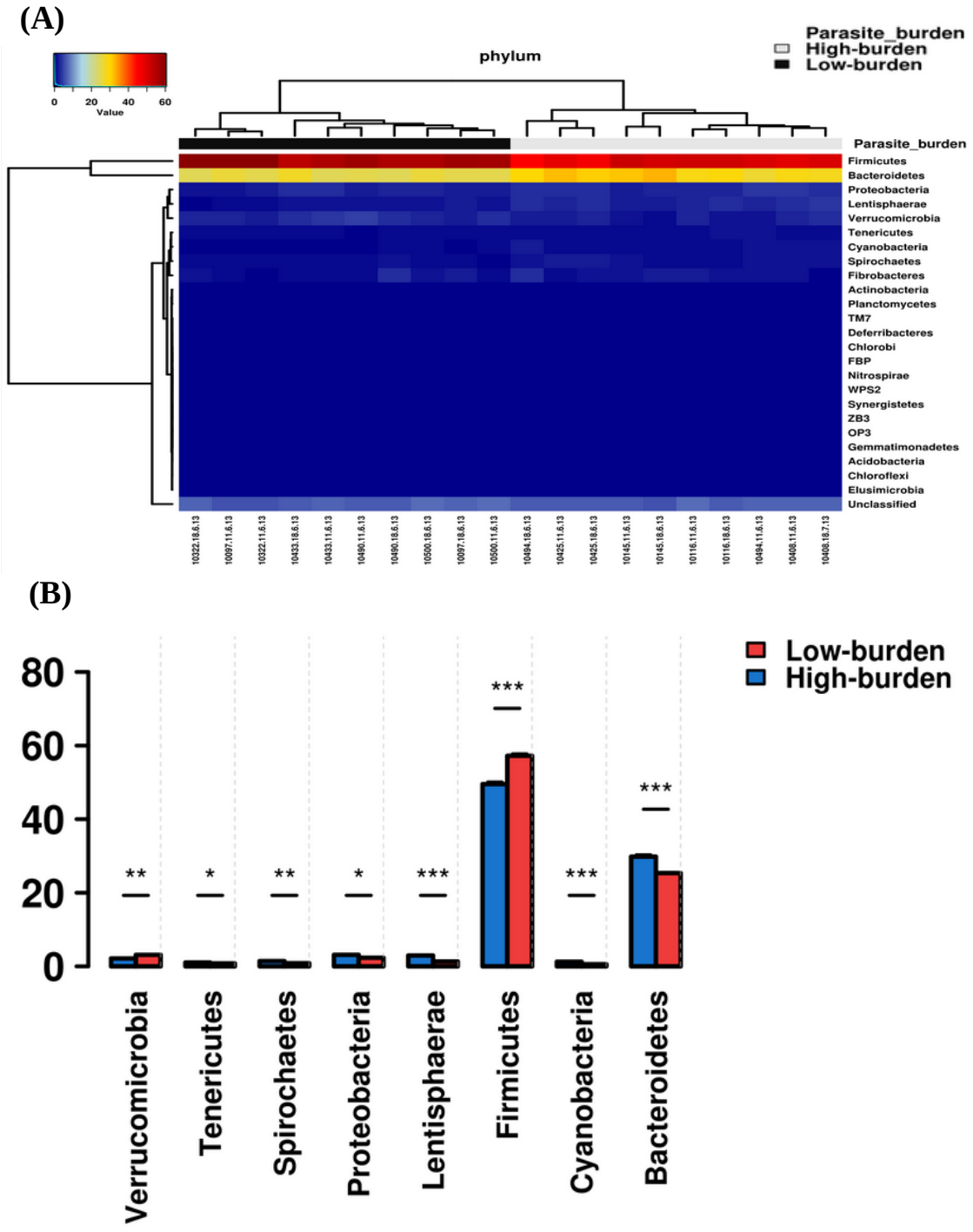


Figure 4.8 Average relative abundances (%) of identified phyla. (A) HeatMap+ of the relative abundances of the identified phyla; (B) the phyla with a significant difference in relative abundances in high and low burden sheep. The Y-axis shows the average relative abundances (%); X-axis shows phyla. Unpaired t-test; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

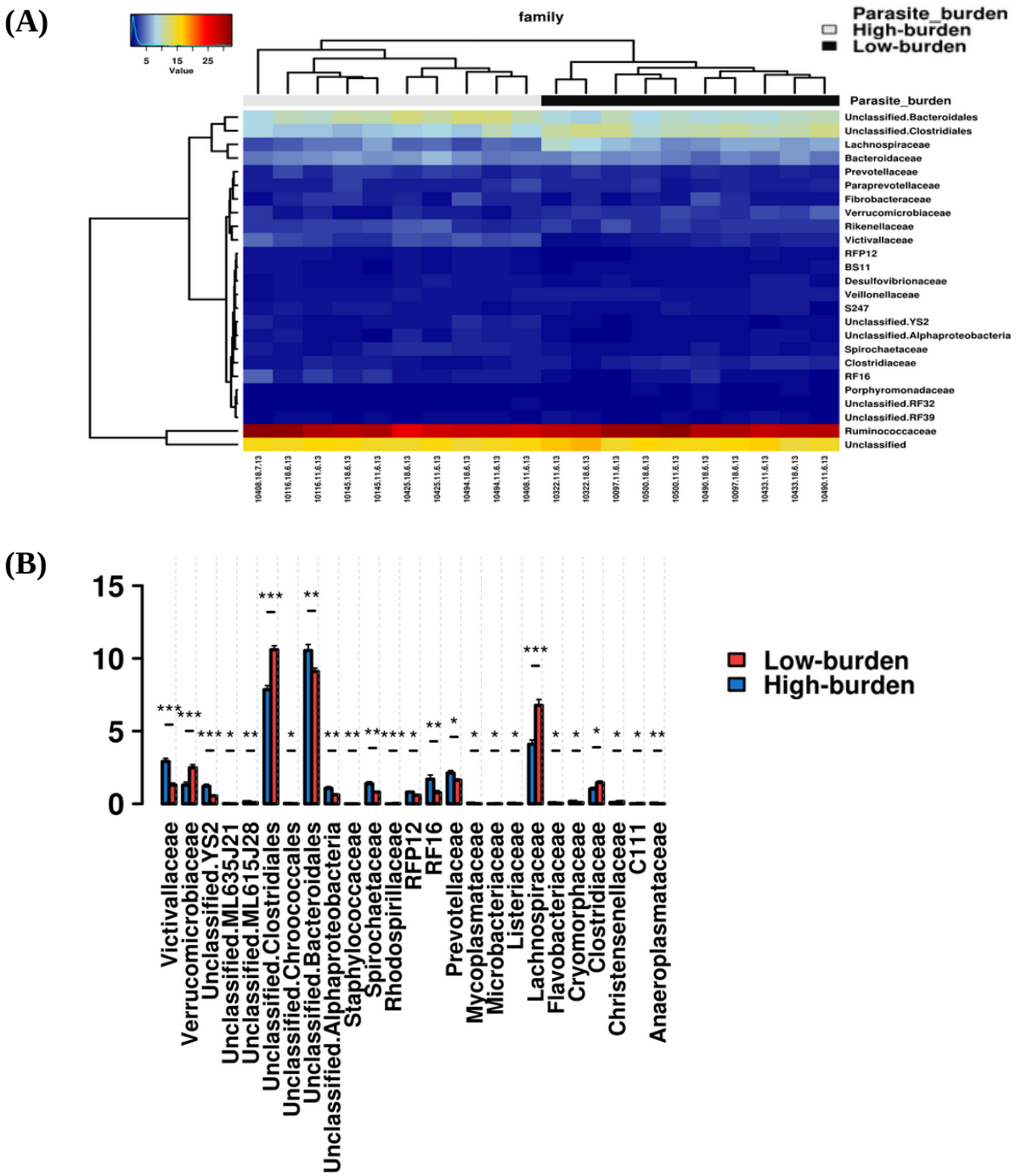


Figure 4.9 Average relative abundances (%) of the dominant families. (A) HeatMap+ of the relative abundances of the 25 dominant families; (B) the family with significant differences ($p \leq 0.05$) in relative abundances in high and low burden sheep (total family=133). The Y-axis shows the average relative abundances (%); X-axis shows family. Unpaired t-test; *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

Table 4.3 Identified genera with significantly ($p \leq 0.05$) different abundances between the high and low burden sheep. Wilcoxon signed-rank test; *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

Taxa (genus)	Mean		Median		P value
	High-burden	Low-burden	High-burden	Low-burden	
Unclassified	19.96	21.62	19.94	21.74	0.001***
Unclassified- Bacteroidales	10.54	9.1	10.38	8.93	0.005**
Unclassified- Clostridiales	7.85	10.6	7.63	10.54	0.00004****
Unclassified- Victivallaceae	2.91	1.29	2.97	1.35	0.00002****
Prevotella	2.12	1.62	2.16	1.54	0.04*
Unclassified.RF16	1.7	0.76	1.26	0.7	0.002**
Treponema	1.38	0.8	1.46	0.78	0.004**
Ruminococcus	1.24	1.83	1.2	1.73	0.0005***
Akkermansia	1.23	2.39	1.16	2.17	0.0007***
Unclassified.YS2	1.2	0.56	1.12	0.54	0.0006***
Unclassified- Alpha-Proteobacteria	1.06	0.62	1.12	0.64	0.01**
Unclassified.RFP12	0.8	0.59	0.87	0.65	0.03*
Dorea	0.56	2.13	0.53	2.22	0.0002***
Clostridium	0.37	0.62	0.39	0.66	0.008***
BF311	0.35	0.041	0.29	0.01	0.007***
Unclassified.RF32	0.35	0.27	0.34	0.24	0.03*
Unclassified- Barnesiellaceae	0.23	0.15	0.23	0.12	0.03*
Fluviicola	0.16	0.081	0.14	0.08	0.04*
Unclassified.ML615J28	0.15	0.085	0.14	0.07	0.009**
Unclassified- Erysipelotrichaceae	0.13	0.067	0.12	0.06	0.005**
Geobacter	0.096	0.19	0.085	0.14	0.01**
Unclassified- Christensenellaceae	0.089	0.16	0.095	0.17	0.02*
Unclassified- Enterobacteriaceae	0.068	0.032	0.035	0	0.05*
Unclassified- Flavobacteriaceae	0.051	0.024	0.04	0.015	0.02*
Anaeroplasma	0.033	0.012	0.035	0.005	0.01**
Unclassified- Chroococcales	0.027	0.011	0.02	0.01	0.03*
Unclassified- Dehalobacteriaceae	0.024	0.054	0.03	0.06	0.05*
Pelotomaculum	0.023	0.081	0.02	0.08	0.03*
Unclassified- ML635J21	0.02	0.006	0.015	0.01	0.05*
Unclassified- Anaeroplasmataceae	0.016	0.002	0.02	0	0.01**
Unclassified- Coriobacteriaceae	0.006	0.018	0.005	0.02	0.03*
Salinicoccus	0.005	0	0.005	0	0.01**
Acholeplasma	0.004	0	0	0	0.03*
Cryocola	0.003	0.013	0	0.01	0.03*
Unclassified- Rhodospirillaceae	0	0.027	0	0.025	0.0002***
Coprobacillus	0	0.007	0	0.01	0.005**

The differences in faecal microbial composition (relative abundance) between high-burden and low-burden sheep was compared using Linear Discriminant Analysis (LDA)

Effect Size (LEfSe) algorithm [225]. Out of 200 OTUs identified to genus level, 30 had significant differences in relative abundances in high-burden sheep relative to low-burden sheep using the stringent cut-off value of absolute LDA score log10 ≥ 2.0 (Figure 4.10). Among these genera, *Treponema* and *Prevotella* were found associated with high-burden sheep; whereas *Dorea*, *Clostridium* and *Akkermansia* were found to be more prevalent in low-burden sheep.

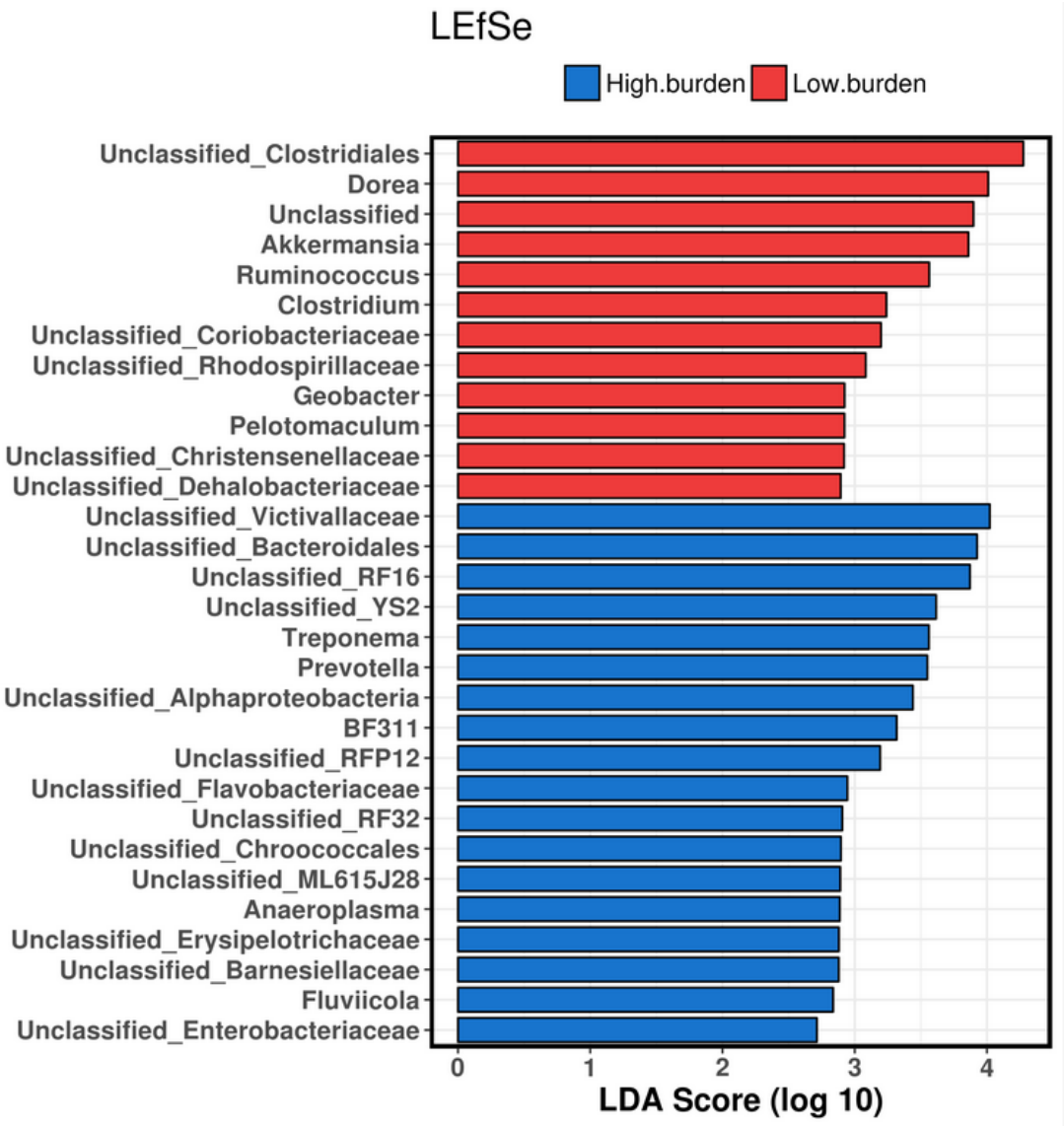


Figure 4.10 Genera with absolute LDA score ≥ 2.0. Taxa (genus) associated with the differences between high and low burden sheep were identified using LEfSe.

Approximately 60% of OTUs were present in a minimum 10% of the samples of both high-burden and low-burden groups (Figure 4.11), whereas 55.04% core OTUs (present in 50% of the samples within the group) were shared between the two groups (Appendix 4, Figure A4.5) but their abundance differed (Appendix 4, Table A4.1). Details of the shared and unique taxa are presented in Table A4.2 (Appendix 4).

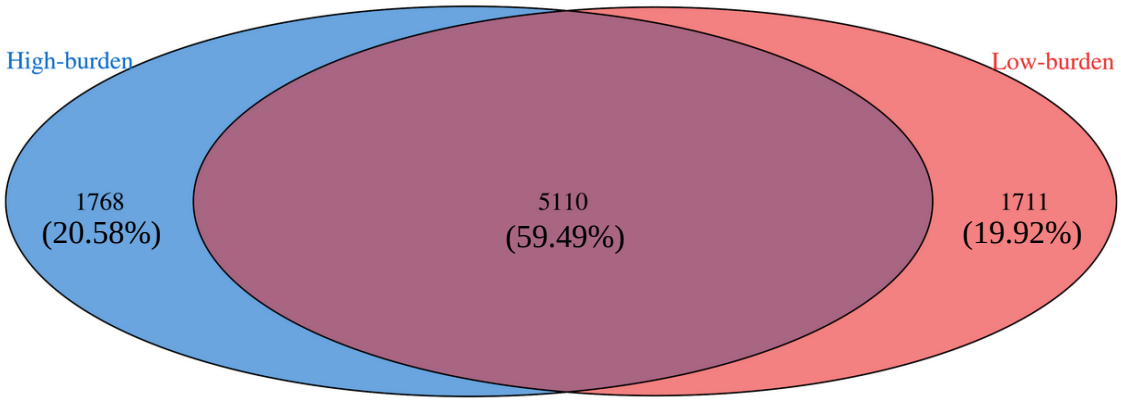


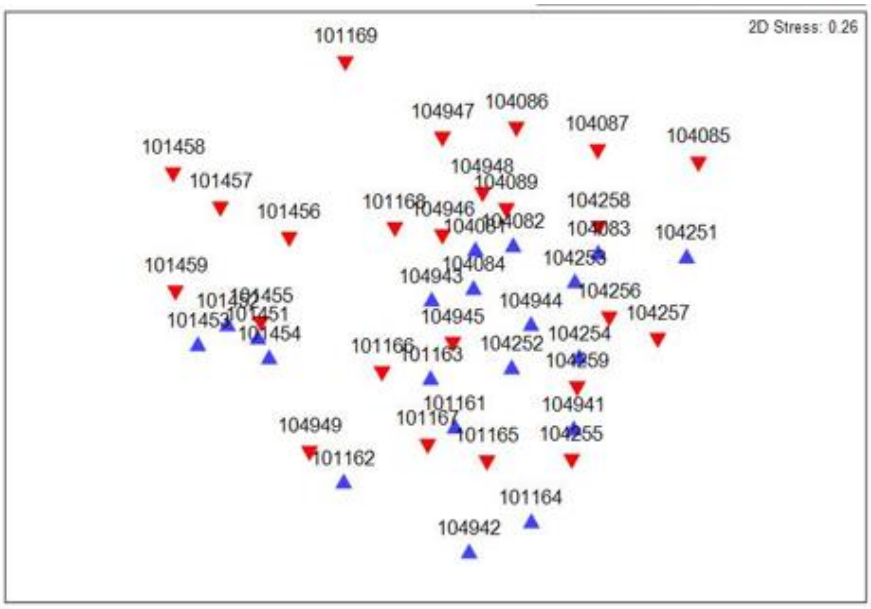
Figure 4.11 Venn diagram representing the shared and unique OTUs in sheep with high and low burdens of parasite. A bacterial group was considered to be present in a sample group if it was identified in at least 10% of the samples within the group.

4.3.4 Comparison of the Community Structure of Faecal Microbiota of High- and Low-Burden Sheep Before and After *H. contortus* Infection Determined by ARISA

Three way ANOSIM revealed little separation ($R \leq 0.243$, $p \leq 0.008$) of gut microbial composition following *H. contortus* infection in high-burden sheep, and no clear separation in low-burden sheep ($R \leq 0.190$, $p \leq 0.04$). An nMDS plot showed a lack of distinct clustering of gut microbiota in uninfected and infected sheep with high worm burden, although there was a predominance of infected sheep samples in the upper left

section of the plot (Figure 4.12A). No clear clustering was observed in low-burden sheep (Figure 4.12B). There were no significant differences in the relative abundances of the dominant OTUs following infection (Figure 4.13).

(A)



(B)

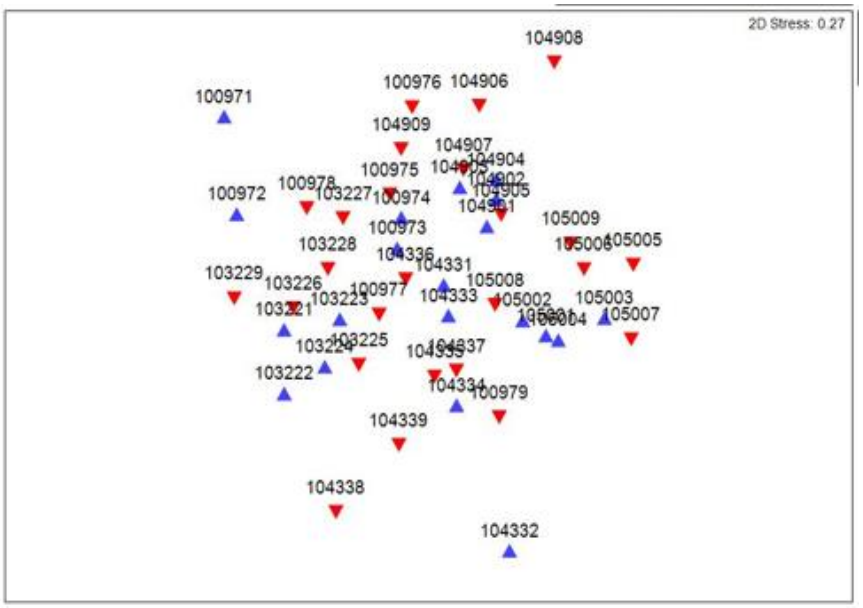


Figure 4.12 Clustering of gut microbiome of uninfected and infected sheep using ARISA. (A): high-burden; (B): low-burden sheep. nMDS plot of sheep based on Bray-Curtis similarity matrix of faecal microbial composition. Blue indicates uninfected whereas red represents infected sheep sample. Sampling were conducted over eight weeks, giving rise to 40 dot points for both high and low burden sheep.

