Investigating early life microbial and host transcriptomic dynamics in the bovine gastrointestinal tract

by

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Abstract

There is increasing concern surrounding the ability of livestock industries to meet the needs of the rising global population. The gastrointestinal microbiota of ruminants plays a critical role in feed degradation, host energy supply, but is also a substantial source of anthropogenic greenhouse gas emissions. It is proposed that dietary intervention during the first weeks of life may offer an opportunity to permanently manipulate microbial colonisation patterns of the rumen, with a view to enhancing host performance whilst mitigating climatic impacts. However, the optimum window for intervention remains to be elucidated. Despite the close relationship between the rumen and its microbes, understanding of the molecular controls of rumen development during early life is limited. In mature animals, microbial fermentation in the rumen is the principle host energy source, but the hindgut and its microbiome may play of increased importance while the rumen develops during early life. However, little is known of the hindgut microbiota and its contribution to animal growth. Study 1 investigated the temporal dynamics of the rumen microbiota in beef calves during early life using 16S rRNA sequencing, to characterise the patterns of microbial establishment in the rumen and identify the most favourable timeframe for dietary manipulation. The microbial community displayed an ordered pattern of succession during the first 3 weeks of life, but settled by day 21, indicating that this may be the limit of any timeframe for early life manipulation. Study 1 also revealed a substantial farm effect on the colonisation of certain microbial groups, including *Methanobrevibacter smithii* ($P<0.05$) and *Dialister* ($P<0.05$). Such an effect has not been reported previously and may have substantial implications in future manipulation efforts. Study 2 characterised the transcriptomic profile of rumen tissue from birth to post weaning, revealing significant enrichment in immune related genes (e.g. *TLR5, LAP, TAP*) and processes following birth ($P<0.05$). This was not associated with any depression in known tight junction genes ($P>0.05$), indicating that rumen permeability was not compromised. Further exploring the relationship between microbial colonisation and rumen immune function may offer an opportunity to manipulate the establishment of certain taxa. Solid

feed allocation was associated with enhanced expression of genes involved in Volatile Fatty Acid (VFA) absorption (*MCT1;* $P<0.05$) and metabolism (*BDH1, ACAT;* $P<0.05$). Understanding the mechanistic control of VFA absorption and how it changes during the life-cycle of the animal will be key for the design of optimal calf nutrition strategies. Study 3 characterised the hindgut microbiota of young ruminants, and its response to fortification of milk replacer with sodium butyrate (SB). The trophic effect of butyrate on calf growth and feed efficiency ($P<0.1$) was associated with increased concentrations of total VFA, propionate and acetate ($P<0.05$) in the hindgut. Native butyrogenic bacteria *Butyrivibrio* and *Shuttleworthia* were decreased by SB ($P<0.05$), while the proportion of the propionate producer *Phascolarctobacterium* was higher ($P<0.05$). *Mogibacterium* is associated with impaired gut health and was reduced in the cecum of SB calves ($P<0.05$). These data show that the beneficial effects of SB on growth and performance occur in tandem with changes in the abundance of important SCFA producing and health-associated bacteria in the hindgut in milk-fed calves, and that SB supplementation may suppress butyrate biosynthesis in the gut. Therefore, efforts to improve animal performance via early life manipulation should also consider the hindgut compartments, as this may offer a method to improve animal performance during the milk-feeding period. In summary, the data presented in this thesis contributes to understanding of rumen microbial composition and molecular development during early life and shows that enhanced activity of the hindgut microbiota may contribute to early life calf growth.

Preface

This thesis is an original work by Eóin O'Hara and is part of a collaborative initiative between Prof. Leluo Guan at the University of Alberta and Dr. Sinéad Waters of Teagasc, Ireland. The Teagasc Animal Ethics Committee (RMIS 6341) and the Irish Health Products Regulatory Authority (AE19132/P016) granted ethical approval for all experimental procedures described in Chapters Two and Three, while the University College Dublin Animal Research Ethics Committee granted approval for the study described in Chapter Four.

Chapter 4 of this thesis has been published as "Effect of a butyrate-fortified milk replacer on gastrointestinal microbiota and products of fermentation in artificially reared dairy calves at weaning. Sci Rep. 2018 Oct 8;8(1):14901". I was responsible for laboratory and data analysis as well as manuscript writing. M.S. McCabe assisted with laboratory analysis. A. Kelly and D.A. Kenny devised the study, supervised data collection and contributed to manuscript writing. L.L. Guan contributed to manuscript writing. S.M. Waters was the supervisory author responsible for concept formation and contributed to manuscript writing.