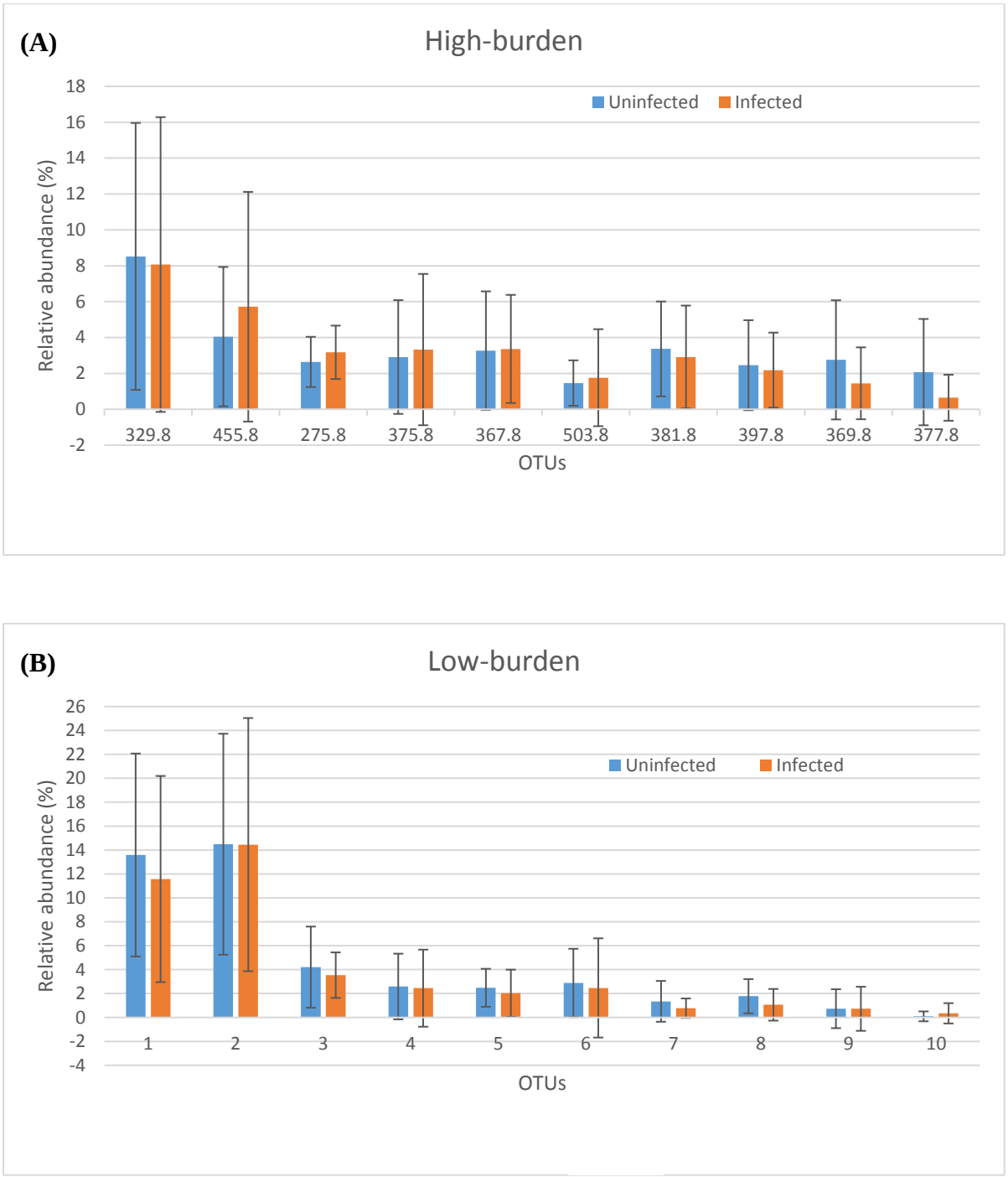


Figure 4.13 Average relative abundances of commonly detected OTUs of uninfected and infected sheep detected by ARISA. (A): high-burden; (B): low-burden sheep. The Y-axis shows the average relative abundances (%). X-axis represents OTUs. (Paired t-test; no significance was observed).

4.3.5 Comparison of the Community Structure and Composition of Faecal Microbiota of High- and Low-Burden Sheep Before and After *H. contortus* Infection Determined by 16S rRNA Sequencing

4.3.5.1 Rarefication

16S rRNA amplicon sequencing analysis was conducted on the selected samples from the five high-burden and five low-burden sheep. Rarefaction analysis suggested that sequencing depth for this study was adequate (Appendix 4, Figure A4.6).

4.3.5.2 Cluster analysis for comparison of microbial composition before and after infection with H. contortus

ANOSIM analysis based on Bray-Curtis, and weighted and unweighted UniFrac distance, were used to determine whether changes in microbial composition had occurred after *H. contortus* infection. Bray-Curis and weighted UniFrac revealed separation of faecal microbial composition in sheep with high worm burden before infection relative to after infection. In sheep with low parasite burden a comparatively smaller separation was detected (Table 4.4).

A distinct difference in clustering patterns was observed in high-burden sheep relative to low-burden sheep in response to *H. contortus* infection (Figure 4.14). In high-burden sheep two distinct clusters were observed: one for uninfected sheep and the other for infected sheep. However, in low-burden sheep there was no such clearly observable difference in infected and uninfected sheep. A similar trend was observed by PCoA based on the weighted and unweighted UniFrac distance (Appendix 4, Figure A4.7)

Table 4.4 ANOSIM of microbial composition of sheep following infection using 16S data using 16S data. **p ≤ 0.01, ***p ≤ 0.001.

Parameter	**R** (Bray-Curtis)	**R** (Weighted UniFrac)	**R** (Unweighted UniFrac)
High-burden (n=5)	0.514*******	0.629***	0.208**
Low-burden (n=5)	0.233***	0.248***	0.235***

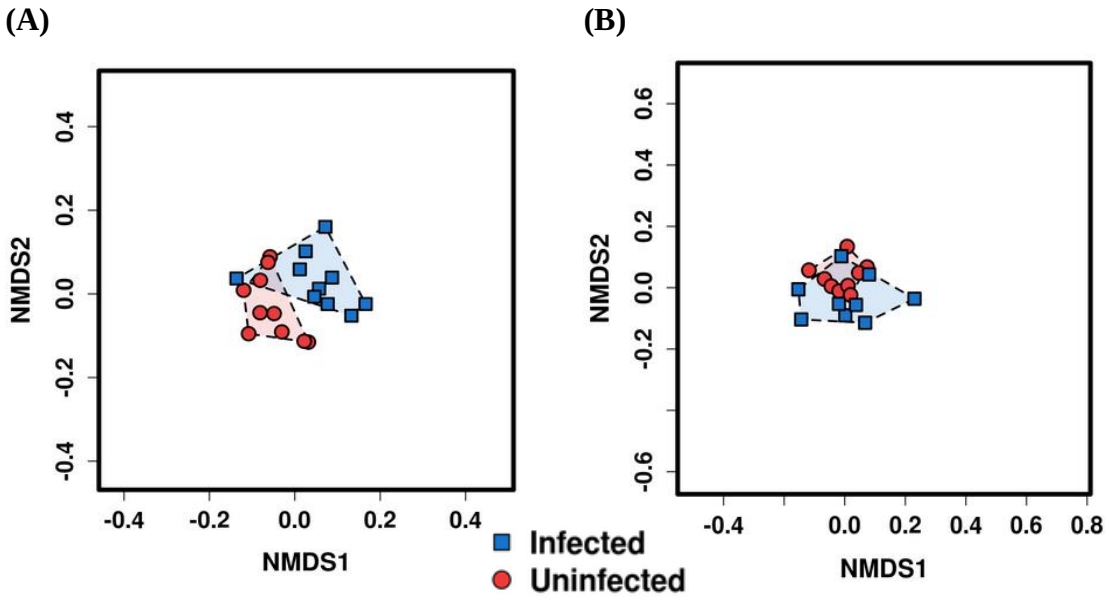


Figure 4.14 Clustering of faecal microbiota of uninfected and infected sheep based on 16S data. **(**A): High-burden; (B): Low-burden sheep. nMDS plot of sheep based on Bray-Curtis distance of faecal microbial composition. Each symbol represents an individual sheep sample.

4.3.5.3 The impact of H. contortus *infection on microbial diversity*

The Shannon, Simpson, Chao 1, richness, evenness and Fisher's alpha diversity indices were used to compare microbial diversity in uninfected and infected sheep (Table 4.5). In low-burden sheep there was a trend of shift towards lower diversity of faecal microbiota following infection with *H. contortus* ($P \leq 0.06$ for each diversity index used except the Chao1 index). In contrast, diversity appeared to increase following infection in high-burden sheep, though the differences were not significant (with the exception of Chao1 and Simpson diversity index).

Table 4.5 Microbial diversity indices of sheep faecal microbiota of uninfected and infected sheep. Significance determined using the nonparametric paired t-test (Wilcoxon matched-pairs signed rank test).

Diversity index	High-burden sheep			Low-burden sheep		
	Uninfected	Infected	p value	Uninfected	Infected	p value
Species richness	2335.50±42.33	2402.30±79.56	0.12	2383.0±30.10	2181.67±159.25	0.06
Species evenness	0.88±0.01	0.89±0.01	0.12	0.88±0.01	0.86±0.03	0.06
Chao1	3849.39±101.84	4004.96±197.77	0.06	3942.78±74.11	3662.09±227.41	0.12
Fisher's alpha	956.86±26.76	1003.67±53.13	0.12	990.12±18.94	863.44±97.55	0.06
Shannon	6.83±0.04	6.89±0.08	0.12	6.86±0.03	6.65±0.26	0.06
Simpson	0.997±0.001	0.998±0.001	0.06	0.997±0.001	0.995±0.004	0.06

4.3.5.4 The impact of H. contortus *infection on microbial community composition*

To determine which organisms differed in abundance before and after infection, sequence data were analysed at the phylum, family and genus level.

The total number of phyla was similar in high-burden (24 phlya) and low-burden (26 phyla) sheep (Appendix 4, Figure A4.8). Significant differences in the relative abundances of four phyla were observed in high-burden sheep following infection, including the dominant phyla Firmicutes and Bacteriodetes. In low-burden sheep the relative abundances of two phyla differed significantly after infection, with both phyla being non-dominant (Figure 4.15).

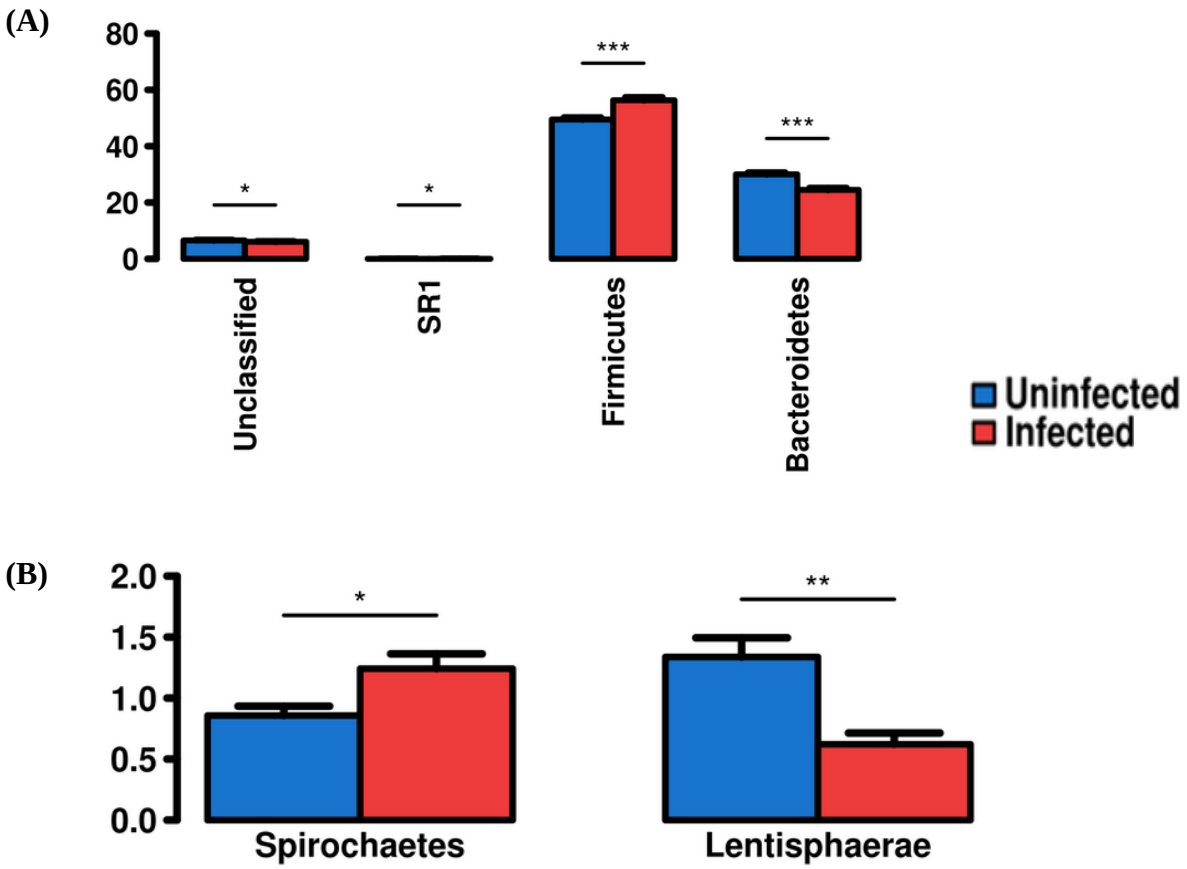


Figure 4.15 The bacterial phyla with significant differences in relative abundances before and after infection. (A): high-burden; (B): low-burden sheep. The Y-axis shows the average relative abundances (%); X-axis shows phyla. Pair-wise comparisons are done by paired t-test; *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

A total of 133 bacterial families were detected. In high-burden sheep, the abundance of 17 families differed significantly after infection; whereas in low-burden sheep the abundance of only seven families differed significantly after infection (Figure 4.16). Moreover, HeatMap+ of the 30 dominant families also indicated the marked differences in relative

abundances between uninfected and infected sheep with high worm burden (Appendix 4, Figure A4.9).

In high burden sheep, 207 genera were detected, with a similar number of genera (200) in low-burden sheep. In high-burden sheep, the relative abundance of 20 genera differed significantly following infection (Table 4.6); whereas in low-burden sheep the relative abundance of only 10 genera differed significantly (Table 4.7). There was no clear directional shift in abundance: some genera increased in abundance following infection, while others decreased in abundance. To further elucidate which genera contributed to the differences in microbial composition following infection, the relative abundances of genera were evaluated using LEfSe**.** In high-burden sheep 18 genera, and in low-burden sheep 10 genera were determined to contribute to the differences in microbial composition following infection (Figure 4.17).

Around 60% of the OTUs detected were present (in a minimum of one sample) both before and after *H. contortus* infection: this was the case for both high-burden and low-burden sheep (Appendix 4, Figure A4.10). Details of the shared and unique taxa between infected and uninfected sheep are documented in Table A4.3 (Appendix 4). When considering the core microbiota, 59% of OTUs were shared between uninfected and infected sheep for both high and low parasite burden groups (Appendix 4, Figure A4.11).

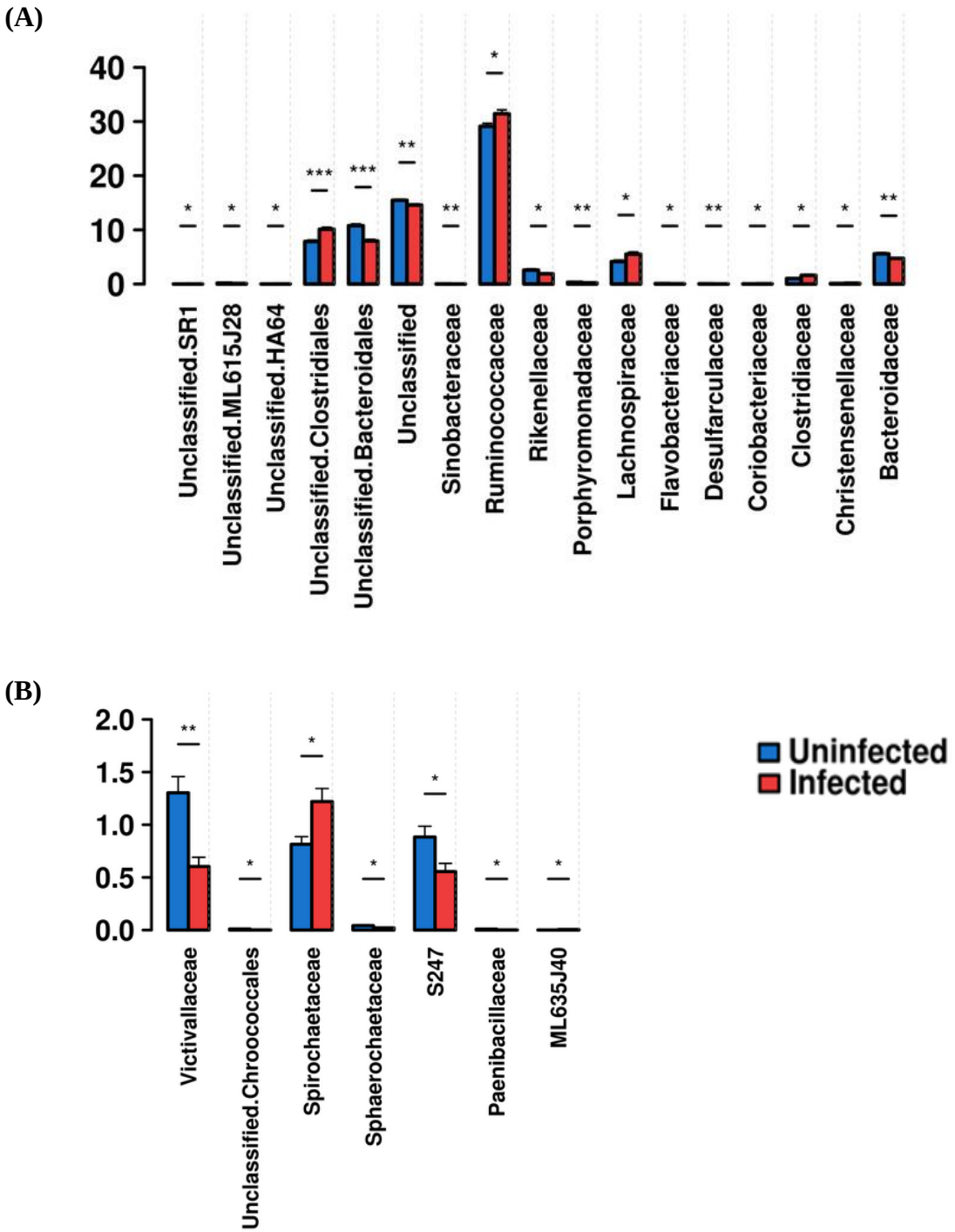


Figure 4.16 The bacterial families with significant differences in relative abundances before and after infection. (A): high-burden; (B): low-burden sheep. The Y-axis shows the average relative abundances (%); X-axis shows family. Pair-wise comparisons are done by unpaired t-test; *p ≤ 0.05, **p ≤ 0.01' ***p ≤ 0.001.

Table 4.6 The genera with significantly ($p \leq 0.05$) different abundances between the uninfected and infected sheep high parasite burden. Statistical comparisons were conducted using Wilcoxon signed-rank test; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.

Taxa (genus)	Mean		Median		P value
	Uninfected	Infected	Uninfected	Infected	
Unclassified- Bacteroidales	10.78	7.95	10.48	7.93	0.00002****
Unclassified- Clostridiales	7.84	10.12	7.61	10.17	0.0005***
Unclassified- Rikenellaceae	2.56	1.87	2.4	1.85	0.04*
CF231	1.31	1	1.06	0.94	0.04*
Ruminococcus	1.23	2.11	1.19	2.14	0.0003***
Bacteroides	1.19	0.86	1.25	0.82	0.004**
Phascolarctobacterium	0.99	0.86	0.98	0.86	0.04*
Dorea	0.54	1.36	0.48	1.27	0.008**
BF311	0.38	0.09	0.29	0.03	0.01**
Clostridium	0.37	0.51	0.39	0.48	0.02*
Unclassified- ML615J28	0.16	0.09	0.14	0.08	0.01**
Unclassified- Christensenellaceae	0.09	0.15	0.095	0.13	0.05*
Unclassified- Flavobacteriaceae	0.05	0.02	0.04	0.01	0.004**
Unclassified -Desulfarculaceae	0.04	0.015	0.03	0.01	0.007**
Fusibacter	0.02	0.005	0.01	0.005	0.003**
Unclassified- Sinobacteraceae	0.02	0.004	0.01	0	0.006**
Unclassified- Dehalobacteriaceae	0.02	0.05	0.02	0.04	0.02*
Unclassified- SR1	0	0.005	0	0	0.03*
Lactococcus	0	0.006	0	0	0.03*
Candidatus hepatoplasma	0	0.004	0	0	0.03*

Table 4.7 The genera with significantly ($p \leq 0.05$) different abundances between the uninfected and infected sheep with low parasite burden (n=20). Wilcoxon signed-rank test; *$p \leq 0.05$, **$p \leq 0.01$.

Taxa (genus)	Mean		Median		P value
	Uninfected	Infected	Uninfected	Infected	
Unclassified- Victivallaceae	1.3	0.6	1.36	0.61	0.006**
CF231	1.07	2.75	1.05	1.69	0.008**
Unclassified.S247	0.88	0.56	0.86	0.55	0.02*
Treponema	0.81	1.22	0.79	1.12	0.005**
Unclassified- Chroococcales	0.01	0.002	0.01	0	0.04*
Coprobacillus	0.008	0.001	0.01	0	0.02*
Unclassified- Aeromonadaceae	0.001	0.013	0	0.01	0.05*
Butyricimonas	0	0.007	0	0	0.03*
Succiniclasticum	0	0.1	0	0	0.03*

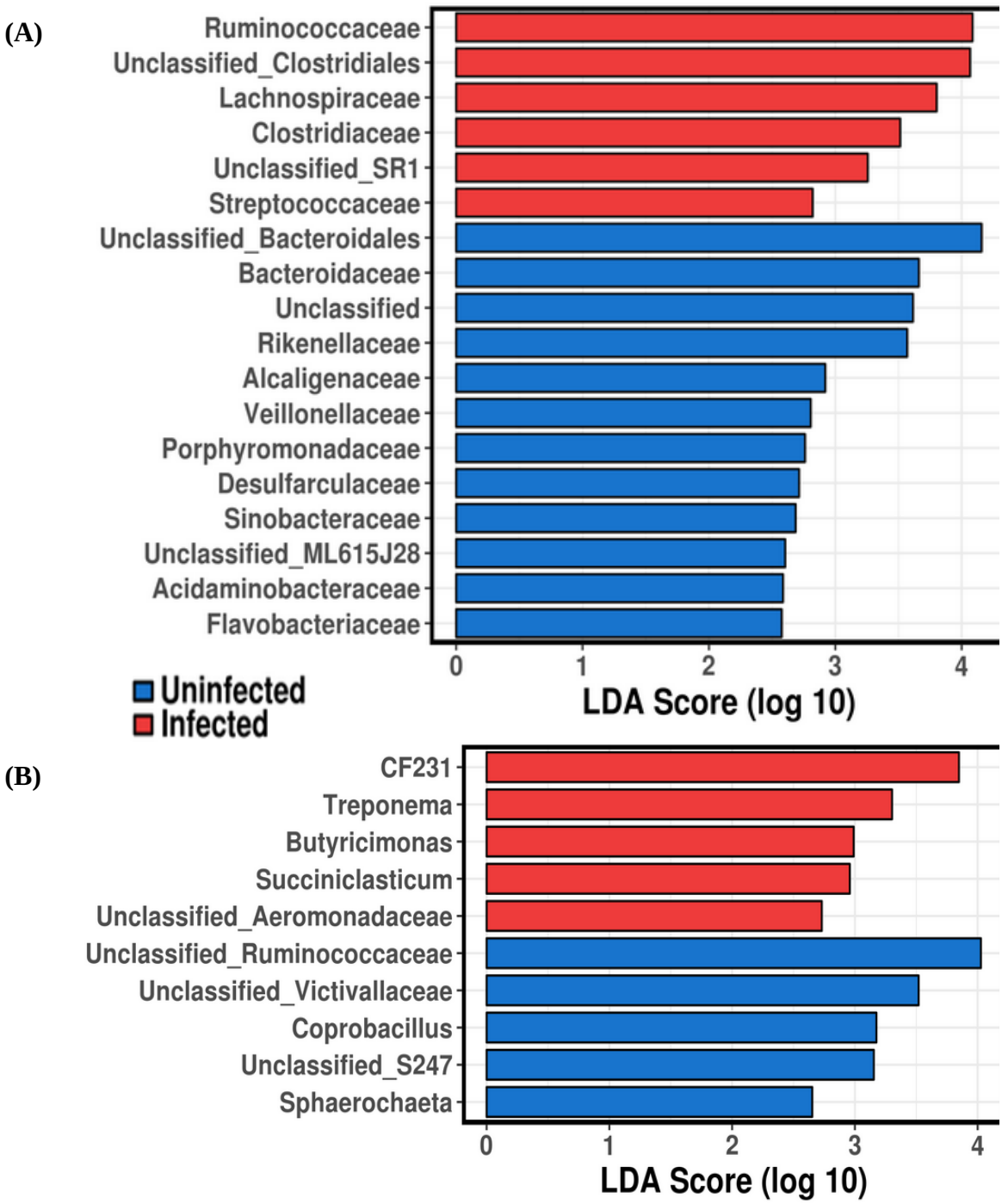


Figure 4.17 Significantly discriminative genera with absolute LDA score ≥ 2.0 between uninfected and infected group of sheep using LEfSe. (A): high-burden; (B): low-burden sheep.

4.4 Discussion

This study identified correlations between sheep faecal microbiota and *H. contortus* infection. Fecundity (faecal egg counts) have been consistently shown to correlate positively with worm burdens in *H. contortus* infections [226-229] and, therefore, in this study, low and high parasite burden sheep were defined by the respective low and high FECs of the sheep following infection.

Two key findings were of particular interest in this study. First, in the absence of infection (prior to experimental infection) sheep that go on to develop a high burden of infection have a faecal microbial composition that differs to sheep that subsequently develop a lower burden of infection. Secondly, following infection with *H, contortus*, sheep with a high-burden infection appear to undergo a greater change in their faecal microbial community structure than do sheep with a low-burden infection (relative to their respective pre-infection microbiota)

.

4.4.1 Prior to Infection with *H. contortus*

There were several noticeable differences in the structure and composition of the faecal microbial communities between high-burden and low-burden sheep, prior to infection with *H. contortus*. When samples were analysed by ARISA and 16S rRNA sequencing, nMDS using Bray-Curtis similarity matrix showed clustering of high-burden sheep separate from low-burden sheep; indicative of differing microbial community structure (Figures 4.5 and 4.7). There are various aspects that impact upon community structure. In terms of which organisms are present or absent, both ARISA and 16S rRNA sequencing revealed a large proportion (>70% in ARISA and ~60% in 16S sequencing) of OTUs to

be shared amongst high-burden and low-burden sheep (Figures 4.6 and 4.11). However, the relative abundance of important taxonomic groups differed in high-burden and low-burden sheep. Differences in abundance were observed in both dominant and sub-dominant taxa. Differences in microbiota were postulated following infection with the parasite, as have been shown for caprine infection with *H. contortus* [68], and are discussed below. However, such a segregation of host microbiota before infection in sheep that develop high-burden and low-burden infection after exposure to *H. contortus*, is of particular interest. To our knowledge, such a comparative study has not been undertaken previously. A hypothesis of such a finding is currently unknown, but this preliminary work, if confirmed in larger cohorts, could have important ramifications in breed selection for parasite resistance and/or resilience.

Firmicutes and Bacteriodetes, which together make up ~ 80% of the total population in both the high- and low-burden groups, dominated the faecal microbiota of sheep in this study. These results are in line with the recent study of sheep faecal microbiota [66]. However, there was a significant difference in relative abundance of Bacteriodetes (higher in high-burden sheep) and Firmicutes (lower in high-burden sheep) between high- and low-burden sheep. Both are known to be important (predominant) phyla in the gut microbiota of various animals, including cattle [67, 139], sheep [66], and humans [230, 231].

In humans, in which the vast majority of gut microbial composition studies have been conducted, both Bacteroidetes and Firmicutes are considered to be important phyla in healthy gut microbial communities. However, their exact role and the importance of their relative abundance has been debated. It has been suggested that the ratio of Firmicutes to Bacteriodetes may play a role in the development of obesity, with obese individuals

having 20% more Firmicutes and almost 90% less Bacteroidetes compared to the lean individuals [232]. While obesity is obviously considered an undesirable state of health in humans, the propensity to gain weight (albeit muscle mass) is a desirable trait in animals reared for meat production. Here we observed more Firmicutes and less Bacteriodetes in low-burden sheep, which corresponds with the obese microbial composition in humans [232]; though it should be noted that differences in proportions were considerable less in sheep than in obese humans. Nonetheless, it may be that the low-burden sheep have a microbial composition that favours their continued weight gain, and perhaps overall health. Interestingly, in the Australian sea lion, an animal with a thick layer of body fat for thermoregulation, there was a notable predominance of of Firmicutes (80%) over Bacteriodes (2%) [69]. In contrast, De Filippo *et al* [233] found children in Africa had "significant enrichment in Bacteroidetes and depletion in Firmicutes" relative to European children. i.e the high burden sheep had the same pattern as children that are more 'susceptible' (at least more exposed) to gastrointestinal pathogens. While the ratio of Firmicutes to Bacteriodetes is of interest, it is difficult to ascertain the importance of the relative abundance of two phyla, given the diversity of ecological and functional roles the many species within each phyla can play. It may be that the presence/absence of less dominant phyla, and the associated species/genera within those phyla, are equally or more important to gastrointestinal function and community structure than merely the ratio of the two dominant phyla.

Differences were detected in the relative abundance of subdominant phyla of bacteria. Significant differences were noted in the relative abundance of Verrucomicrobia, Tenericutes, Spirochaetes, Proteobacteria, Lentisphaerae, and Cyanobacteria. Proteobacteria are important as they are the *Escherichia coli*, *Campylobacter*, and related Gram negative bacilli. In humans it is often considered undesirable to have them in high

numbers [234]; and here we see the Proteobacteria to be more abundant in high-burden sheep.

There were differences in relative abundances in some interesting genera. *Akkermansia* and *Dorea* were identified as the dominant groups in low burden sheep, whereas *Prevotella* was dominant in high burden sheep. The genus *Akkermansia* is of particular interest; a total of 50 different OTUs were associated this genus (data not shown) with an average relative abundance of 1.13% and 2.39% in high and low parasite burden sheep, respectively. *Akkermansia* is a Gram negative anaerobe in the human gut and has also been detected in the gastrointestinal tract of various other mammals [235]. *Akkermansia* uses mucin, a key component of mucous, as a source of energy. Thus, the bacterium is commonly associated with the mucous lining that covers the epithelial cells of much of the gastrointestinal tract. This mucous layer also acts as an adhesive surface for numerous microbes, facilitating host-microbe interactions. *A. muciniphila* colonises the intestine, protecting the gut from pathogens by means of competitive exclusion [236]. This bacterium colonises the human intestine at a very young age, possibly through the birthing process, or through feeding as it is found at low concentrations in breast milk and formula [237]. A low concentration of this species in human gut could indicate a thin mucous layer, thereby resulting in a weakened gut barrier function. Patients suffering from IBD, obesity and Type II diabetes tend to have lower concentrations of *A. muciniphila* [238]. Considering the development of third larval stage (L3) of *H. contortus* (which burrow into the gastric pits), and the key role mucous and associated molecules such as host galectins plays in resistance to infection [36], there may be a role for *Akkermansia* in maintenance of a healthy mucosa in sheep. Such modulation of mucin may hinder the development, establishment and feeding of the larval stage of *H. contortus*, by direct or indirect mechanisms. Such a scenario would ultimately impede the

development of adult stage establishment and egg laying. The validity of such a hypothesis is yet to be established, but warrants further consideration and investigation.

In addition to cluster analysis and difference in relative abundance of some taxa of interest, microbial diversity indices were also suggestive of a different community composition in high-burden and low-burden sheep prior to infection. Species richness, Chao1 and Fisher's alpha tests were all suggestive of greater diversity in low-burden sheep. The exact role and/or importance of diversity in healthy gut function is unknown. In broad ecological terms, diversity is usually considered a desirable trait in natural ecosystems. Diversity has been assumed to be desirable in gut microbial communities too, notably in humans when comparing the gut microbiota of people living in industrialised countries (reduced diversity) relative people living a traditional lifestyle in non-industrialised countries such and Papua New Guinea [223, 239]. However, considering diversity alone, in the absence of species composition, is likely to be an overly simplistic measure of gut bacterial community health.

4.4.2 After Infection with *H. contortus*

This study has demonstrated that infection of sheep with *H. contortus* clearly impacts upon the faecal microbial composition, and that the impact is greatest in high-burden sheep. To some degree, this finding is perhaps unsurprising, given the pathology that is likely to impact environmental conditions of the abomasum (e.g. pH change, presence of blood, mucosal damage) following infection [240]. However, what is of interest is the clear directional shift in community composition (Figure 4.15), and the extent of change in community composition (Table 4.6) in high-burden sheep.

In high-burden sheep a change in relative abundance in dominant taxa Firmicutes and Bacteriodetes was seen; with the shift going in the direction of what low-burden sheep have in the absence of infection (increase in Firmicutes and decrease in Bacteroidetes). However, though not significant, species richness was higher following infection in high burden sheep. Similarly, in goats species richness was observed to be higher in animals infected with *H. contortus* [68]. This might be due to the alterations in the composition of the major phyla like Firmicutes and Bacteriodetes, and subsequently, introduction of less dominant species to fill the vacated niches. In contrast, the only significant difference in abundance of phyla in low-burden sheep was in sub-dominant phyla, suggesting a lesser overall impact on community composition. ANOSIM based on weighted and unweighted UniFrac suggested that number of OTUs rather than the abundance of OTUs played a major role in the differences between infected and uninfected groups of sheep. This premise is supported to some extent by diversity indices. For all indices tested, there was a trend (P=0.06) towards lesser species diversity in low-burden sheep following infection. It could be hypothesized that the presence of less adult parasites in low-burden sheep made less physiological changes in the gut, conferring a reduced alteration of community composition. Conversely, the high burden of adult parasites could result in large physiological changes in the gut; conferring increased alteration of community composition.

Beneficial organisms are an integral part of host physiology and have significant roles in ruminant metabolism [193, 195]. This study has highlighted an area of research which is currently lacking. Parasite infection clearly altered the gut microbiota of infected sheep. The initial work presented here also suggests that microbiota may vary between sheep with differing disease susceptibility/resistance to the most globally significant nematode

parasite of small ruminants. Further studies are clearly warranted and suggest parasite-microbe interactions may have important impacts on productivity in small ruminants.

Chapter 5

General Discussion

Interactions between microbes and animals are complex and multifaceted; however, improving our understanding of such interactions is likely to be beneficial to humankind. In recent years there has been an explosion of interest in the gut microbiota of humans, largely with the aim of improving human health [241]. The important role the gut microbiota is thought to play in human health has provided an impetus for research in livestock. Two key facets are now driving this, especially in ruminant research: 1) the intimate relationship with productivity and microbiota in ruminants allowing the utilisation of various food sources, including difficult to digest substrates such as cellulose; and 2) the central importance of these animals to the world economy, especially in low-income communities [41]. To this point, few researchers have focused on microbial communities associated with sheep, despite their obvious agricultural (and thus economic) importance. Hence, this study has contributed to the small existing body of work that has recently been published on the gut microbiota of sheep [66, 140, 205], providing an insight into the composition and stability of the faecal microbiota. In addition, parasites, especially *H. contortus* in small ruminant animals (goats/sheep), limit effective productivity. Therefore, this study also sought to understand aspects between faecal microbial composition and the infection in sheep with the parasite, *H. contortus.*

Only one recent study has used 16S rRNA sequencing to investigate the faecal microbiota of sheep [66]. At the higher taxonomic levels the findings of this study are consistent with the findings of the recent study by Tanca and colleagues [66]. Firmicutes and Bacteroidetes dominate, and many of the less dominant phyla and families were also present in both studies (see Chapter 3). Creating this 'base knowledge' of the sheep faecal (gut) microbiota is essential to enable further work to build upon it. The data generated in this study enabled us to then consider changes to the microbiota (see below and Chapter 4). Moreover, this base knowledge enables consideration of other potential research in

sheep, such as seeking to improve nutritional status and immunomodulation. The research conducted in this thesis on the normal microbiota of sheep has also demonstrated that the sheep faecal microbiota is stable over at least 4 weeks; though there is some changes to the microbiota over approximately 6 months.

Historically, sheep developed in countries where large commercial livestock enterprises are common and have been intensively selected for increased productivity traits such as meat, wool and milk; not generally in tandem with resistance to parasite infection. Such animals, with lower genetic resistance, have survived and reproduced in intensive farming practice due to the widespread availability of highly effective drugs [41].The advent of resistance by parasites against all classes of these drugs has led to targeted breeding programs for parasite resistance. Intriguingly in this study, the microbial community composition of faeces of uninfected sheep differs in sheep that go on to develop a high burden infection compared to those that develop a low-burden infection. As this was an observational study, we cannot ascertain whether the difference in microbial composition directly impacts the burden of infection. However, the finding is of considerable interest, and highlights the need for future research in this area.

Currently, estimated breeding values (EBVs) for parasite resistance are an industry standard used in Australia. FECs, which in this study segregated sheep into high or low-burden sheep, is the phenotypic marker used to assess sheep breed resistance and make informed breeding choices for future breeding programs (i.e. typically to selectively breed for lower FECs and, therefore, increased resistance to parasite infection). However, currently there is a poor correlation between susceptibility and EBV for parasite resistance. Known heritable genetic traits account for ~30% of whether an animal will actually have reduced susceptibility to parasite infection; i.e. there are unknown factors

that also contribute to parasite resistance/susceptibility. The mechanisms behind the observed correlation between faecal community composition and resistance/susceptibility to *H. contortus* infection are unknown, and given the small sample size of this study (which focused on 10 sheep) a larger study is warranted. Future studies should include a variety of breeds, as differences in parasite resistance have been demonstrated amongst different breeds [242]. In a larger study, it may be possible to further elucidate the significance of the faecal microbiota in moderate-burden sheep, which were not included in our statistical analyses. If further studies were to reveal a similar outcome as this study, the use the faecal microbial profile as a marker for resistance/susceptibility may be a possibility given the low heritability of current EBVs. With the increased high throughput technologies, decreased costs and potential portable on-farm systems (e.g. https://nanoporetech.com/products/minion) this may be a commercial reality in the future.

The implication of this finding for broader gastrointestinal parasite resistance is difficult to ascertain at this stage. While there are general similarities in the cellular immune response to *H. contorus* and other gastrointestinal parasites of ruminants such as *Ostertagia* and *Trichostrongylus* (obviously antibody development is specific for each parasite), some lifecycle and feeding traits of these parasites differ. An individual can have reduced susceptibility to infection due to various mechanisms, such as immune function, resistance to colonisation and damage in the abomasum, or improved nutrition. In addition, whether microbiota plays a role in resistance (the ability to counteract or prevent infection) and/or resilience (the ability to survive and thrive despite infection) mechanisms is also currently unknown.

This study has demonstrated that the gut microbiota changes after infection with *H. contortus*. Similar findings to this study have been reported by a small number of other

gastrointestinal parasite-gut microbiota studies conducted to date. Li *et al.* [68] investigated *H. contortus* infection and gut microbiota of goats, and found that the rumen microbiota of infected goats differed from that of uninfected controls. Duarte *et al*. [243] compared the faecal microbiota of cats naturally infected with *Toxocara cati* to cats without any gastrointestinal parasite infection. They found that many of the predominant taxa remained largely unchanged, but genera including *Dorea* and *Enterococcus* were more abundant in cats infected with *T. cati*. In humans, there is some evidence of differences in faecal microbial composition in people naturally infected with helminths [138]. While these studies were all well conducted and interpretations appear appropriate, it is perhaps not surprising most studies show at least subtle differences in the gut microbiota of animals (including humans) infected with gastrointestinal parasites relative to uninfected individuals. Gastrointestinal parasites often cause mucosal damage and an inflammatory response; thus altering the habitat of many gut microorganisms. In the case of *H. contortus*, the pH of the abomasum is known to increase in infected animals [68, 240]. Of interest in this study is that the faecal microbiota in the same individuals were compared, before and after infection; not as in other studies where individuals with infection were compared to different individuals without infection. This study provides clear evidence of changes in the faecal microbiota of individual animals following parasite infection.

Future studies to further investigate the mechanisms responsible for, and implications of, differences in faecal microbiota are desirable. However, there is also potential for further analysis of the current dataset, or additional analysis of currently archived samples, which could improve our current understanding of the faecal microbiota in relation to resistance/susceptibility of sheep to *H. contortus*. In particular, the application of the Kyoto Encyclopedia of Genes and Genomes database (commonly referred to as KEGG)

for functional analysis could provide an insight into relationships between community composition and metabolic processes in the high-burden and low-burden sheep, both before and after infection. Similar functional analysis was conducted by Li *et al*. when investigating the impact of *H. contortus* infection on the microbiome in goat's rumens, with eight pathways predicted to be significantly affected by infection, including essential cellular functions such as ATP synthesis (ABC transporters), carbohydrate metabolism and amino acid synthesis [68]. Functional analysis should be conducted on the current dataset before future studies are conducted, as it may help generate more targeted hypotheses to test in the future.

Conventional statistical approaches to microbiota analyses may either miss true correlations between taxa, or recognise spurious correlations [244]. Network analysis is well suited to sizable datasets with temporal or spatial variation [245], and has been applied to pig faecal microbiota [246] to investigate growth traits. Such analysis would require sequencing of additional archived samples, and in doing so re-sequencing samples already analysed for consistency. Nonetheless, network analysis may reveal important relationships between taxa that have not yet been detected, thus potentially revealing additional relationships in the faecal microbiota of low- and high-burden sheep.

Another consideration is the application of metagenomic approaches targeting microbes other than bacteria. Archaea and some protists are known to play an important role in the overall community composition and metabolic activity of the gut microbiota of ruminants.

Throughout this study, we used faecal pellets for analysis on the assumption that bacteria in faeces represent the microbial community of the digestive tract. Faeces has been used to gain an insight into digestive tract community composition in enumerable studies in

humans and other animals. Faeces is an opportunistic sample, but is also highly suitable in that it is non-invasive and is unlikely to induce excessive stress in the animal. Stress has been linked to gut microbiota perturbations [247, 248]; thus stress could bias results.

In comparison, the two most common ways of obtaining samples for the digestive tract *per se* is by euthanising the experimental animal, or the insertion of a fistula into the stomach. Given this was a longitudinal cohort study, euthanizing animals was not an option. A large cohort study would have enabled sheep to be euthanised at various stages of the study; however, that would not have allowed for the direct comparison of microbiota in the same individuals before and after infection. The use of fistula's is likely to cause physiochemical changes and inflammatory responses in the digestive tract, thus impacting on the microbiota present. Moreover, this procedure requires surgical intervention and is cost prohibitive to perform in larger sample population [188]. Thus, it was determined that faeces would be the best sample for this study.

The use of faecal samples in a study such as this does have some limitations. The faecal microbiota of the leopard seal was more similar to the microbiota from sections of the large intestine than the small intestine [149], and this is likely to hold true for all higher animals: microbial composition of faeces is likely to most closely resemble that of the lower digestive tract. *H. contortus* infects the abomasum, thus the microbial community structure of that section of the gastrointestinal tract may be of most relevance when investigating susceptibility or resistance to *H. contortus* infection. However, compartments of the digestive tract are intimately connected via various physiological body systems. Hence, changes in one compartment are likely to cause physiological and immunological changes in other areas of the gastrointestinal tract [249]. Such changes would be expected to cause perturbations in respective microbial communities. Due to

this interconnectivity of the digestive tract, as well as the physical linear nature, where microbes present in the upper digestive tract exit the body through the lower digestive tract, faeces is a good specimen for studies such as this.

In an additional experiment (Appendix 5) using ARISA and 16S rRNA gene sequencing, microbiota of abomasal samples were compared with the microbiota of faecal samples. At the conclusion of our experimental infection study (Chapter 4), faecal samples were collected from nine sheep and then immediately euthanized to collect abomasal samples. Not surprisingly, ANOSIM, nMDS and PCoA (Appendix 5: Table A5.1, Figure A5.1 and Figure A5.2) were indicative of a marked separation of microbial composition of the two sites. However, 74.07%, 59.06% and 47.05% of the phyla, families and genera respectively, were shared between the faecal and abomasal samples (Appendix 5, Table A5.2). Similar results were observed when considering the core microbiota (Appendix 5, Table A5.3). This overlap of taxa in the two sites suggests that faecal samples may be adequate at detecting major changes in microbioal community composition in the abomasum. Not surprisingly, relative abundances of the core taxa differed between the sites (Appendix 5: Table A5.4, Figure A5.5 and Figure A5.6). For example, *Prevotella* accounted for ~25% of the total bacteria detected in abomasal samples, but constituted only ~2% of the faecal microbiota. Indeed, the Bacteriodetes, the phylum in which *Prevotella* belongs, dominate the abomasum microbiota, accounting for 60% of all detected bacteria at that site compared to only ~26% in faeces. Despite these differences in relative abundance, it is noteworthy that the ten most abundant genera in the abomasum were all readily detectable in faeces. This suggests that with appropriate analysis, it may be possible to use faecal samples to detect changes to the dominant genera in the abomasum, though changes in the subdominant genera may be difficult to detect.

This study has made important and novel advances in our understanding of the faecal microbiota of sheep. It is the largest study of the faecal microbiome of this important production animal, and one of the few studies in any species to adequately investigate stability of the faecal microbiota at different time points. The faecal microbiota and resistance/susceptibility to *H. contortus* infection reveal a correlation, but the study was not designed to determine causation. On the basis of these findings, further studies should be conducted to determine whether the gut microbiota of sheep could be used or manipulated to lower the burden of *H. contortus* in sheep and other production ruminants.

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Appendices

Appendix 1

Table A1.1 List of mammalian species (supplement to Table 1.1, Ley *et al*. [60]).