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Table of Contents

Abstract ii

Preface iv

Acknowledgements v

Table of contents vi

List of tables xii

List of figures xiii

Abbreviations xv

Chapter 1: Literature Review 1

1.1 Introduction 1

1.2 The ruminant digestive tract 2

1.2.1 Development of the rumen during early life 3

1..3 Rumen microbial diversity 4

1.3.1 Rumen bacteria 6

1.3.2 Rumen archaea 8

1.3.3 Rumen protozoa 9

1.3.4 Rumen fungi 11

1.3.5 Rumen viruses 12

1.3.6 Hindgut microbiota in ruminants 13

1.4 Role of the rumen microbiome in animal production 14

1.4.1 The rumen microbiome and feed efficiency 14

1.4.2 The rumen microbiome and methane production 15

1.5 Early life – the “window of opportunity” for effective manipulation of the rumen microbiome? 17

1.5.1 Microbial colonisation of the pre-functional rumen 18

1.5.2 Early life rumen microbial dynamics 18

1.5.3 Functional achievement of the rumen microbiota in early life 21

1.5.4 Factors influencing rumen microbial colonisation and development in early life 21

1.6 Methods to study the rumen microbiome 23

1.6.1 Terminology 23

1.6.2 Classic cultivation-based methods 23

1.6.3 Molecular methods to evaluate the rumen microbiome 24

1.6.4 Pioneer molecular techniques 25

1.6.5 Modern technologies to study the rumen microbiome 26

1.6.5.1 Amplicon sequencing 27

1.6.5.2 Metagenomic shotgun sequencing 28

1.6.5.3 New frontiers in omics technologies to study the rumen 29

1.7 Knowledge gaps, hypotheses, and objectives 30

1.8 Literature cited 31

1.9 Figures 55

Chapter 2: Diet, age, and farm environment influence rumen microbial establishment patters during early life 57

2.1 Introduction 57

2.2 Materials and methods 58

2.2.1 Ethical statement 58

2.2.2 Experimental animal trial 58

2.2.3 DNA isolation 59

2.2.4 16S rRNA gene amplicon library preparation and sequencing 60

2.2.5 Bioinformatic analysis 60

2.2.6 Statistical analysis 61

2.3 Results 62

2.3.1 Sequence data information 62

2.3.2 Rumen microbial diversity and structure during early life 62

2.3.2.1 α-Diversity 62

2.3.2.2 β-Diversity 62

2.3.3 Microbial composition in the rumen digesta during early life 63

2.3.4 Effect of rumen fraction on microbial composition during early life 63

2.3.5 Temporal development of the rumen microbiota during early life 64

2.3.5.1 Rumen liquid digesta 64

2.3.5.2 Rumen solid digesta 67

2.3.6 Influence of farm on microbial community composition in the rumen during early life 68

2.4 Discussion 69

2.5 Conclusion 76

2.6 Literature cited 76

2.7 Tables and figures 81

Chapter 3: Temporal dynamics of the rumen transcriptome of beef calves from birth to post-weaning 96

3.1 Introduction 96

3.2 Materials and methods 97

3.2.1 Ethical statement 97

3.2.2 Experimental animal model 97

3.2.3 Rumen tissue sampling 98

3.2.4 RNA isolation 98

3.2.5 RNA-seq library construction and sequencing 98

3.2.6 Bioinformatic and statistical analysis 99

3.3 Results and Discussion 100

3.3.1 Mechanisms underpinning physical development of the rumen 101

3.3.2 Transcriptional dynamics of genes and pathways underpinning VFA absorption in the rumen 103

3.3.3 Genes and pathways involved in the host immune response are enriched in the rumen during early life 106

3.3.4 Upregulation of genes involved in stress response and collagen formation in the rumen of naturally delivered calves compared to calves delivered via elective caesarean section 111

3.4 Conclusions and summary 112

3.5 Literature cited 113

3.6 Tables and figures 117

Chapter 4: Effect of a butyrate-fortified milk replacer on gastrointestinal microbiota and products of fermentation in artificially reared dairy calves at weaning 144