Order	Family	Genus/species	Common name
Artiodactyla	Bovidae	*Antidorcas marsupialis*	Springbok
Artiodactyla	Bovidae	*Bos javanicus*	Banteng
Artiodactyla	Bovidae	*Budorcas taxicolor*	Takin
Artiodactyla	Bovidae	*Gazella spekei*	Speke's Gazelle
Artiodactyla	Bovidae	*Ovis ammon*	Argali Sheep
Artiodactyla	Bovidae	*Ovis canadensis*	Bighorn Sheep
Artiodactyla	Bovidae	*Ovis vignei*	Trancaspian Urial Sheep
Artiodactyla	Giraffidae	*Giraffa camelopardalis reticulata*	Reticulated Giraffe
Artiodactyla	Giraffidae	*Okapia johnstoni*	Okapi
Artiodactyla	Suidae	*Babyrousa babyrussa*	Babirusa
Artiodactyla	Suidae	*Potamochoerus porcus*	Red River Hog
Artiodactyla	Suidae	*Sus cebifons*	Visayam Warty Pig
Carnivora	Canidae	*Speothos venaticus*	Bushdog
Carnivora	Felidae	*Acinonyx jubatus*	Cheetah
Carnivora	Hyaenidae	*Crocuta crocuta*	Spotted Hyena
Carnivora	Pantherinae	*Panthera leo*	Lion
Carnivora	Ursidae	*Ailuropoda melanoleuca*	Giant Panda
Carnivora	Ailuridae	*Ailurus fulgens*	Red Panda
Carnivora	Ursidae	*Tremarctos ornatus*	Spectacled Bear
Carnivora	Ursidae	*Ursus americanus*	North American Black Bear
Carnivora	Ursidae	*Ursus maritimus*	Polar Bear
Chiroptera	Phyllostomidae	*Carollia perspicillata*	Seba's Short-tailed Bat
Chiroptera	Pteropodidae	*Pteropus giganteus*	Flying Fox
Hyracoidea	Procaviidae	*Procavia capensis*	Rock Hyrax
Insectivora	Erinaceidae	*Atelerix albiventris*	Hedgehog
Lagomorpha	Leporidae	*Oryctolagus cuniculus*	European Rabbit
Perissodactyla	Equidae	*Equus asinus*	Somali Wild Ass
Perissodactyla	Equidae	*Equus equus*	Horse
Perissodactyla	Equidae	*Equus grevyi*	Grevy's Zebra
Perissodactyla	Equidae	*Equus hartmannae*	Hartmann's Mountain Zebra
Perissodactyla	Rhinocerotidae	*Diceros bicornis*	Black Rhinoceros
Perissodactyla	Rhinocerotidae	*Rhinoceros unicornis*	Indian Rhinoceros

Table A1.1 (*cont.*) List of mammalian species (supplement to Table 1.1, Ley *et al*. [60]).

Order	Family	Genus/species	Common name
Primates	Atelidae	*Ateles geoffroyi*	Black-handed Spider Monkey
Primates	Callitrichidae	*Callithrix geoffroyi*	Geoffrey's marmoset
Primates	Cebidae	*Callimico goeldii*	Goeldi's Marmoset
Primates	Cercopithecidae	*Colobus angolensis*	East Angolan Colobus
Primates	Cercopithecidae	*Colobus guereza*	Eastern Black and White Colobus
Primates	Cercopithecidae	*Papio hamadryas*	Hamadryas Baboon
Primates	Cercopithecidae	*Presbytis francoisi*	Francois Langur
Primates	Cercopithecidae	*Pygathrix nemaeus*	Douc langur
Primates	Hominidae	*Gorilla gorilla*	Western lowland Gorilla
Primates	Hominidae	*Pan paniscus*	Bonobo
Primates	Hominidae	*Pan troglodytes*	Chimpanzee
Primates	Hominidae	*Pongo pygmaeus abelii*	Sumatran Orangutan
Primates	Lemuridae	*Eulemur macaco macaco*	Black Lemur
Primates	Lemuridae	*Eulemur mongoz*	Mongoose Lemur
Primates	Lemuridae	*Lemur catta*	Ring-tailed Lemur
Primates	Pitheciidae	*Pithecia pithecia*	White-faced Saki
Proboscidae	Elephantidae	*Elephas maximus*	Asiatic Elephant
Proboscidae	Elephantidae	*Loxodonta africana*	African Elephant
Rodentia	Bathyergidae	*Heterocephalus glaber*	Naked Molerat
Rodentia	Caviidae	*Hydrochaeris hydrochaeris*	Capybara
Rodentia	Sciuridae	*Callosciurus prevosti*	Prevost's squirrel
Xenarthra	Dasypodidae	*Tolypeutes matacus*	Southern three-banded Armadillo
Diprotodontia	Macropidae	*Macropus rufus*	Red Kangaroo
Monotremata	Tachyglossidae	*Tachyglossidae aculeatus*	Short-beaked Echidna
Artiodactyla	Bovidae	*Bos taurus*	Dairy Cow (Holstein)
Rodentia	Muridae	*Rattus norvegicus*	Norway Rat (Wistar)
Primates	Hominidae	*Homo sapiens*	Human
Primates	Hominidae	*Gorilla beringei*	Bwindi Gorilla

Appendix 2

Table A2.1 DNA yield (ng/µL) of six faecal samples of sheep by using three different genomic DNA extraction techniques.

SL No.	Tag no of sheep	DNA yield (ng/µL) - Final elute:100µL		
		Qiagen DNA stool kit	Power soil kit	Qiagen DNA stool kit with EconoSpin column
1	01 10343 01	22.8	22.0	11.8
2	01 10343 02	41.2	22.4	31.3
3	01 10116 01	29.0	32.1	11.0
4	01 10116 02	37.0	35.6	21.5
5	01 10460 01	25.8	25.6	15.5
6	01 10460 02	30.0	26.4	13.4

Appendix 3

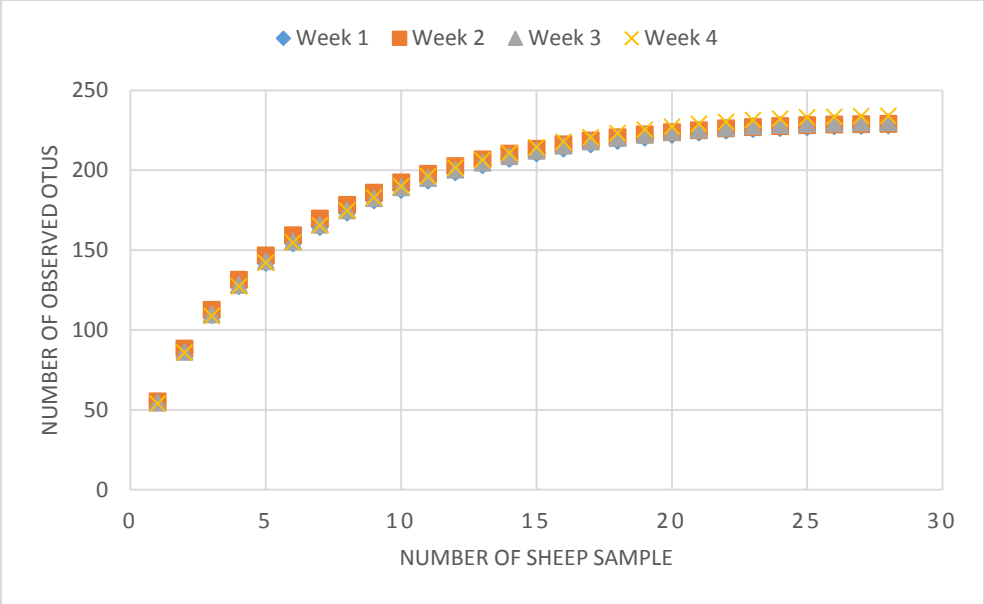


Figure A3.1 OTU accumulation plot. Weekly samples curves are overlapping each other. These curves plot the cumulative number of OTUs as a function of the number of individual samples. The curve become asymptotic as the number samples increase, and each sample adds an increasingly smaller number of new OTUs.

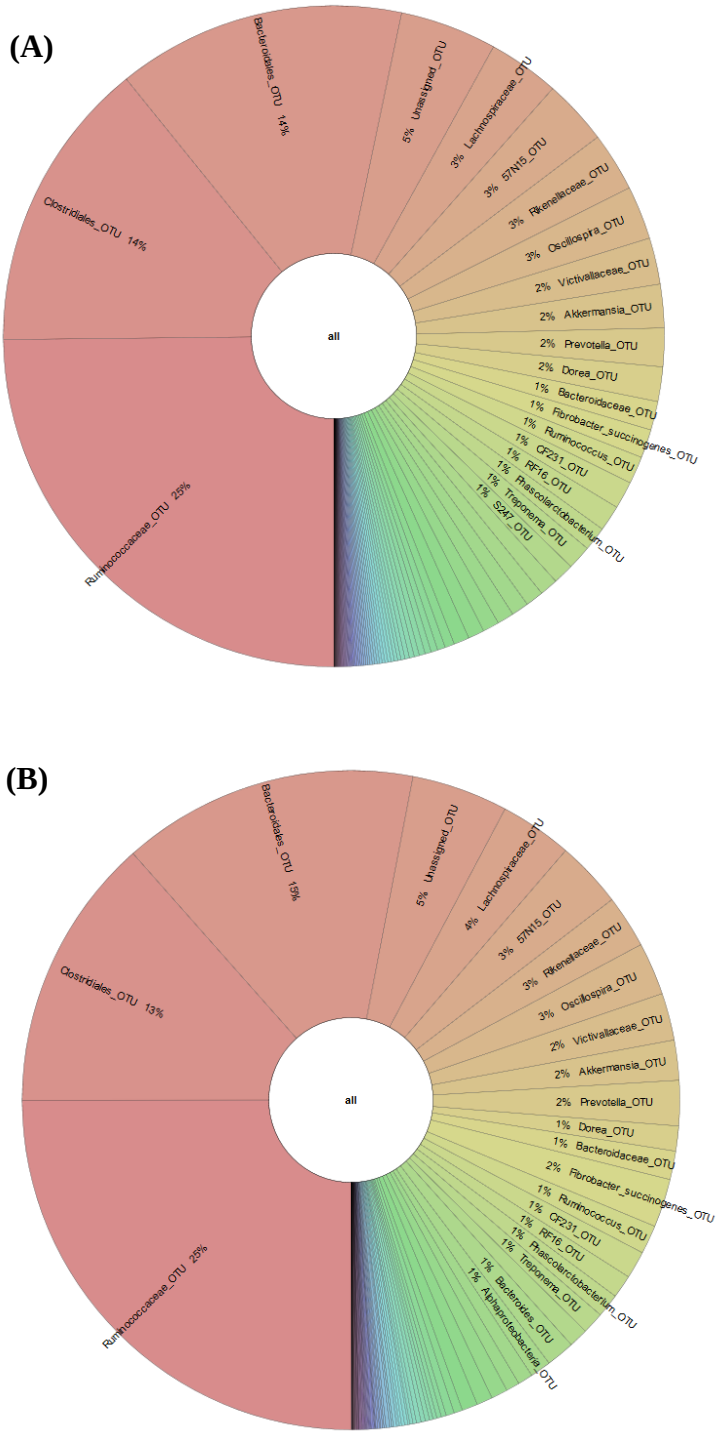


Figure A3.2 Visualization of microbial community composition at genus level using hierarchical trees shown as pie charts (Krona charts). (A): week 24; (B): week 25.

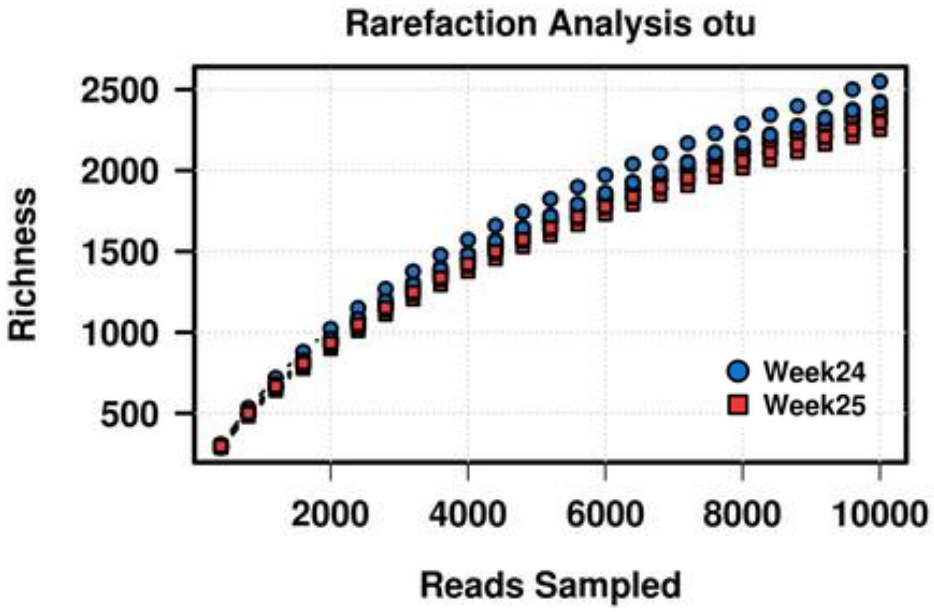


Figure A3.3 Rarefaction curve based on OTU richness values from 16S rRNA sequencing analysis.

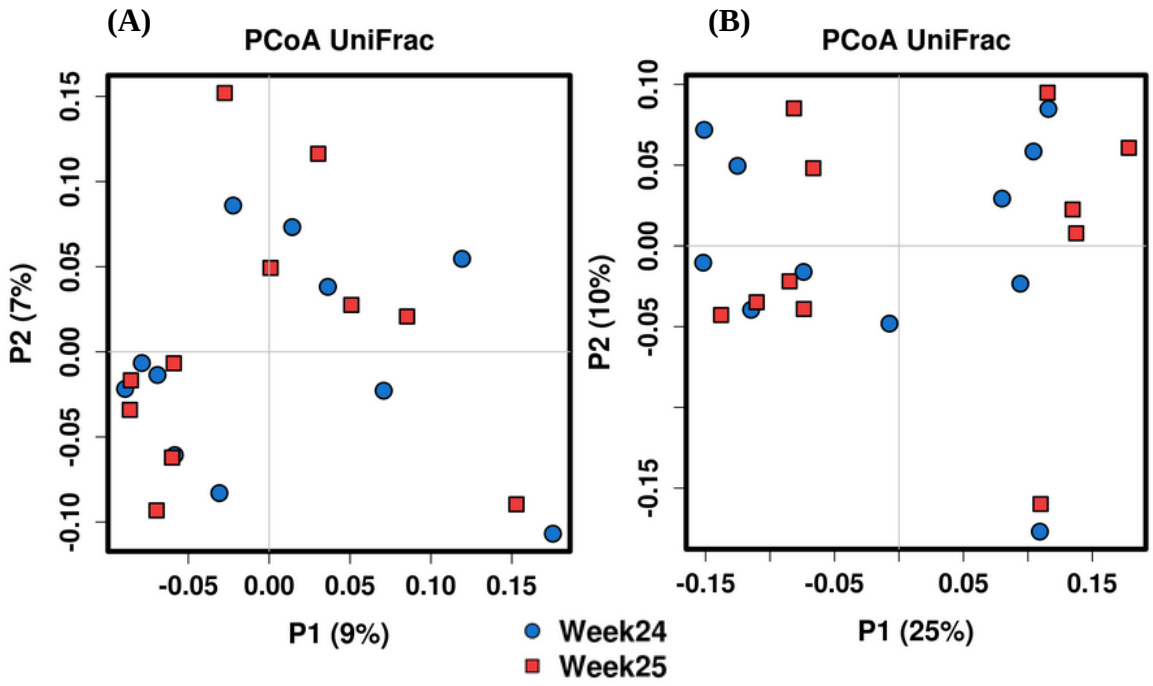


Figure A3.4 Clustering of microbiome between week 24 and week 25 sheep samples. PCoA plot of sheep based on weighted UniFrac (A) and un-weighted UniFrac (B). Each sample is represented by a single point.

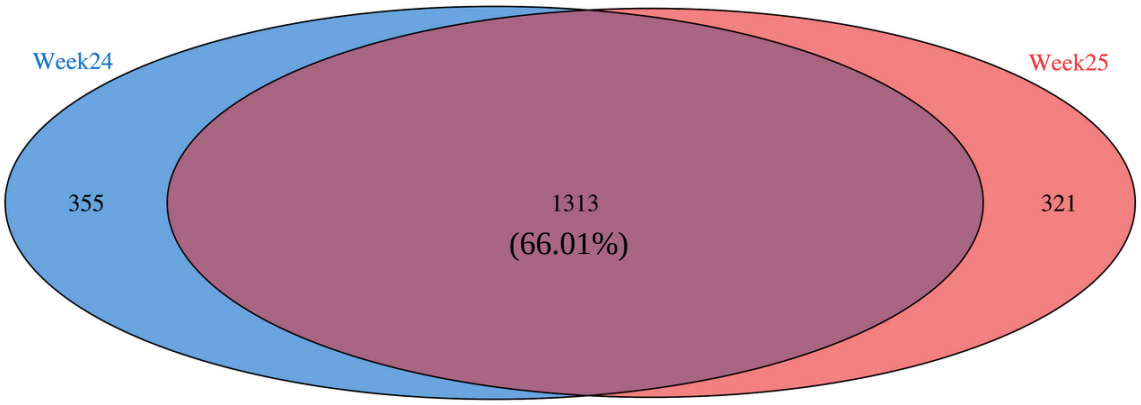


Figure A3.5 The stability of the core microbiota of sheep over a 2-week period. Venn diagrams representing the shared and unique OTUs in weeks 24 and 25 samples from 11 sheep. Approximately 66% of all core OTUs were present in both weeks. A bacterial group was marked as present in a sample group if it was detected in at least 50% of the samples within the group.

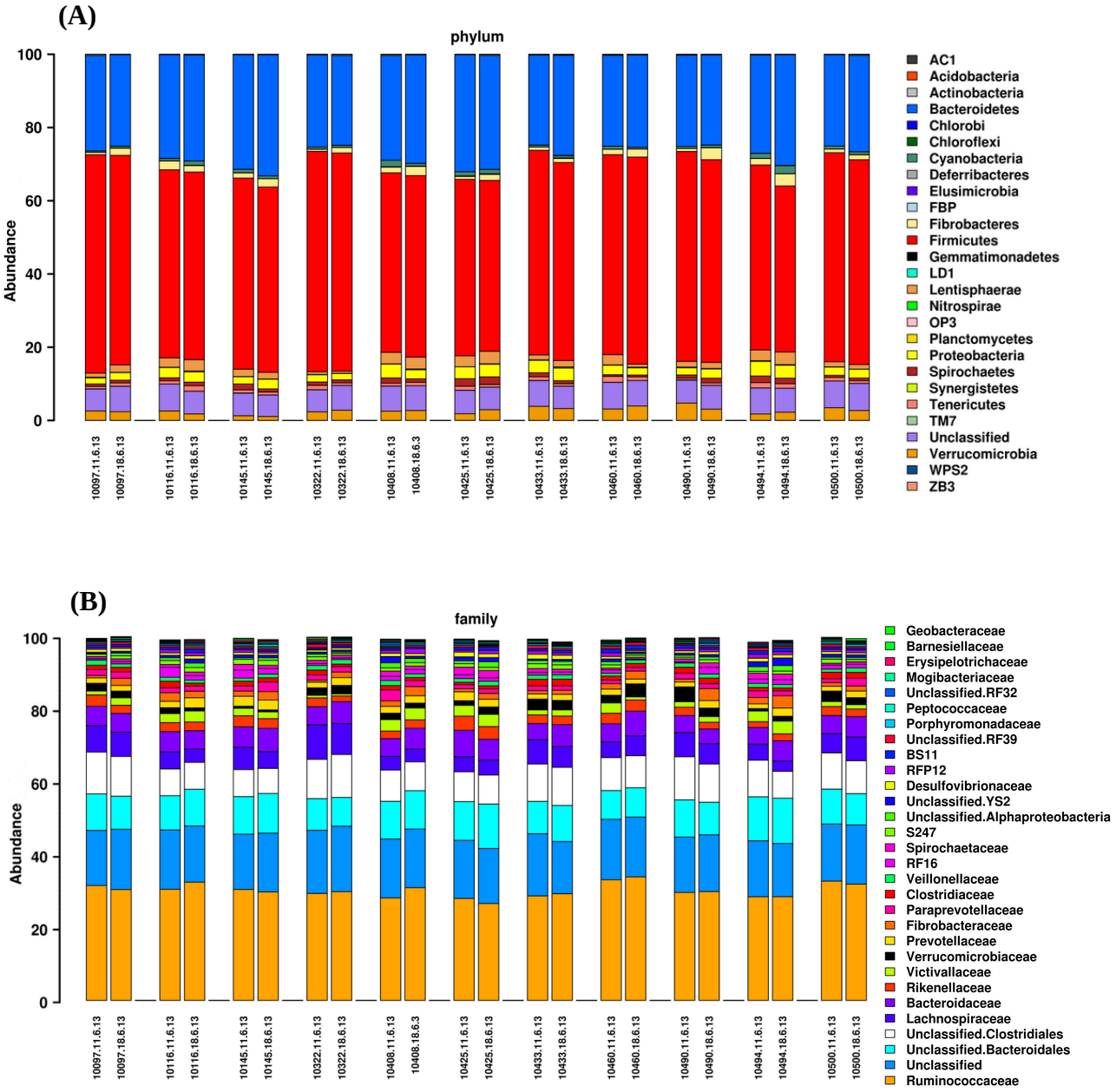


Figure A3.6 Average relative abundances (%) of 30 most abundant taxa from 11 sheep using 16S rRNA sequencing. (A): Phyla; (B): Family. The Y-axis shows the average relative abundances (%); X-axis shows sample identification (five digit identification code followed by the date of sample collection). Each pair of columns indicate same sheep in weeks 24 (left column of the pair) and week 25 (right column).

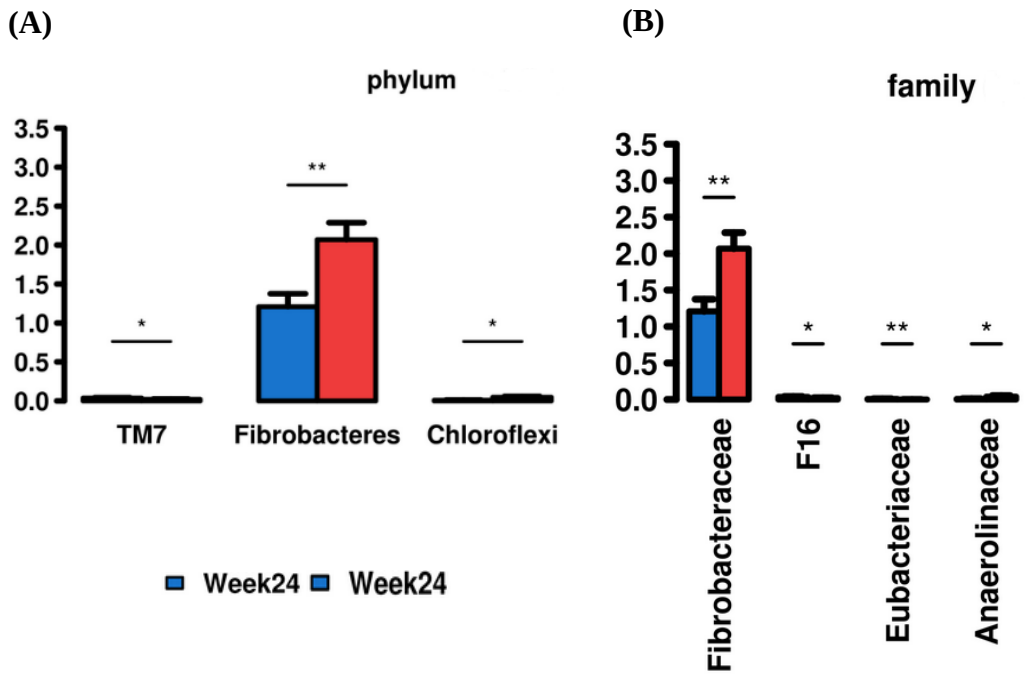


Figure A3.7 Significant differences in relative abundances of taxa of faecal bacteria from 11 sheep samples collected in weeks 24 and 25 and analysed by 16S rRNA sequencing. (A): Phyla; (B): Family. The Y-axis shows the average relative abundances (%). Pair-wise comparisons are done by paired t-test; *$p \leq 0.05$, **$p \leq 0.01$.

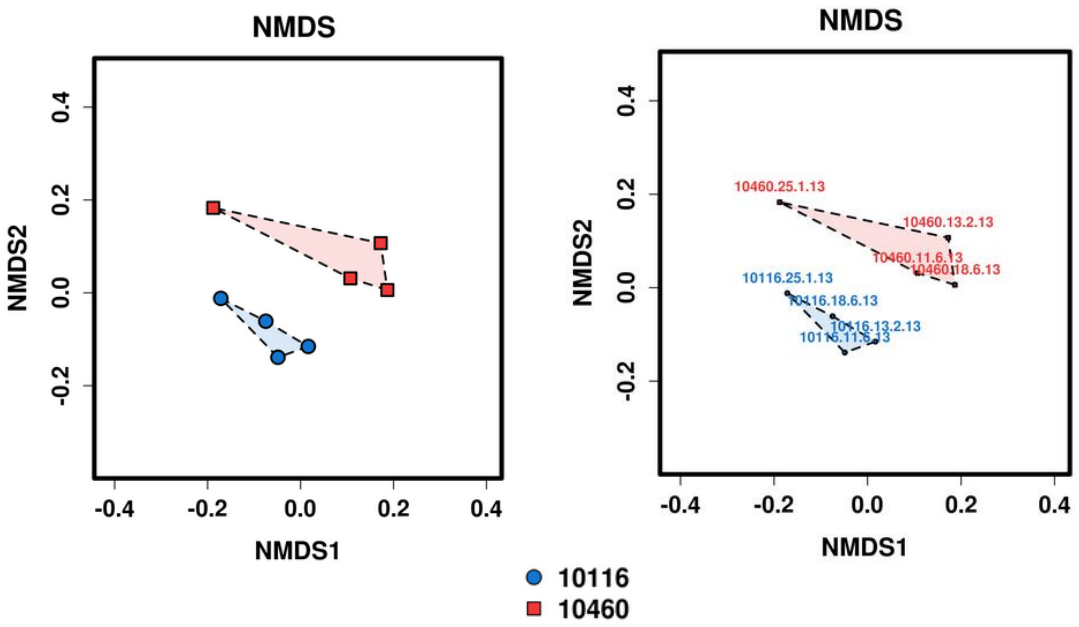


Figure A3.8 nMDS plot based on the Bray-Curtis distance matrix representing the faecal microbial composition of different sheep samples (n=2). Each sample is represented by a single point. Right figure: first five digit indicate the sheep tag; next digits indicate the date of sample collection.

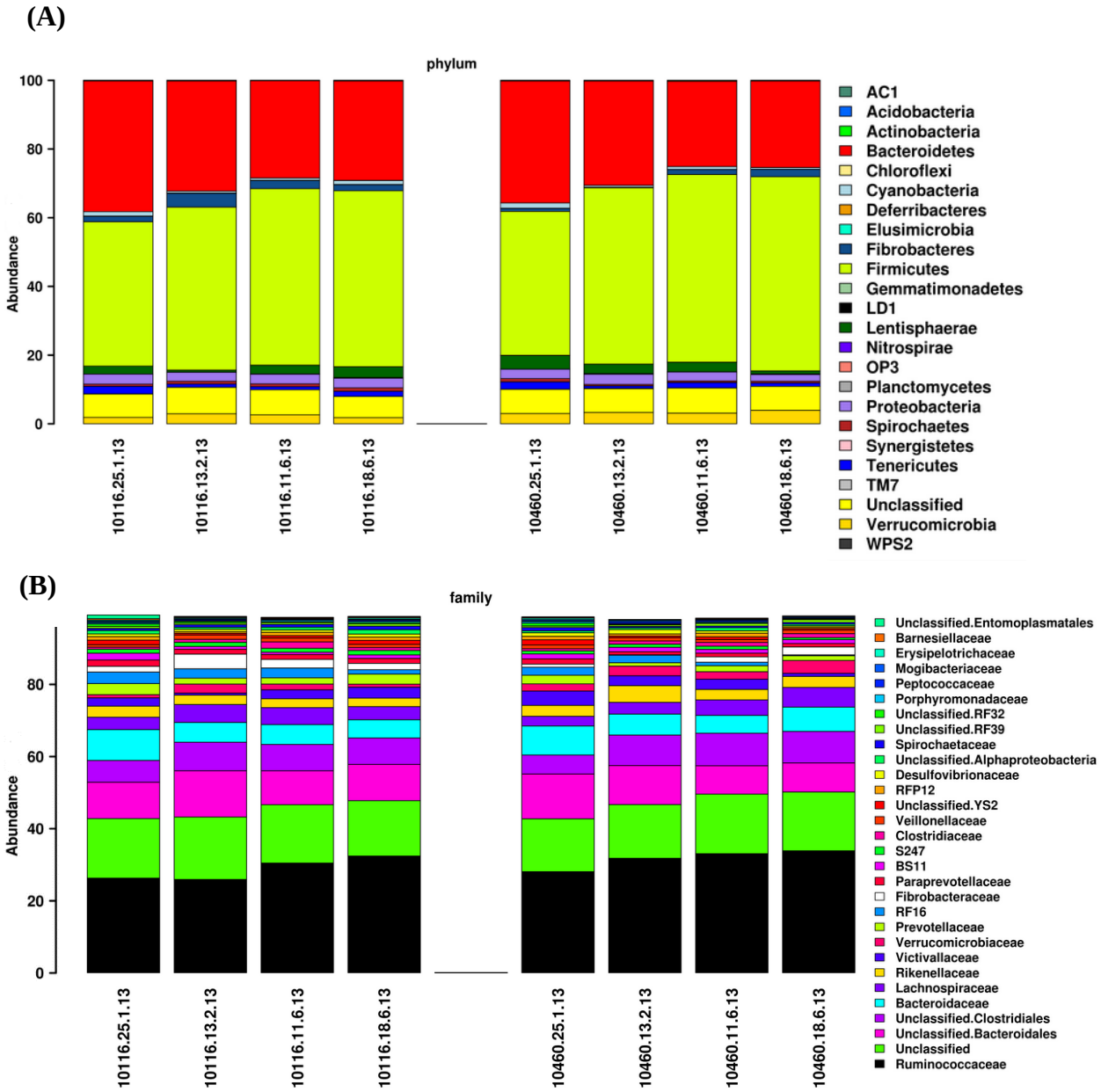


Figure A3.9 Average relative abundances (%) of 30 most abundant taxa from 2 sheep using 16S rRNA sequencing over a 6 months period. (A): Phyla; (B): Family. The Y-axis shows the average relative abundances (%); X-axis shows sample identification (five digit identification code followed by the date of sample collection). First four columns indicate same sheep in week 1, 3, 24 and 25 (left to right) and the second four column indicate another sheep with similar pattern of week samples.

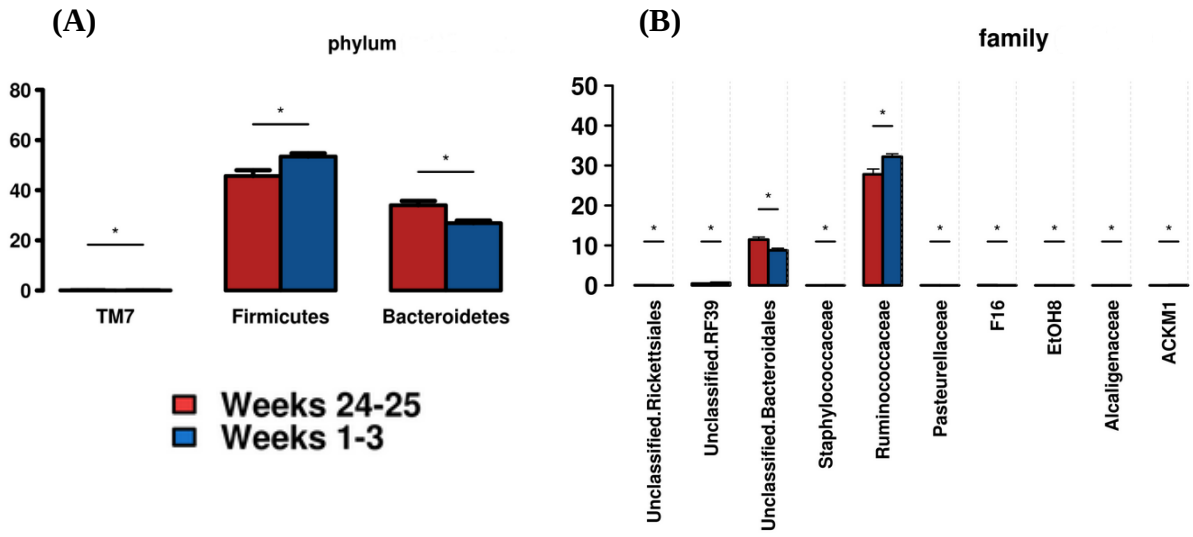


Figure A3.10 Significant differences in relative abundances of taxa of faecal bacteria from 2 sheep samples collected over a 6 months period and analysed by 16S rRNA sequencing. (A): Phyla; (B): Family. The Y-axis shows the average relative abundances (%). Pair-wise comparisons are done by paired t-test; *$p \leq 0.05$.

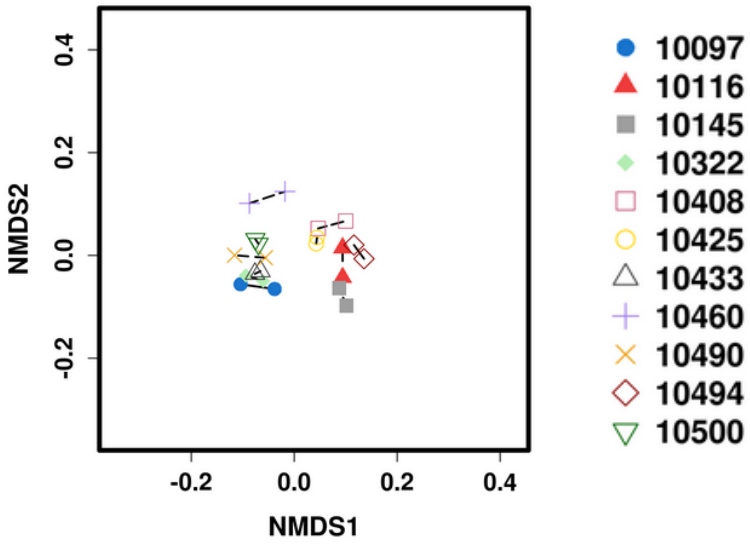


Figure A3.11 nMDS plot based on the Bray-Curtis distance matrix representing the faecal microbial composition of different sheep samples (n=11). Each sample is represented by a single point. Here, different number indicates different sheep tag.

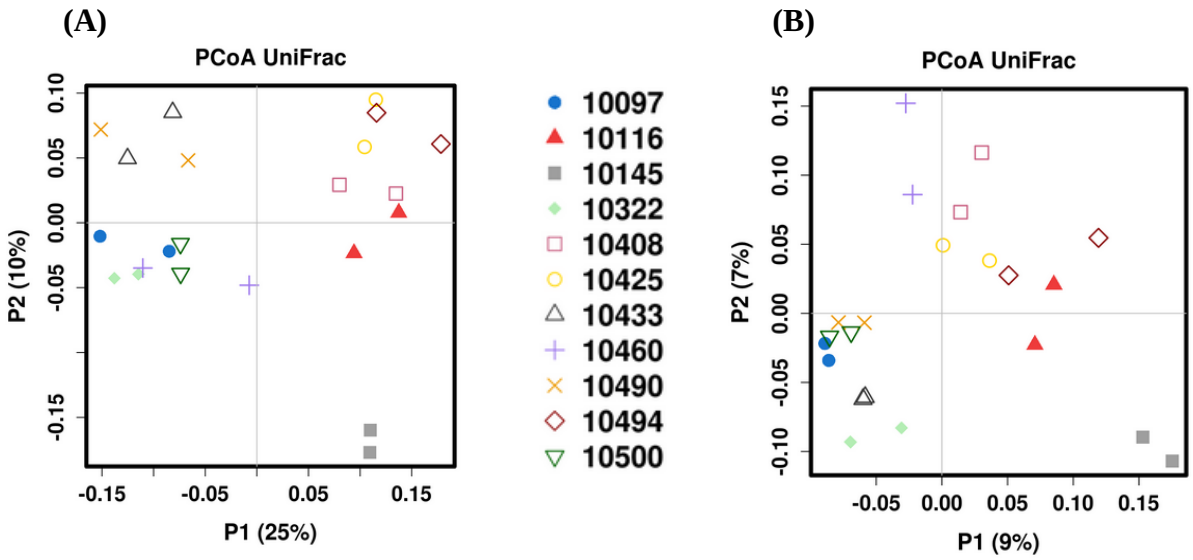


Figure A3.12 Clustering of microbiome between the sheep samples (n=11). PCoA plot of sheep based on weighted Unifrac (A) and un-weighted unifrac (B) distance of faecal microbial composition. Each sample is represented by a single point. Here, different number indicates different sheep tag.

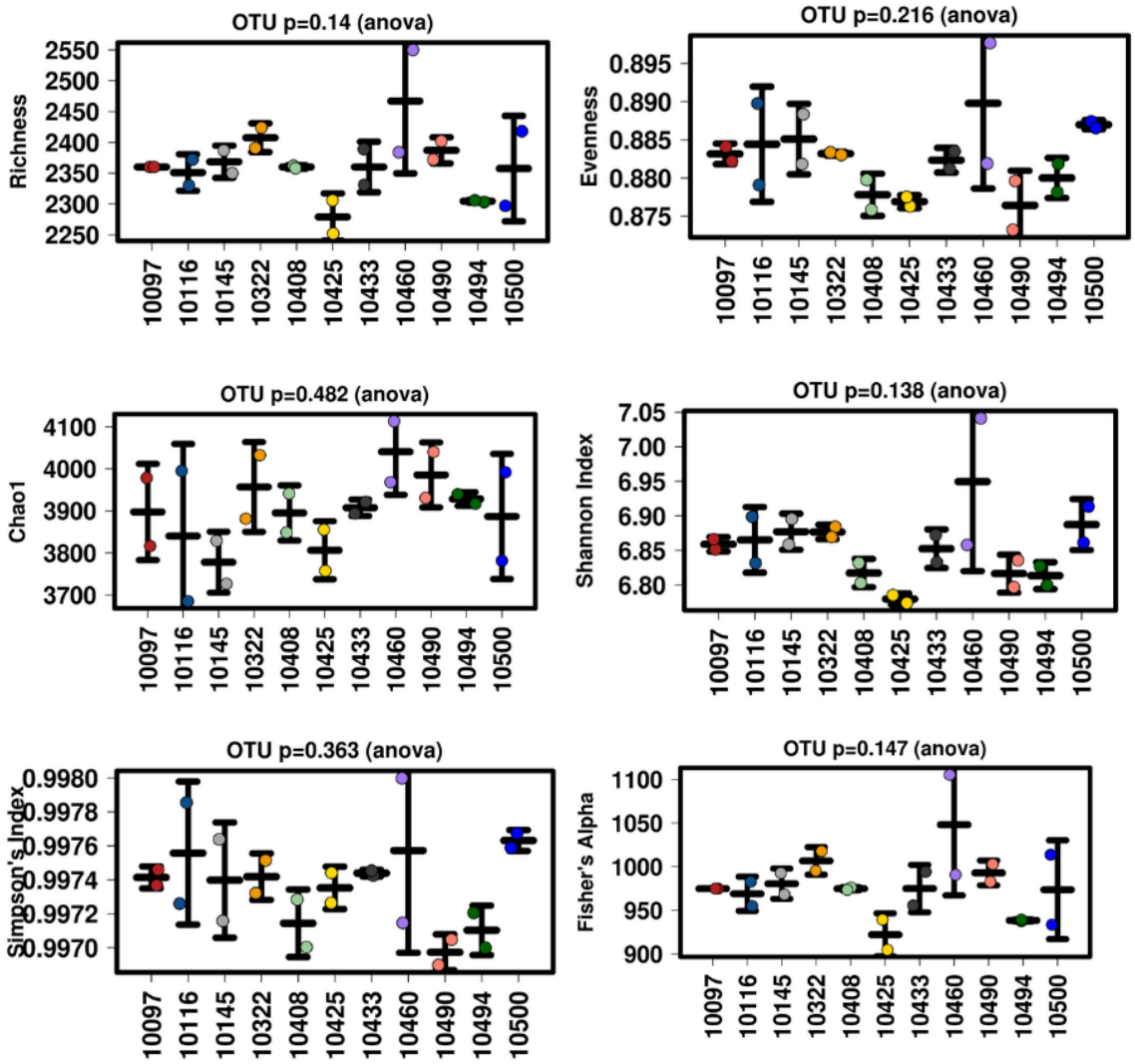


Figure A3.13 Diversity of gut microbiota of sheep (n=11). OTU richness, Evenness, Chao1, Shannon, Simpson and Fisher's alpha of each sheep sample was calculated. ANOVA was conducted to test the significance between the sheep. No significance was recorded.

Table A3.1 Average relative abundances (%) of 50 most abundant family (core bacteria) present in minimum 50% of the samples (n=11) identified by using 16S rRNA sequencing.

Taxa	Type	Abundance (%)	
		Week24	Week 25
Ruminococcaceae	core	29.777	30.044
Unclassified	core	15.824	15.69
Unclassified.Bacteroidales	core	9.731	9.779
Unclassified.Clostridiales	core	9.453	8.915
Lachnospiraceae	core	5.66	5.138
Bacteroidaceae	core	5.135	5.583
Rikenellaceae	core	2.587	2.339
Victivallaceae	core	2.015	2.057
Verrucomicrobiaceae	core	1.983	1.862
Prevotellaceae	core	1.695	2.021
Paraprevotellaceae	core	1.512	1.554
Clostridiaceae	core	1.316	1.12
Fibrobacteraceae	core	1.208	2.068
RF16	core	1.113	1.005
Veillonellaceae	core	1.081	1.063
Spirochaetaceae	core	1.06	0.982
S247	core	0.934	0.895
Desulfovibrionaceae	core	0.834	0.742
Unclassified.YS2	core	0.814	0.819
Unclassified.Alphaproteobacteria	core	0.757	0.896
RFP12	core	0.72	0.716
BS11	core	0.685	0.691
Unclassified.RF39	core	0.605	0.534
Porphyromonadaceae	core	0.312	0.325
Unclassified.RF32	core	0.293	0.281
Peptococcaceae	core	0.275	0.313
Mogibacteriaceae	core	0.273	0.274
Erysipelotrichaceae	core	0.257	0.238
Barnesiellaceae	core	0.215	0.152
Cryomorphaceae	core	0.155	0.113
Geobacteraceae	core	0.143	0.152
Campylobacteraceae	core	0.137	0.137
Christensenellaceae	core	0.129	0.12
Unclassified.ML615J28	core	0.122	0.129
Pirellulaceae	core	0.11	0.152
Enterobacteriaceae	core	0.068	0.031
Unclassified.GMD14H09	core	0.068	0.038
Dehalobacteriaceae	core	0.059	0.051
Flavobacteriaceae	core	0.052	0.06
Sphaerochaetaceae	core	0.044	0.042
Desulfarculaceae	core	0.043	0.036
F16	core	0.035	0.02
ACKM1	core	0.033	0.032
Anaeroplasmataceae	core	0.031	0.028
Coriobacteriaceae	core	0.03	0.027
Mycoplasmataceae	core	0.029	0.028
Unclassified.Mollicutes	core	0.029	0.025
Unclassified.Endomicrobia	core	0.028	0.015
Acidaminobacteraceae	core	0.026	0.039
Listeriaceae	core	0.025	0.031

Table A3.2 Average relative abundances (%) of 50 most abundant species (core bacteria) present in minimum 50% of the samples (n=11) identified by using 16S rRNA sequencing.

Taxa	Type	Abundance (%)	
		Week24	Week25
Unclassified.Ruminococcaceae__Unclassified.Ruminococcaceae	core	23.615	23.761
Unclassified	core	21.258	21.235
Unclassified.Bacteroidales__Unclassified.Bacteroidales	core	9.731	9.779
Unclassified.Clostridiales__Unclassified.Clostridiales	core	9.453	8.915
X57N15__Unclassified.57N15	core	2.822	2.895
Unclassified.Rikenellaceae__Unclassified.Rikenellaceae	core	2.585	2.326
Oscillospira__Unclassified.Oscillospira	core	2.567	2.517
Unclassified.Lachnospiraceae__Unclassified.Lachnospiraceae	core	2.542	2.508
Unclassified.Victivallaceae__Unclassified.Victivallaceae	core	2.015	2.057
Akkermansia__Unclassified.Akkermansia	core	1.904	1.776
Dorea__Unclassified.Dorea	core	1.545	1.139
Prevotella__Unclassified.Prevotella	core	1.539	1.908
Fibrobacter_succinogenes	core	1.208	2.068
CF231__Unclassified.CF231	core	1.172	1.189
Unclassified.RF16__Unclassified.RF16	core	1.113	1.005
Ruminococcus__Unclassified.Ruminococcus	core	1.099	1.155
Treponema__Unclassified.Treponema	core	1.06	0.982
Phascolarctobacterium__Unclassified.Phascolarctobacterium	core	1.028	1.011
Unclassified.S247__Unclassified.S247	core	0.934	0.895
Unclassified.Bacteroidaceae__Unclassified.Bacteroidaceae	core	0.828	0.794
Unclassified.YS2__Unclassified.YS2	core	0.814	0.819
Bacteroides__Unclassified.Bacteroides	core	0.813	1.255
Unclassified.Alphaproteobacteria__Unclassified.Alphaproteobacteria	core	0.757	0.896
Unclassified.RFP12__Unclassified.RFP12	core	0.72	0.716
Unclassified.BS11__Unclassified.BS11	core	0.685	0.691
Unclassified.Clostridiaceae__Unclassified.Clostridiaceae	core	0.644	0.529
Unclassified.RF39__Unclassified.RF39	core	0.605	0.534
Clostridium__Unclassified.Clostridium	core	0.531	0.468
Coprococcus__Unclassified.Coprococcus	core	0.312	0.25
Unclassified.RF32__Unclassified.RF32	core	0.293	0.281
BF311__Unclassified.BF311	core	0.257	0.166
Unclassified.Mogibacteriaceae__Unclassified.Mogibacteriaceae	core	0.219	0.184
Unclassified.Barnesiellaceae__Unclassified.Barnesiellaceae	core	0.215	0.152
Unclassified.Peptococcaceae__Unclassified.Peptococcaceae	core	0.176	0.185
Fluviicola__Unclassified.Fluviicola	core	0.145	0.1
Geobacter__Unclassified.Geobacter	core	0.143	0.152
Campylobacter__Unclassified.Campylobacter	core	0.137	0.137
Unclassified.Christensenellaceae__Unclassified.Christensenellaceae	core	0.129	0.12
Unclassified.ML615J28__Unclassified.ML615J28	core	0.122	0.129
Unclassified.Pirellulaceae__Unclassified.Pirellulaceae	core	0.11	0.152
Ruminococcus_flavefaciens	core	0.105	0.157
Unclassified.Desulfovibrionaceae__Unclassified.Desulfovibrionaceae	core	0.105	0.093
Unclassified.Erysipelotrichaceae__Unclassified.Erysipelotrichaceae	core	0.094	0.089
Paludibacter__Unclassified.Paludibacter	core	0.093	0.092
Unclassified.Verrucomicrobiaceae__Unclassified.Verrucomicrobiaceae	core	0.079	0.083
Desulfovibrio__Unclassified.Desulfovibrio	core	0.077	0.075
Unclassified.Enterobacteriaceae__Unclassified.Enterobacteriaceae	core	0.068	0.031
Unclassified.GMD14H09__Unclassified.GMD14H09	core	0.068	0.038
Butyrivibrio__Unclassified.Butyrivibrio	core	0.061	0.039
Pelotomaculum__Unclassified.Pelotomaculum	core	0.056	0.051

Table A3.3 ANOSIM pairwise comparison of R values of 28 sheep samples based on the Bray-Curtis similarity matrix.

	10097	10106	10116	10145	10169	10237	10261	10269	10272	10275	10322	10323	10343	10368	10381	10400	10408	10425	10431	10433	10455	10460	10474	10490	10494	10500	10538
10097																											
10106	**0.7**																										
10116	**0.8**	**0.7**																									
10145	**1.0**	**1.0**	**0.9**																								
10169	**0.5**	**0.7**	**0.9**	**1.0**																							
10237	0.3	**0.9**	**1.0**	**1.0**	**0.6**																						
10261	**0.4**	**0.9**	**0.6**	**0.9**	**0.8**	**0.6**																					
10269	0.4	**0.2**	**0.7**	**1.0**	0.2	**0.5**	0.5																				
10272	**0.9**	**0.9**	**0.9**	**0.9**	**1.0**	**0.9**	**0.7**	0.6																			
10275	**0.5**	**0.4**	**0.6**	**1.0**	**0.7**	**0.7**	**0.5**	**0.5**	**0.8**																		
10322	**0.6**	**0.9**	**0.9**	**1.0**	**1.0**	**1.0**	**0.8**	**0.6**	**0.7**	**0.8**																	
10323	**0.5**	**0.6**	**0.7**	**0.9**	**0.5**	0.1	**0.4**	**0.4**	**0.7**	**0.7**	**0.7**																
10343	0.4	**0.4**	**0.4**	**0.7**	0.4	0.5	0.4	0.3	**0.5**	0.3	**0.4**	0.2															
10368	**0.5**	**0.6**	**0.6**	**1.0**	**0.7**	**0.8**	0.4	0.3	**0.8**	**0.7**	**0.6**	**0.6**	**0.4**														
10381	**0.5**	**0.5**	**0.6**	**1.0**	0.4	**0.5**	**0.6**	0.3	**0.7**	0.2	**0.7**	**0.4**	0.3	**0.6**													
10400	**0.7**	**0.9**	**0.7**	**1.0**	**0.9**	**0.8**	**0.6**	**0.6**	**0.5**	**0.8**	**1.0**	**0.6**	**0.6**	0.5	**0.6**												
10408	**0.7**	**0.8**	**0.5**	**1.0**	**0.9**	**0.8**	**0.7**	**0.6**	**0.8**	**0.6**	**1.0**	**0.6**	**0.5**	**0.6**	**0.6**	**0.8**											
10425	0.4	**0.8**	**0.6**	**1.0**	**0.8**	**0.7**	**0.5**	0.5	**0.9**	**0.4**	**0.8**	**0.5**	0.3	**0.5**	0.4	**0.7**	0.5										
10431	**0.6**	**0.7**	**0.7**	**1.0**	**0.8**	**0.8**	**0.6**	**0.4**	**0.6**	**0.6**	**1.0**	**0.4**	**0.4**	**0.6**	**0.4**	**0.9**	**0.9**	**0.7**									
10433	0.3	**0.4**	**0.5**	**1.0**	**0.5**	**0.6**	**0.5**	0.2	**0.7**	**0.4**	**0.6**	**0.5**	0.4	**0.6**	0.4	**0.7**	**0.4**	**0.6**	**0.4**								
10455	**0.7**	**0.6**	**0.6**	**1.0**	**0.9**	**0.8**	**0.5**	**0.5**	**0.8**	0.4	**0.9**	**0.5**	0.3	**0.5**	**0.5**	**0.8**	**0.9**	**0.5**	**0.8**	**0.5**							
10460	**0.7**	**0.8**	**0.8**	**1.0**	**0.6**	**0.7**	**0.7**	0.3	**0.7**	**0.6**	**1.0**	**0.4**	0.4	**0.7**	**0.6**	**0.9**	**0.8**	**0.8**	**0.9**	**0.4**	**0.7**						
10474	0.4	**0.7**	**0.8**	**1.0**	**0.4**	**0.8**	**0.7**	0.1	**0.9**	**0.9**	**0.9**	**0.5**	0.4	**0.7**	0.4	**0.9**	**0.9**	**0.8**	**0.8**	0.4	**0.8**	**0.8**					
10490	**0.4**	**0.9**	**0.8**	**1.0**	**0.5**	**0.6**	**0.7**	0.4	**0.9**	**0.8**	**1.0**	**0.5**	0.3	**0.6**	**0.6**	**0.9**	**0.9**	**0.7**	**0.9**	**0.5**	**1.0**	**0.8**	0.1				
10494	**0.6**	**0.5**	**0.4**	**1.0**	**0.8**	**0.7**	**0.7**	**0.5**	**0.8**	**0.8**	**0.8**	**0.6**	**0.4**	**0.6**	**0.6**	**0.6**	**0.5**	**0.5**	**0.7**	**0.6**	**0.7**	**0.7**	**0.6**	**0.7**			
10500	**0.5**	**0.8**	**0.8**	**1.0**	**0.5**	**0.7**	**0.8**	**0.5**	**1.0**	**0.8**	**1.0**	**0.5**	0.4	**0.8**	**0.6**	**1.0**	**1.0**	**0.7**	**1.0**	**0.5**	**0.9**	**0.8**	**0.7**	**0.6**	**0.7**		
10538	0.2	**0.5**	**0.6**	**0.8**	0.3	0.2	0.0	0.1	**0.7**	**0.6**	**0.5**	0.1	0.3	0.1	0.4	**0.5**	**0.4**	0.3	0.3	0.2	**0.4**	0.4	0.2	**0.4**	**0.6**	**0.5**	
10563	**0.4**	**0.6**	**0.8**	**1.0**	**0.7**	0.4	**0.5**	0.3	**0.7**	**0.5**	**0.9**	0.3	0.3	0.3	0.4	**0.7**	**0.8**	0.5	**0.5**	**0.4**	0.3	0.4	**0.6**	**0.8**	**0.6**	**0.7**	0.1

R values which are significant ($p \leq 0.05$) are green shaded with bold numbers.

Appendix 4

H. contortus larvae (L3) culture

To collect *H. contortus* L3 for subsequent experimental infection (Section 4.2.3), 5-6 month old Merino crossbreed lambs were transported to the Monash Animal Facility, Gippsland. After arrival, animals were treated with anthelmintic (Cydectin®) and fed *ad libitum* with no outdoor access for grazing, and left for 2 weeks for acclimatization. After animals were confirmed as uninfected by individual FEC, each animal was infected with 8000 *H. contortus* L3 larvae /animal, using Novartis Haecon-5 strain of *H. contortus*. To do so, L3 larvae were suspended in 4ml dH_2O and dispensed into a biodegradable gelatin capsule (7ml capacity capsules; Torpac®). The lambs were appropriately restrained and a bolus gun containing the gelatin capsule with the L3 larvae was inserted into the side of the mouth of the sheep and dispensed into the oesophagus.

At 28 days post-infection, FEC were performed twice a week by using modified McMaster technique to monitor the infection level (Appendix 4). Once FEC were >1000 EPG, faeces collection occurred daily. Faecal collection involved securely fitting a faecal collection bag around the animal's rear to collect the faecal pellets. The bag was emptied every 12 hours. After collection, faecal material was transferred into 50 x 30 cm plastic trays, lightly dampened with water from a spray bottle and covered with clear sheets of plastic. The trays containing the faeces were set up close to a light source and were placed on heated pet mats (28^oC, Paws N Claws, 43 cm x 58 cm) to help maintain a suitable temperature and stimulate egg hatching (Figure 4.1).

Water mist was sprayed every 24 hrs to maintain the moisture of the faeces. After 10 days evidence of egg hatching and L3 larval development was assessed (peanut colour like

deposition on the surface of the plastic sheets). If any such deposition was observed on the plastic sheets, water was sprayed to droplet off the plastic sheets into a plastic beaker and was transfered the liquid to a sterile 175cm^2 flask (BD Falcon^{TM}, USA). The flask was placed under light microscope at 40 × magnification to check for *H. contortus* L3 and stored at 4ºC until use.

The stored *H. contortus* L3 were filtered at 4 week intervals to separate dead or non-viable L3, restrain bacterial/fungal contamination, and remove general debris. The details of the cleanning and filtering *H. contortus* L3 described below in Appendix 4.

Securely fitted faecal collection bag

Collection of faeces from the bag

Faeces in the plastic tray for hatching and larval development

Figure A4.1 Collection of faeces from sheep and culture of larvae in the wet laboratory. Photo courtesy: Md Shakif-Ul-Azam.

Cleaning and filtering of L3

- A circular plastic container was filled up with cold tap water and on top of it, placed a piece of 20 µm nylon cloth sieve wedged between the two circular plastic containers.
- The Falcon flask containing L3 was poured on the top of the sieve.
- A light source was fixed to shine the sieve which caused the L3 to actively migrate away from the light towards the bottom of the container.
- After 24 hours, L3 were collected by removing the nylon cloth sieve and carefully emptying the water from the top of the container by suction.
- When approximately 150 ml of water was left in the container, the migrated L3 were collected by transferring liquid into new sterile 175cm^2 flask and stored at 4°C. The nylon sieve containing dead L3 and fungal spores, after 24 hour incubation with 1% Iodine (Fronine Laboratory supplies, Australia) washed with fresh cold water several times and autoclave for future uses.

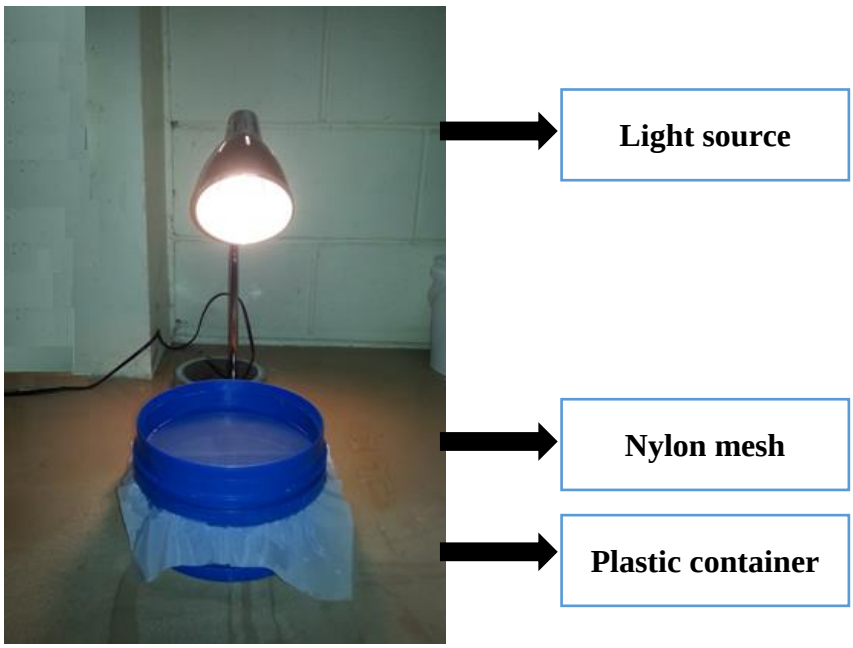


Figure A4.2 Filtration of collected and stored *H. contortus* L3. Photo courtesy: Md Shakif-Ul-Azam

Modified McMaster method

- Fresh faecal sample (around 2 grams) were directly collected from sheep rectum.
- The faecal sample were broken up until particles were evenly distributed.
- 60 ml of saturated salt solution were added and mixed uniformly using a special bit on an electric drill (min rpm 2000).
- The suspension was filtered through a tea strainer, and kept for 5 minutes.
- Filtrate was stirred and by using a sterile Pasteur pipette suspension was placed in a Whitlock McMaster egg counting chamber (JA Whitlock & Co, NSW, Australia).
- Two separate chambers were counted to provide an average count. The chamber holds 0.5 ml of solution and the dilution factor from the mixture is 1:25. Mean chamber counts were multiplied by 50 to gain the measurement of eggs per gram of faeces (EPG).

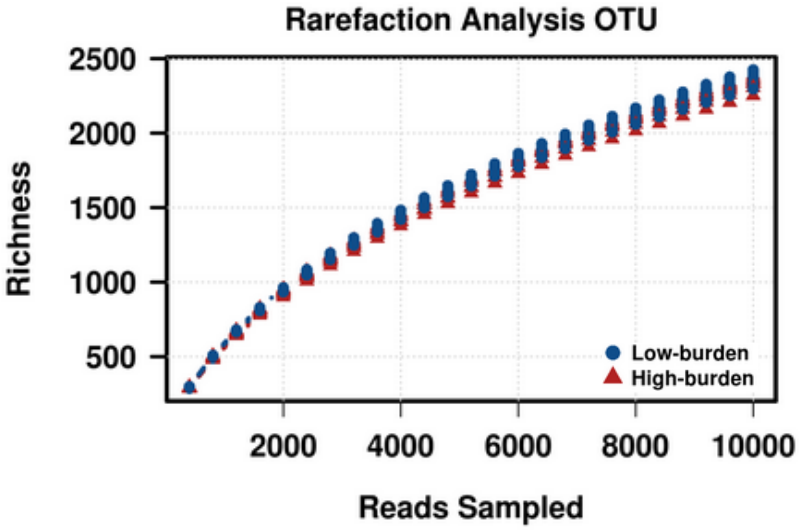


Figure A4.3 Rarefaction curve based on OTU richness values.

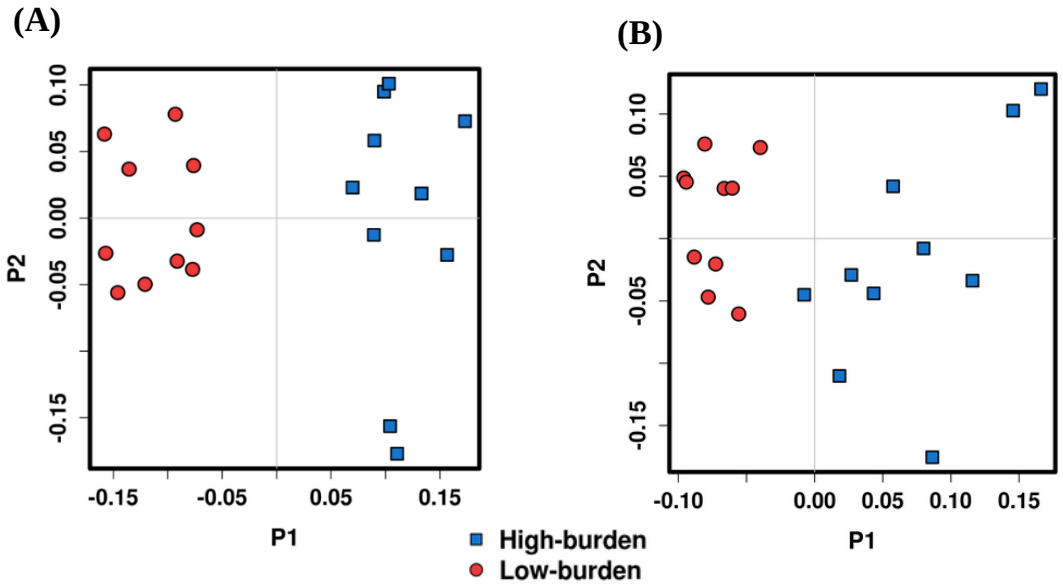


Figure A4.4 Distinct clustering of microbiome between high and low worm burden sheep. PCoA plot of sheep based on weighted UniFrac (A) and un-weighted UniFrac (B) distance of faecal microbial composition. Each symbol represents an individual sheep sample.

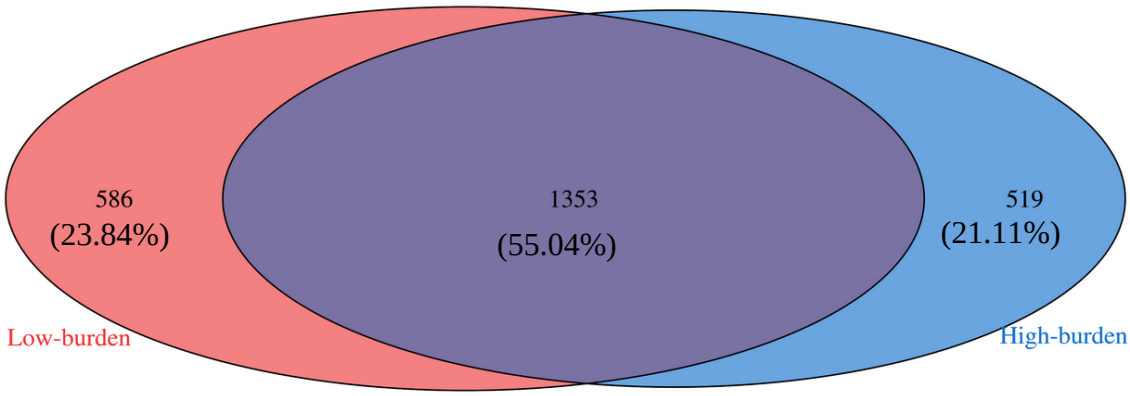


Figure A4.5 Venn diagram representing the core OTUs in group of sheep with high and low burden of parasite. A bacterial group was considered to be present in a sample group if it was identified in at least 50% of the samples within the group.

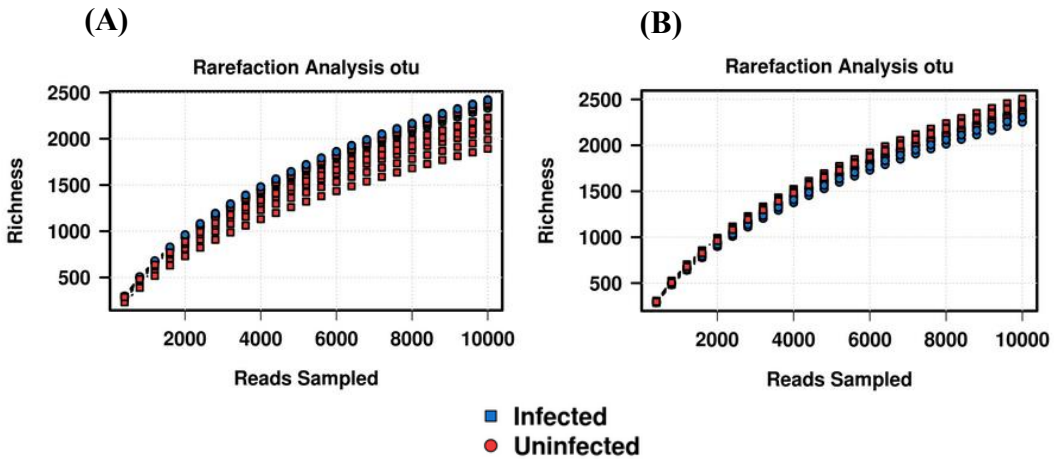


Figure A4.6 Rarefaction curve based on OTU richness values. (A): High-burden sheep; (B): low-burden sheep.

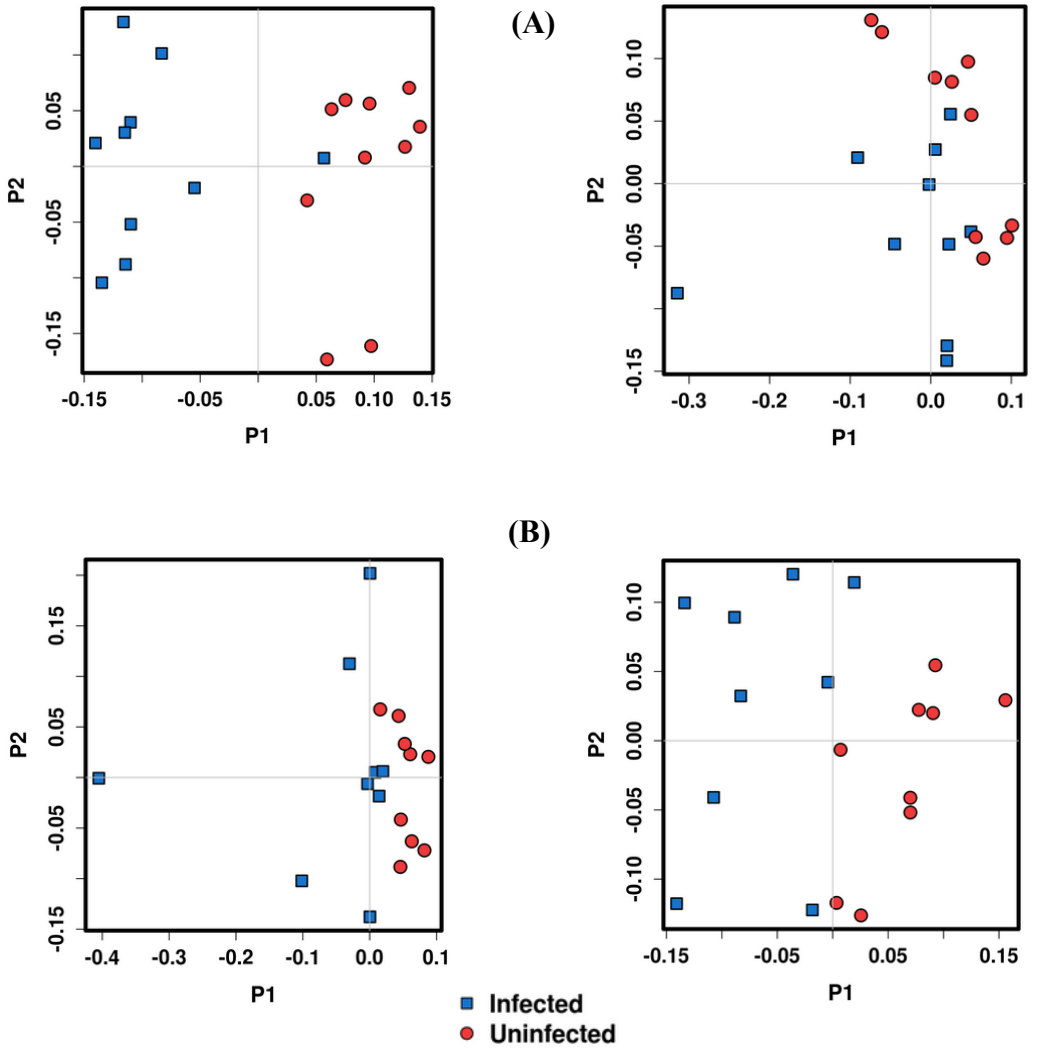


Figure A4.7 Clustering of microbiome of infected and uninfected sheep. (A): high-burden; (B): low-burden sheep. PCoA plot of sheep based on weighted (left) and unweighted (right) UniFrac distance of gut microbial composition. Each symbol represents an individual sheep sample.

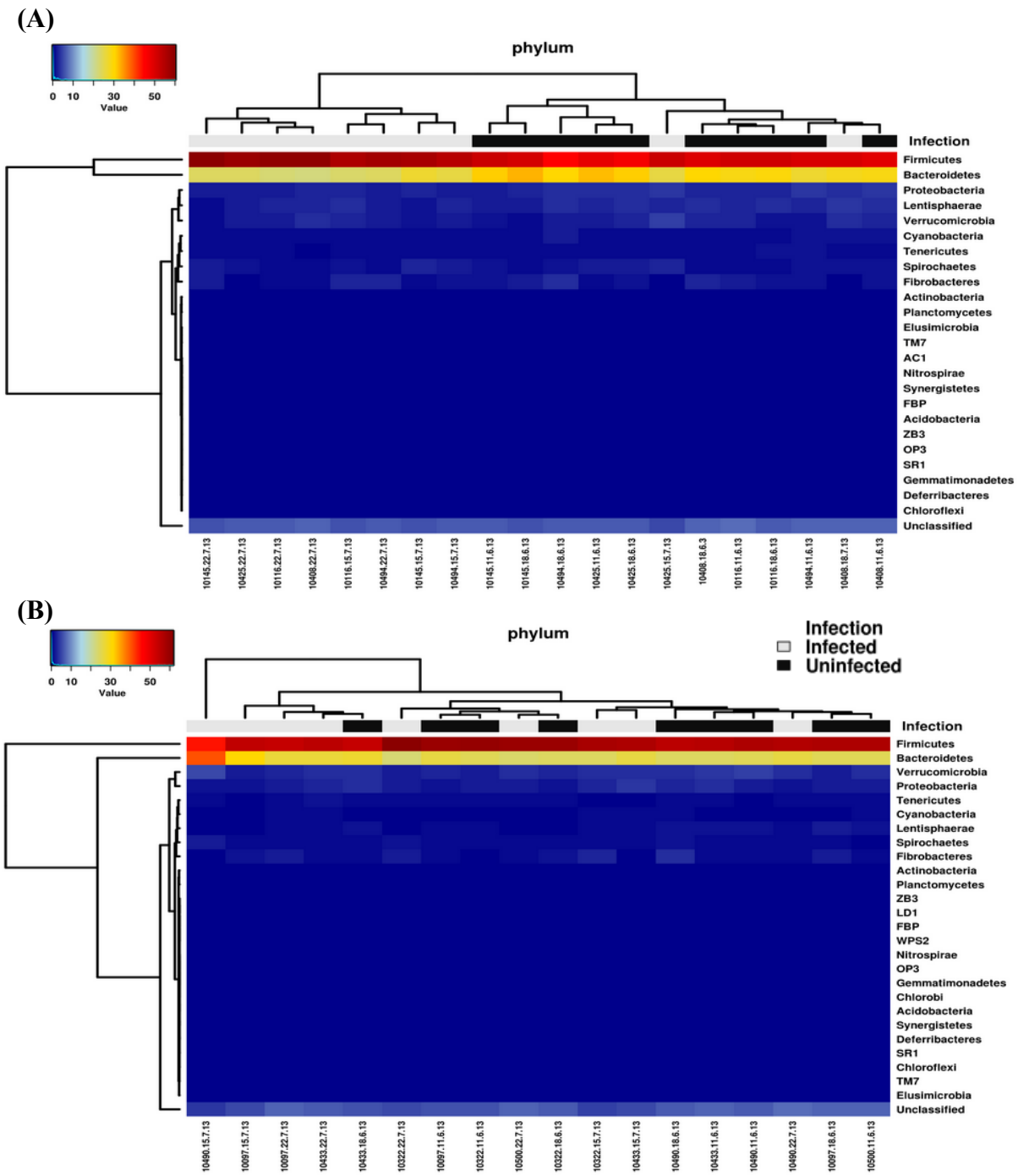


Figure A4.8 HeatMap+ of the relative abundances of the identified phyla in uninfected and infected sheep. (A): high-burden; (B): low-burden sheep. The maps showed marked differences in relative abundances in high-burden sheep compared to low-burden sheep.

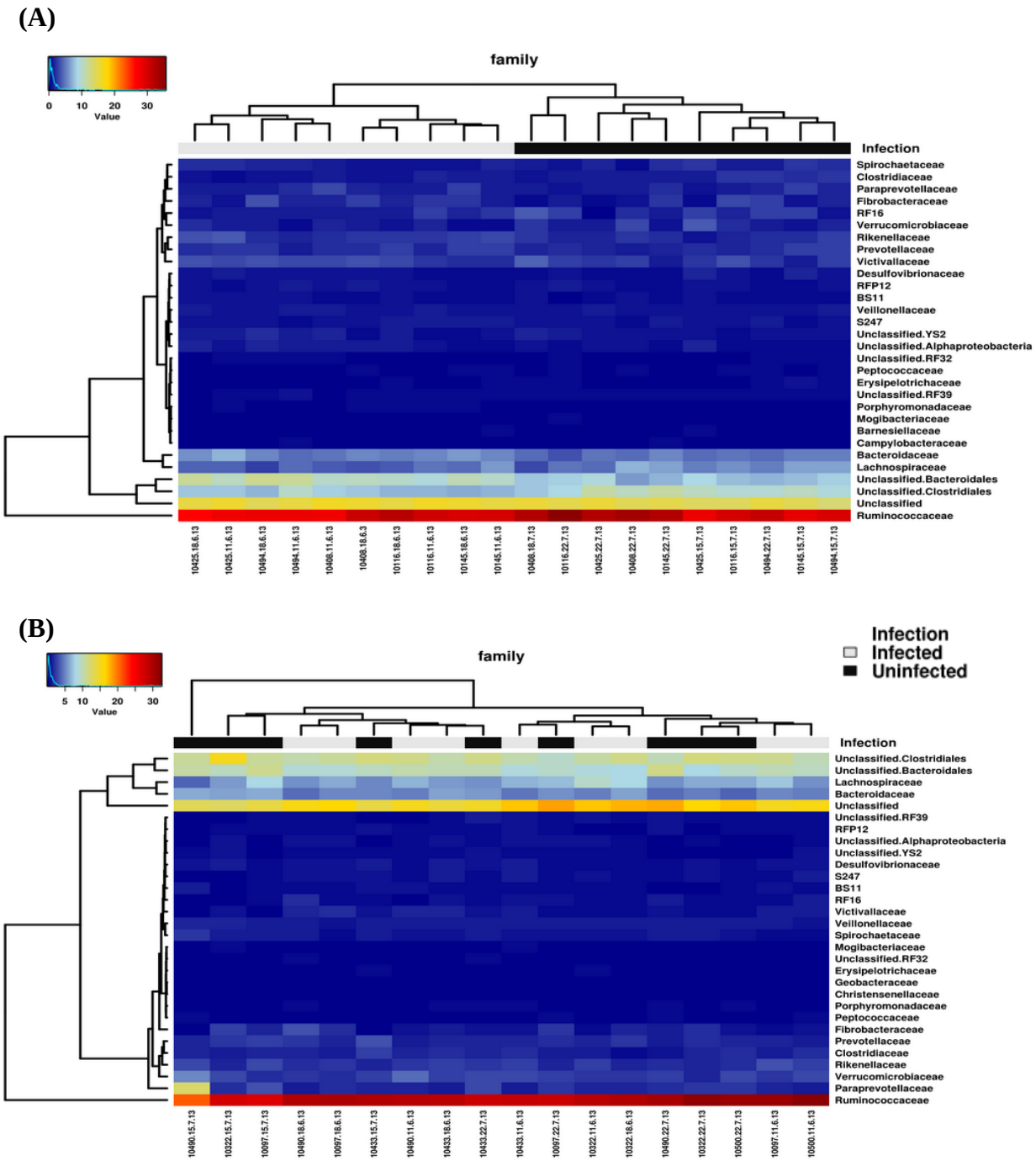


Figure A4.9 HeatMap+ of the relative abundances of the 30 dominant family in uninfected and infected sheep. (A): high-burden; (B): low-burden sheep. The maps showed marked differences in relative abundances in high-burden sheep compared to low-burden sheep.

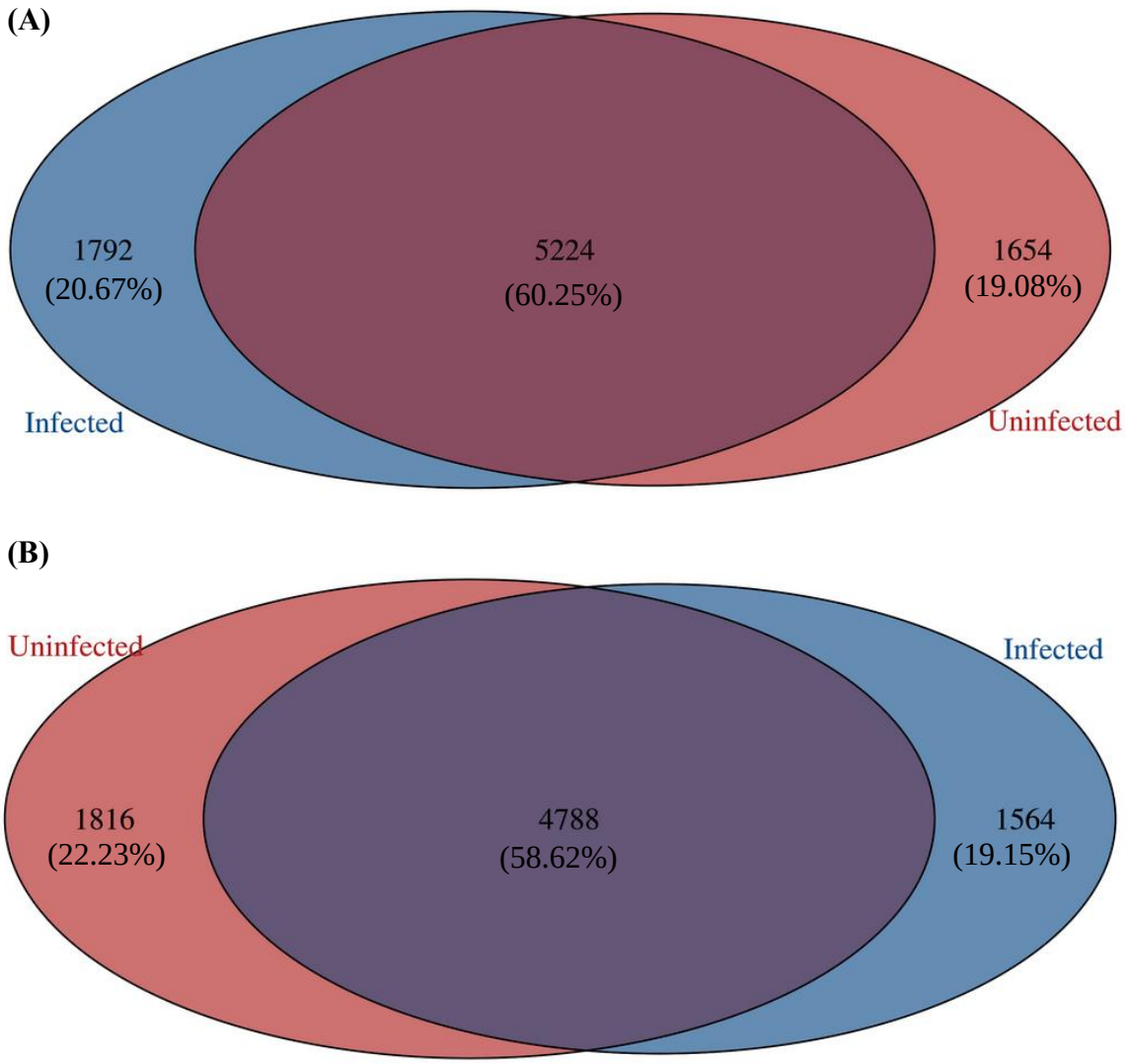


Figure A4.10 Venn diagram representing the shared and unique OTUs present in sheep before and after infection. (A): high-burden sheep; (B) low-burden sheep. A bacterial group was marked as present in a sample group if it was identified in at least 10% of the samples within the group.

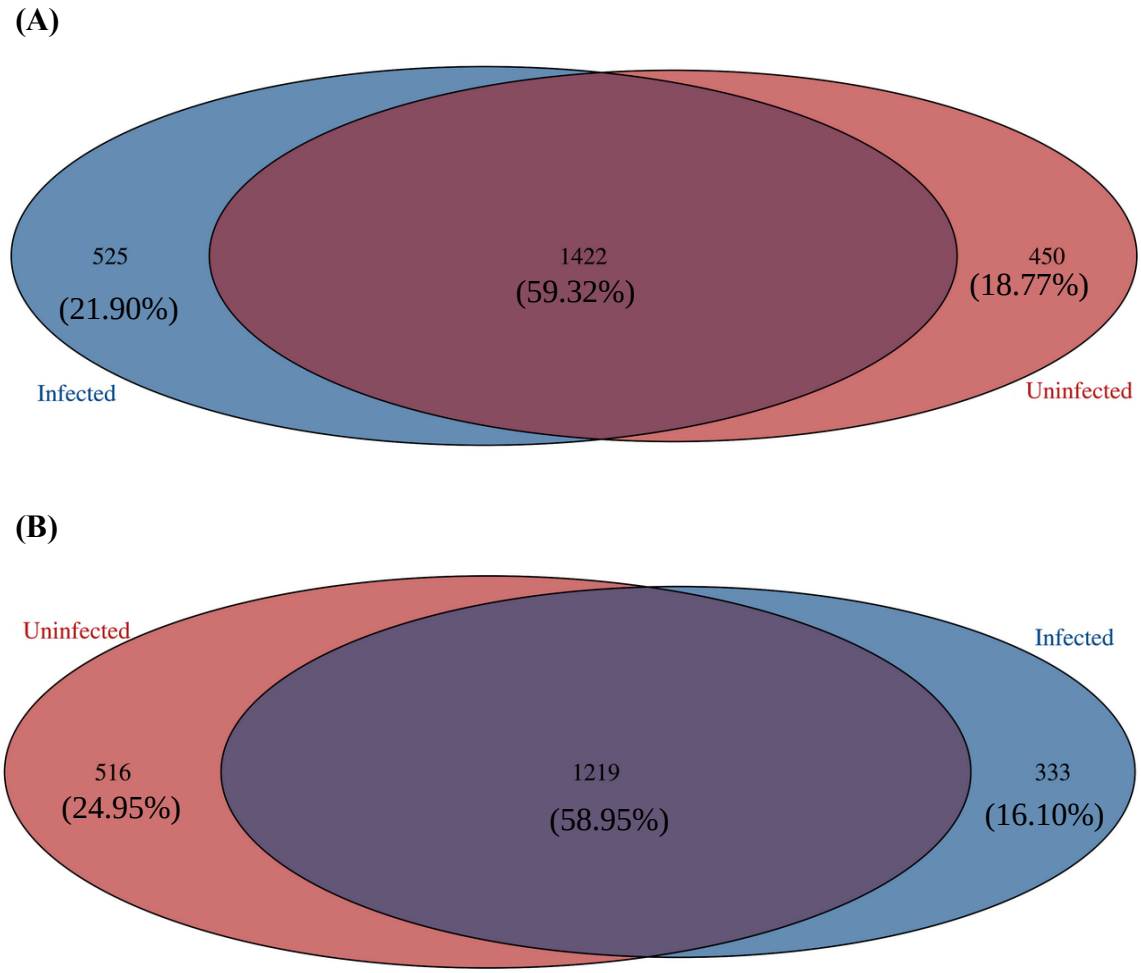


Figure A4.11 Venn diagram representing the core OTUs present in sheep before and after infection. (A): high-burden sheep; (B) low-burden sheep. An OTU was considered as core if it was identified in at least 50% of the samples within the group.

Table A4.1 The 50 most abundant core OTUs with different abundances between the high and low parasite burden sheep.

Taxa	Relative abundance (%)	
	High-burden	**Low-burden**
p__Fibrobacteres__g__Fibrobacter__s__succinogenes_OTU_1	1.495	0.943
p__Bacteroidetes__g__CF231_OTU_2	1.133	1.016
p__Bacteroidetes__g__57N15_OTU_5	0.890	0.803
p__Bacteroidetes__g__Prevotella_OTU_747	0.828	0.629
p__Bacteroidetes__o__Bacteroidales_OTU_11	0.826	0.337
p__Bacteroidetes__o__Bacteroidales_OTU_3	0.824	0.987
p__Bacteroidetes__o__Bacteroidales_OTU_15	0.751	0.527
p__Bacteroidetes__o__Bacteroidales_OTU_9310	0.697	0.773
p__Bacteroidetes__g__57N15_OTU_4	0.686	0.773
p__Proteobacteria__f__Desulfovibrionaceae_OTU_18	0.658	0.579
p__Lentisphaerae__f__Victivallaceae_OTU_31	0.637	0.317
p__Bacteroidetes__f__Rikenellaceae_OTU_23	0.613	0.504
p__Firmicutes__f__Ruminococcaceae_OTU_29	0.538	0.303
p__Bacteroidetes__f__Rikenellaceae_OTU_39	0.528	0.393
p__Bacteroidetes__o__Bacteroidales_OTU_285	0.526	0.33
p__Bacteroidetes__g__Bacteroides_OTU_33	0.499	0.327
p__Firmicutes__o__Clostridiales_OTU_42	0.494	0.602
p__Firmicutes__g__Phascolarctobacterium_OTU_8	0.492	0.524
p__Bacteroidetes__o__Bacteroidales_OTU_82	0.482	0.252
p__Firmicutes__g__Phascolarctobacterium_OTU_7	0.475	0.52
p__Bacteroidetes__o__Bacteroidales_OTU_40	0.472	0.372
p__Spirochaetes__g__Treponema_OTU_16	0.467	0.263
p__Firmicutes__f__Ruminococcaceae_OTU_17	0.454	0.447
p__Bacteroidetes__o__Bacteroidales_OTU_58	0.423	0.301
p__Bacteroidetes__g__57N15_OTU_19	0.420	0.424
p__Firmicutes__g__Oscillospira_OTU_20	0.414	0.399
p__Bacteroidetes__o__Bacteroidales_OTU_35	0.412	0.301
p__Bacteroidetes__f__BS11_OTU_59	0.404	0.296
p__Proteobacteria__c__Alphaproteobacteria_OTU_52	0.399	0.283
p__Bacteroidetes__f__RF16_OTU_28	0.398	0.126
p__Bacteroidetes__o__Bacteroidales_OTU_149	0.386	0.327
p__Firmicutes__f__Ruminococcaceae_OTU_147	0.371	0.325
p__Firmicutes__f__Ruminococcaceae_OTU_80	0.364	0.376
p__Bacteroidetes__f__Bacteroidaceae_OTU_25	0.363	0.331
p__Bacteroidetes__f__Rikenellaceae_OTU_22	0.360	0.366
p__Lentisphaerae__f__Victivallaceae_OTU_168	0.355	0.187
p__Firmicutes__f__Clostridiaceae_OTU_27	0.348	0.352
p__Firmicutes__f__Ruminococcaceae_OTU_72	0.339	0.128
p__Bacteroidetes__g__57N15_OTU_48	0.330	0.400
k__Unassigned_OTU_41	0.326	0.436
p__Bacteroidetes__g__Bacteroides_OTU_47	0.325	0.418
p__Bacteroidetes__o__Bacteroidales_OTU_12	0.324	0.403
p__Lentisphaerae__f__Victivallaceae_OTU_117	0.318	0.157
p__Bacteroidetes__f__RF16_OTU_37	0.308	0.22
p__Firmicutes__o__Clostridiales_OTU_200	0.305	0.209
p__Bacteroidetes__o__Bacteroidales_OTU_152	0.298	0.181
p__Verrucomicrobia__g__Akkermansia_OTU_9	0.297	0.657
p__Bacteroidetes__f__RF16_OTU_65	0.278	0.164
p__Bacteroidetes__g__Prevotella_OTU_30	0.278	0.212
p__Firmicutes__f__Ruminococcaceae_OTU_112	0.277	0.093

Table A4.2 Shared and unique taxa present in high and low parasite burden sheep. A bacterial group was considered to be present in a sample group if it was identified in at least 10% of the samples within the group.

Taxa	Unique taxa		Shared	Total count
	High-burden	Low-burden		
Phyla	0	6	19	25
Class	1	6	38	45
Order	6	11	63	80
Family	17	16	100	133
Genus	30	26	144	200
Species	31	32	146	209
OTU	1768	1711	5110	8589

Table A4.3 Shared and unique taxa present in uninfected and infected group of sheep with high and low parasite burden. A bacterial group was considered to be present in a sample group if it was identified in at least 10% of the samples within the group.

Taxa	High-burden sheep				Low-burden sheep			
	Unique taxa		Shared	Total count	Unique taxa		Shared	Total count
	Un-infected	Infected			Un-infected	Infected		
Phyla	0	6	19	25	0	4	23	27
Class	2	7	37	46	2	6	41	49
Order	5	10	64	79	4	10	67	81
Family	8	15	109	132	9	20	104	133
Genus	19	33	155	207	18	32	150	200
Species	23	37	154	214	23	36	152	211
OTU	1654	1792	5224	8670	1816	1564	4788	8168

Table A4.4 The 30 most abundant genera (out of 214) with different abundances between the uninfected and infected sheep with high parasite burden (n=20). Wilcoxon signed-rank test; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

Taxa (genus)	Mean		Median		P value
	Uninfected	Infected	Uninfected	Infected	
Unclassified.Ruminococcaceae	23.65	24.86	23.84	24.77	0.28
Unclassified	19.97	19.91	19.98	19.88	0.82
Unclassified.Bacteroidales	10.78	7.95	10.48	7.93	0.0002***
Unclassified.Clostridiales	7.84	10.12	7.61	10.17	0.0004***
Unclassified.Victivallaceae	2.82	2.45	2.97	2.49	0.25
X57N15	2.76	2.7	2.82	2.7	1
Unclassified.Rikenellaceae	2.56	1.87	2.4	1.85	0.038*
Oscillospira	2.52	2.44	2.59	2.43	0.52
Unclassified.Lachnospiraceae	2.37	2.61	2.4	2.63	0.24
Prevotella	2.17	1.85	2.16	1.74	0.17
Fibrobacter	1.93	1.37	1.75	0.98	0.14
Unclassified.RF16	1.45	2.01	1.21	2.24	0.16
Treponema	1.36	1.51	1.46	1.4	0.55
CF231	1.31	1	1.06	0.94	0.045
Ruminococcus	1.23	2.11	1.19	2.14	0.0003***
Bacteroides	1.19	0.86	1.25	0.82	0.004**
Akkermansia	1.13	1.74	1.16	1.44	0.14
Unclassified.YS2	1.12	0.93	1.1	0.88	0.6
Unclassified.Alphaproteobacteria	1.07	0.75	1.16	0.53	0.11
Unclassified.S247	1	0.86	0.96	0.85	0.12
Phascolarctobacterium	0.99	0.86	0.98	0.86	0.05*
Unclassified.RFP12	0.85	0.82	0.87	0.82	0.91
Unclassified.Bacteroidaceae	0.82	0.67	0.8	0.64	0.13
Unclassified.BS11	0.7	0.58	0.68	0.57	0.20
Unclassified.Clostridiaceae	0.55	0.86	0.49	0.6	0.27
Dorea	0.54	1.36	0.48	1.27	0.008**
Unclassified.RF39	0.5	0.51	0.48	0.46	0.79
BF311	0.38	0.097	0.29	0.03	0.015*
Clostridium	0.37	0.51	0.39	0.48	0.017*
Unclassified.RF32	0.34	0.32	0.34	0.32	0.88

Table A4.5 The 30 most abundant OTUs with different abundances between the uninfected and infected sheep with high parasite burden (n=20). Wilcoxon signed-rank test; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

OTUs	Mean		Median		P value
	Un-infected	Infected	Un-infected	Infected	
p__Fibrobacteres__g__*Fibrobacter*__s__*succinogenes*_OTU_1	1.5	1.07	1.58	0.68	0.11
p__Bacteroidetes__g__CF231_OTU_2	1.13	0.77	0.92	0.78	0.04*
p__Bacteroidetes__g__57N15_OTU_5	0.89	0.78	0.8	0.78	0.65
p__Bacteroidetes__g__*Prevotella*_OTU_747	0.83	0.63	0.84	0.64	0.27
p__Bacteroidetes__o__Bacteroidales_OTU_3	0.82	0.61	0.7	0.53	0.12
p__Bacteroidetes__o__Bacteroidales_OTU_11	0.83	0.55	0.86	0.51	0.01*
p__Proteobacteria__f__Desulfovibrionaceae_OTU_18	0.66	0.68	0.68	0.54	0.91
p__Bacteroidetes__g__57N15_OTU_4	0.69	0.64	0.66	0.66	0.54
p__Lentisphaerae__f__Victivallaceae_OTU_31	0.64	0.58	0.6	0.56	0.47
p__Bacteroidetes__o__Bacteroidales_OTU_9310	0.7	0.52	0.72	0.54	0.07
p__Spirochaetes__g__*Treponema*_OTU_16	0.47	0.65	0.47	0.5	0.62
p__Bacteroidetes__o__Bacteroidales_OTU_15	0.75	0.34	0.73	0.34	0.03*
p__Bacteroidetes__f__RF16_OTU_28	0.4	0.64	0.26	0.5	0.47
p__Bacteroidetes__f__Rikenellaceae_OTU_39	0.53	0.42	0.44	0.35	0.24
p__Bacteroidetes__f__Rikenellaceae_OTU_23	0.61	0.34	0.62	0.32	0.001***
p__Firmicutes__o__Clostridiales_OTU_42	0.49	0.45	0.5	0.45	0.45
p__Firmicutes__f__Ruminococcaceae_OTU_17	0.45	0.49	0.46	0.44	0.88
p__Firmicutes__f__Ruminococcaceae_OTU_29	0.54	0.4	0.5	0.42	0.08
p__Verrucomicrobia__g__Akkermansia_OTU_13	0.26	0.66	0.28	0.47	0.001***
p__Firmicutes__g__*Phascolarctobacterium*_OTU_8	0.49	0.42	0.51	0.4	0.08
p__Bacteroidetes__o__Bacteroidales_OTU_285	0.53	0.38	0.47	0.39	0.4
p__Firmicutes__g__*Dorea*_OTU_10	0.17	0.72	0.16	0.78	0.002**
p__Firmicutes__g__*Phascolarctobacterium*_OTU_7	0.48	0.41	0.43	0.4	0.13
p__Bacteroidetes__g__57N15_OTU_19	0.42	0.45	0.4	0.45	0.52
p__Firmicutes__f__Clostridiaceae_OTU_27	0.35	0.52	0.28	0.28	0.76
p__Bacteroidetes__g__*Bacteroides*_OTU_33	0.5	0.36	0.47	0.35	0.08
p__Bacteroidetes__o__Bacteroidales_OTU_40	0.47	0.37	0.46	0.34	0.34
p__Bacteroidetes__f__RF16_OTU_37	0.31	0.51	0.34	0.32	0.68
p__Firmicutes__g__*Oscillospira*_OTU_20	0.41	0.37	0.42	0.36	0.2
p__Bacteroidetes__g__*Prevotella*_OTU_30	0.28	0.5	0.26	0.3	0.34

Table A4.6 The 30 most abundant genera (out of 211) with different abundances between the uninfected and infected sheep with low parasite burden (n=20). Wilcoxon signed-rank test; *$p \leq 0.05$, **$p \leq 0.01$.

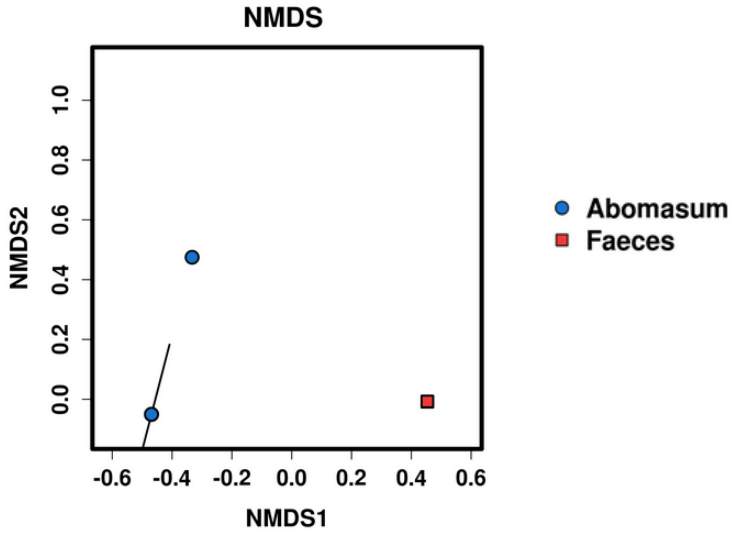
Taxa (genus)	Mean		Median		P value
	Uninfected	Infected	Uninfected	Infected	
Unclassified.Ruminococcaceae	23.02	21.05	22.78	21.22	0.04*
Unclassified	21.55	21.05	21.46	20.93	0.44
Unclassified.Clostridiales	10.78	11.43	10.69	11.56	0.27
Unclassified.Bacteroidales	9.17	9.94	9	9.67	0.14
X57N15	2.88	3.2	2.92	3.31	0.19
Unclassified.Lachnospiraceae	2.83	2.69	2.58	2.94	0.76
Oscillospira	2.56	2.91	2.57	2.94	0.14
Akkermansia	2.45	2.61	2.28	2.44	0.86
Unclassified.Rikenellaceae	2.28	1.93	2.34	1.7	0.13
Dorea	2.12	1.56	2.28	1.51	0.22
Ruminococcus	1.8	2.02	1.73	2.02	0.22
Prevotella	1.6	1.91	1.54	1.8	0.23
Unclassified.Victivallaceae	1.3	0.6	1.36	0.61	0.006**
Fibrobacter	1.3	1.25	1.02	1.17	0.93
Phascolarctobacterium	1.08	1.1	1.07	1.24	0.2
CF231	1.07	2.75	1.05	1.69	0.008**
Bacteroides	0.95	0.86	0.74	0.79	1
Unclassified.S247	0.88	0.56	0.86	0.55	0.02**
Treponema	0.81	1.22	0.79	1.12	0.004**
Unclassified.RF16	0.74	0.61	0.65	0.63	0.69
Unclassified.Bacteroidaceae	0.71	0.71	0.67	0.62	0.79
Unclassified.Clostridiaceae	0.66	0.61	0.62	0.54	0.72
Unclassified.Alphaproteobacteria	0.63	0.45	0.73	0.36	0.1
Unclassified.BS11	0.62	0.57	0.63	0.51	0.34
Unclassified.RF39	0.61	0.6	0.59	0.55	0.6
Clostridium	0.61	0.64	0.61	0.57	0.8
Unclassified.RFP12	0.57	0.55	0.62	0.55	0.76
Unclassified.YS2	0.54	0.49	0.53	0.4	0.63
Coprococcus	0.34	0.23	0.28	0.24	0.11
Unclassified.RF32	0.27	0.25	0.24	0.24	1

Table A4.7 The 30 most abundant OTUs with different abundances between the uninfected and infected sheep low parasite burden (n=20). Wilcoxon signed-rank test; *p ≤ 0.05, **p ≤ 0.01.

OTUs	Mean		Median		P Value
	Un-infected	Infec-ted	Un-infected	Infec-ted	
p__Firmicutes__g__*Dorea*_OTU_10	1.23	0.95	1.08	0.94	0.23
p__Bacteroidetes__o__Bacteroidales_OTU_3	1.06	0.81	0.84	0.86	0.55
p__Verrucomicrobia__g__*Akkermansia*_OTU_13	0.97	0.97	0.88	0.86	0.63
p__Bacteroidetes__g__CF231_OTU_2	0.95	2.54	0.97	1.51	0.01**
p__Fibrobacteres__g__*Fibrobacter*__s__*succinogenes*_OTU_1	0.91	0.93	0.74	0.95	1
p__Bacteroidetes__g__57N15_OTU_5	0.8	0.94	0.79	0.98	0.16
p__Bacteroidetes__o__Bacteroidales_OTU_9310	0.78	0.61	0.72	0.63	0.2
p__Bacteroidetes__g__57N15_OTU_4	0.77	0.87	0.72	0.9	0.31
p__Verrucomicrobia__g__*Akkermansia*_OTU_9	0.68	0.78	0.62	0.68	0.6
p__Firmicutes__o__Clostridiales_OTU_42	0.63	1	0.69	1	0.01**
p__Bacteroidetes__g__*Prevotella*_OTU_747	0.62	0.68	0.66	0.7	0.73
p__Firmicutes__o__Clostridiales_OTU_60	0.62	0.45	0.58	0.41	0.19
p__Proteobacteria__f__Desulfovibrionaceae_OTU_18	0.59	0.61	0.56	0.6	0.89
p__Firmicutes__o__Clostridiales_OTU_24	0.54	0.42	0.46	0.5	0.6
p__Firmicutes__g__*Phascolarctobacterium*_OTU_8	0.53	0.46	0.53	0.48	0.57
p__Firmicutes__g__*Phascolarctobacterium*_OTU_7	0.51	0.61	0.55	0.68	0.03
p__Bacteroidetes__o__Bacteroidales_OTU_15	0.51	0.57	0.44	0.59	0.48
p__Bacteroidetes__f__Rikenellaceae_OTU_23	0.51	0.33	0.46	0.3	0.03*
p__Firmicutes__f__Ruminococcaceae_OTU_17	0.46	0.44	0.4	0.42	0.79
k__Unassigned_OTU_41	0.44	0.37	0.47	0.32	0.2
p__Bacteroidetes__g__*Bacteroides*_OTU_47	0.43	0.14	0.29	0.09	0.02*
p__Firmicutes__g__*Oscillospira*_OTU_20	0.41	0.52	0.41	0.54	0.19
p__Bacteroidetes__g__57N15_OTU_48	0.41	0.38	0.45	0.39	0.6
p__Firmicutes__f__Ruminococcaceae_OTU_68	0.41	0.36	0.33	0.31	0.69
p__Bacteroidetes__o__Bacteroidales_OTU_12	0.4	0.97	0.26	0.4	0.08
p__Bacteroidetes__g__57N15_OTU_19	0.4	0.43	0.44	0.45	0.51
p__Bacteroidetes__f__Rikenellaceae_OTU_39	0.4	0.3	0.31	0.25	0.13
p__Bacteroidetes__o__Bacteroidales_OTU_32	0.39	0.47	0.33	0.23	0.43
p__Firmicutes__o__Clostridiales_OTU_109	0.39	0.25	0.35	0.28	0.16
p__Bacteroidetes__f__Rikenellaceae_OTU_22	0.38	0.37	0.39	0.3	0.16

Appendix 5

(A)



(B)

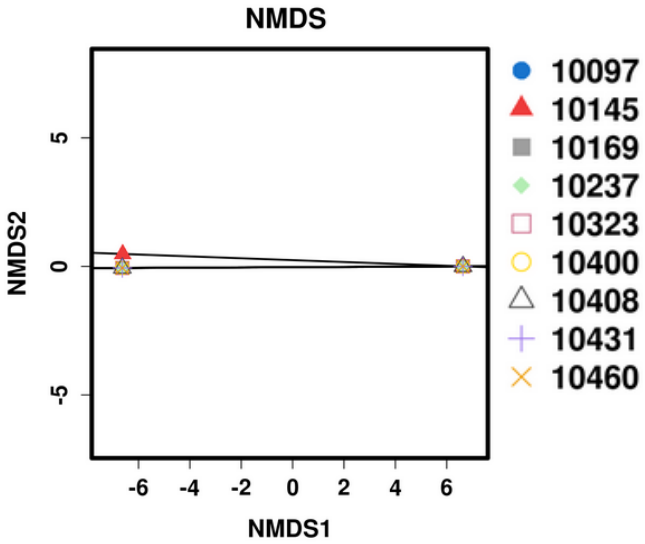


Figure A5.1 Distinct clustering of faecal and abomasal microbiota of sheep. nMDS plot of sheep based on Bray-Curtis distance matrix of microbial composition. (A): Sample source; (B): sheep tag. Each symbol represents an individual sheep sample at a given time. As can be seen, most of the samples from the same sampling source (abomasum/faeces) are overlapping each other.

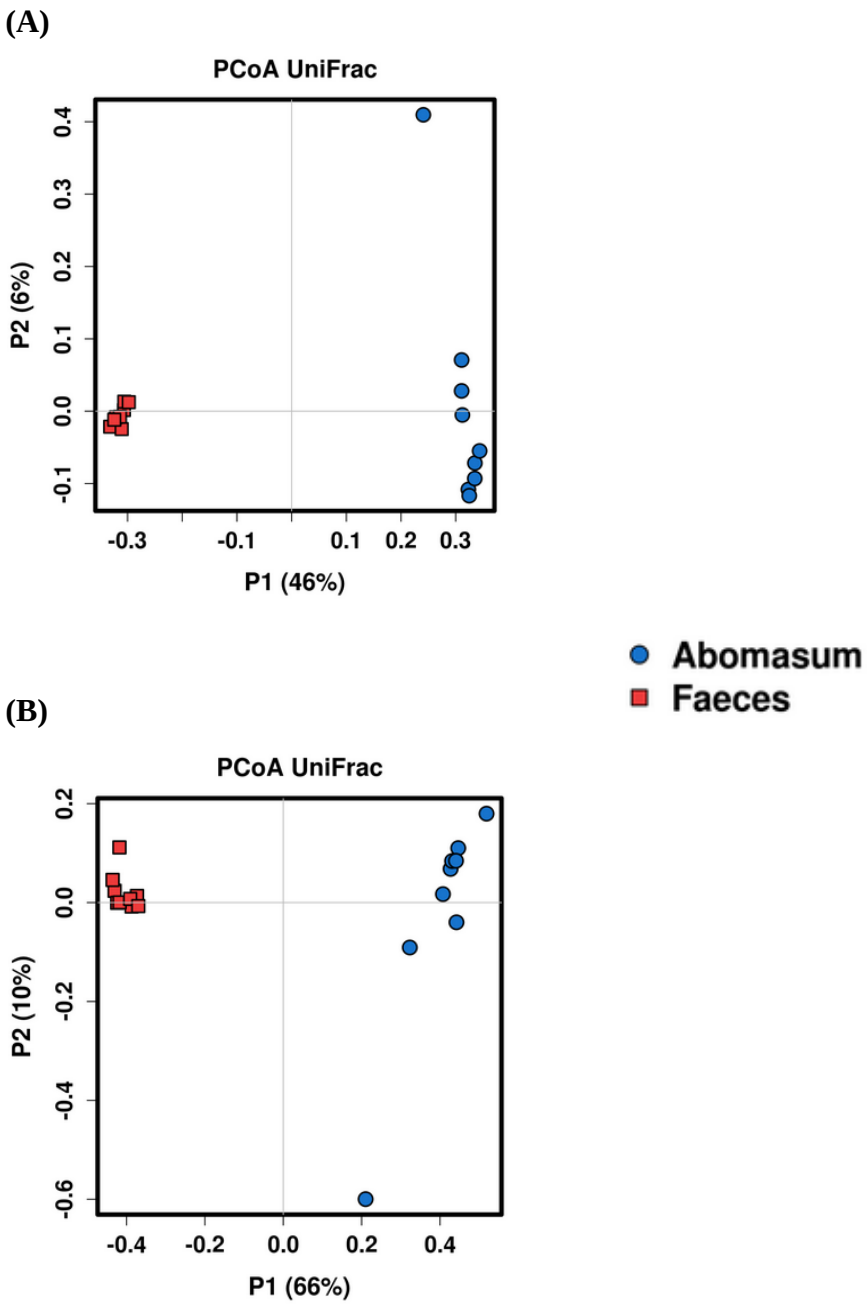


Figure A5.2 Distinct clustering of faecal and abomasal microbiota of sheep. PCoA plots were generated based on weighted (A) and unweighted (B) UniFrac distance matrix of microbial composition. Each symbol represents an individual sheep sample at a given time.

Table A5.1 ANOSIM of microbial composition of samples collected from the abomasum (n=9) and faeces (n=9) of sheep. ***$p \leq 0.001$.

Parameter	Bray-Curtis	Weighted UniFrac	Unweighted UniFrac
R Value	1	0.996	1
P value	0.001***	0.001***	0.001***

Table A5.2 Shared and unique taxa present in abomasum (n=9) and faecal samples (n=9) of sheep. A bacterial group was considered to be present in a sample group if it was identified in at least 10% of the samples (i.e. at least one of nine) within the group.

Taxa	Unique taxa		Shared (%)	Total count
	Abomasum	Faeces		
Phyla	2	5	20 (74.07)	27
Class	5	10	36 (70.58)	51
Order	13	17	63 (67.74)	93
Family	23	38	88 (59.06)	149
Genus	43	73	113 (49.34)	229
Species	49	77	112 (47.05)	238

Table A5.3 Shared and unique core taxa present in abomasum (n=9) and faecal samples (n=9) of sheep. A bacterial group was considered to be present in a sample group if it was identified in at least 50% of the samples within the group.

Taxa	Unique taxa		Shared (%)	Total count
	Abomasum	Faeces		
Phyla	0	5	14 (73.68)	19
Class	8	6	24(63.15)	38
Order	12	14	33(55.93)	59
Family	16	25	46(52.87)	87
Genus	31	42	57(43.84)	130
Species	32	42	58(43.93)	132

Table A5.4 Average relative abundances (%) of identified phyla (core bacteria) present in minimum 50% of the abomasal (n=9) and faecal samples (n=9) of sheep identified by 16S rRNA sequencing.

Phylum	**Abundance (%)**	
	Abomasum	**Faeces**
Bacteroidetes	60.187	26.121
Firmicutes	24.138	53.594
Fibrobacteres	4.521	1.267
Unclassified	2.789	6.394
Actinobacteria	1.787	0.182
Tenericutes	1.64	0.993
Proteobacteria	1.372	4.063
Verrucomicrobia	1.186	2.481
Lentisphaerae	0.634	2.392
Spirochaetes	0.469	1.009
Cyanobacteria	0.436	1.187
Synergistetes	0.222	0.003
Chloroflexi	0.16	0.013
Elusimicrobia	0.138	0.078
TM7	0.086	0.061
SR1	0.074	0.008
Planctomycetes	0.073	0.118
WPS2	0.054	0.003
Armatimonadetes	0.013	0

Table A5.5 Average relative abundances (%) of 30 most abundant families (core bacteria) present in minimum 50% of the abomasal (n=9) and faecal samples (n=9) of sheep identified by 16S rRNA sequencing.

Family	Abundance (%)	
	Abomasum	Faeces
Prevotellaceae	24.489	2
Unclassified.Bacteroidales	11.296	8.374
Unclassified	9.023	15.018
Ruminococcaceae	7.234	28.751
Lachnospiraceae	7.207	5.101
Bacteroidaceae	6.181	5.728
Fibrobacteraceae	4.521	1.267
Unclassified.Clostridiales	4.31	9.221
S247	4.147	0.784
BS11	3.906	0.752
Paraprevotellaceae	3.131	1.397
Erysipelotrichaceae	1.977	0.324
Bifidobacteriaceae	1.726	0.01
Anaeroplasmataceae	1.357	0.032
RF16	1.318	1.092
RFP12	1.048	0.771
Veillonellaceae	0.869	1.057
Victivallaceae	0.57	2.37
Porphyromonadaceae	0.546	0.306
Mogibacteriaceae	0.431	0.259
Unclassified.YS2	0.407	1.143
Spirochaetaceae	0.334	0.974
Christensenellaceae	0.331	0.153
Succinivibrionaceae	0.306	0.004
Clostridiaceae	0.24	1.031
Barnesiellaceae	0.224	0.174
Dethiosulfovibrionaceae	0.222	0.002
Unclassified.Alphaproteobacteria	0.143	1.057
Anaerolinaceae	0.132	0.008
Unclassified.RF32	0.122	0.348

Table A5.6 Average relative abundances (%) of 30 most abundant genera (core bacteria) present in minimum 50% of the abomasal (n=9) and faecal samples (n=9) of sheep identified by 16S rRNA sequencing.

Genus	Abundance (%)	
	Abomasum	Faeces
Prevotella	25.457	2.002
Unclassified	13.616	19.74
Unclassified.Bacteroidales	11.296	8.374
Unclassified.Ruminococcaceae	6.033	23.512
Bacteroides	5.711	0.857
Fibrobacter	4.519	1.267
Unclassified.Clostridiales	4.31	9.221
Unclassified.S247	4.147	0.784
Unclassified.BS11	3.906	0.752
Unclassified.Lachnospiraceae	2.3	2.399
Butyrivibrio	1.94	0.056
Anaeroplasma	1.357	0.027
Unclassified.RF16	1.318	1.092
Unclassified.RFP12	1.048	0.771
Unclassified.Paraprevotellaceae	0.954	0.043
Ruminococcus	0.861	1.521
Unclassified.Victivallaceae	0.57	2.37
Paludibacter	0.437	0.151
Coprococcus	0.422	0.243
Unclassified.YS2	0.407	1.143
BF311	0.393	0.184
Unclassified.Christensenellaceae	0.331	0.153
Unclassified.Mogibacteriaceae	0.328	0.206
Treponema	0.301	0.974
Unclassified.Barnesiellaceae	0.213	0.174
Unclassified.Alphaproteobacteria	0.143	1.057
Unclassified.RF32	0.122	0.348
Oscillospira	0.113	1.968
Clostridium	0.103	0.393
Akkermansia	0.012	1.627