4.1 Introduction 144

4.2 Materials and methods 145

4.2.1 Ethical statement 145

4.2.2 Animal study 145

4.2.3 DNA isolation 146

4.2.4 Microbial profiling using amplicon sequencing 146

4.2.5 Sequence data quality control and pre-processing 147

4.2.6 Bioinformatic analysis 147

4.2.7 Statistical analysis 148

4.3 Results 148

4.3.1 Animal performance 148

4.3.1 Fermentation profiles in the rumen and hindgut at weaning 148

4.3.3 Microbial structure and diversity in the rumen and hindgut in response to SB 148

4.3.4 Microbial composition in the rumen and hindgut in response to SB......149

4.3.4.1 Rumen 149

4.3.4.2 Hindgut 149

4.4 Discussion 150

4.4.1 Sodium butyrate does not induce substantial changes in the rumen microbiota or fermentation profile 151

4.4.2 Sodium butyrate modifies the hindgut microbiota and fermentation profiles in early life 151

4.4.3 The rumen and hindgut harbour significantly different microbial communities at weaning 154

4.5 Conclusions 155

4.6 Literature cited 156

4.7 Tables and figures 162

Chapter 5: General Discussion 168

5.1 Background 168

5.2 Understanding microbial establishment and development in the developing rumen 169

5.2.1 Does mode of delivery influence the rumen and its resident microbes? 169

5.2.2 Understanding colonisation and succession among the early rumen microbiota 169

5.2.3 Understanding ruminal transcriptomic dynamics during early life 169

5.2.4 Butyrate supplementation modifies hindgut microbiota and fermentation 173

5.3 Caveats, implications, and future directions 173

5.4 Literature cited 177

Bibliography 181

Appendices 205

Appendix A 205

Appendix B 206

Appendix C 208

Appendix D 217

Appendix E 219

Appendix F 229

List of Tables

Table 2.1 PERMANOVA results based on Bray-Curtis dissimilarities for bacterial and archaeal communities during early life 81

Table 2.2 Details of differentially abundant taxa according to rumen digesta fraction during early life.. 82

Table 3.1 Details of selected DEG within each statistical contrast performed in EdgeR.....117

Table 3.2 Top Gene Ontology (GO) terms enriched among the DEG in each contrast.......122

Table 3.3 Top Canonical Pathways enriched among the DEG using Ingenuity Pathway Analysis..126

Table 3.4 Top Molecular and Cellular Functions of the DEG predicted by Ingenuity Pathway Analysis 131

Table 4.1 The effect of SB inclusion in milk replacer on SCFA profiles in the rumen and colon..162

Table 4.2 Comparisons of alpha diversity metrics in the rumen, cecum, and colon of calves at weaning 163

Table 4.3 Comparing microbial communities between treatments and gastrointestinal region in the rumen, cecum and colon using PERMANOVA 164

List of Figures

Figure 1.1 Schematic representation of the gastrointestinal tract of ruminant animals 55

Figure 1.2 Rumen epithelial structure and major pathways of rumen microbial fermentation 56

Figure 2.1 Experimental animal trial design and calf management........ 83

Figure 2.2 Temporal dynamics of bacterial and archaeal alpha diversity metrics during early life 84

Figure 2.3 Effect of farm environment on bacterial and archaeal alpha diversity during early life 85

Figure 2.4 NMDS plots of bacterial and archaeal communities during early life 86

Figure 2.5 Effect of farm environment on bacterial and archaeal community composition and beta-dispersion 87

Figure 2.6 Stacked barchart of bacterial phyla composition in the rumen solid and liquid digesta during early life 88

Figure 2.7 Details of the 10 most abundant bacterial taxa detected in rumen liquid and rumen solid digesta during early life 89

Figure 2.8 Barcharts depicting relative abundances of bacterial genera and archaeal species across age, fraction and farm during early life 90

Figure 2.9 Line charts showing temporal dynamics of selected transient taxa in the rumen liquid and rumen solid digesta during early life........91

Figure 2.10 Line charts depicting the predominance of *Prevotella* and the decline of *Bacteroides* in the rumen from day 7 onward, and the dynamics of lactate-producing and -utilising bacteria in the rumen digesta during early life 92

Figure 2.11 Line charts depicting temporal dynamics of selected VFA producers and starch-utilising groups during early life in the rumen digesta 93

Figure 2.12 Line charts depicting temporal dynamics of selected fibrolytic taxa during early life in the rumen digesta 94

Figure 2.13 Visual representation of taxa influenced by farm during early life 95

Figure 3.1 Venn diagram showing numbers of expressed genes in the rumen wall in each age group, and numbers of DEG in each statistical contrast 135

Figure 3.2 Cluster analysis plots generated using the Bray-Curtis Dissimilarity matrix 136

Figure 3.3 Volcano plots of differential gene expression profiles within each statistical contrast 137

Figure 3.4 Boxplots depicting the expression profiles of selected genes involved in the cell cycle process in the rumen wall during early life... 138

Figure 3.5 Boxplots depicting the expression profiles of VFA transporter genes in the rumen wall during early life 139

Figure 3.6 Boxplots depicting the expression profiles of genes involved in ketogenesis detected in the rumen wall during early life 140

Figure 3.7 Boxplots depicting the expression profiles of tight junction protein genes detected in the rumen wall during early life 141

Figure 3.8 Boxplots depicting the expression profiles of pattern recognition receptor genes detected in the rumen wall during early life 142

Figure 3.9 Boxplots depicting the expression profiles of defensin-encoding genes detected in the rumen wall during early life.. 143

Figure 4.1 PCA and cluster dendrogram generated based on the Bray-Curtis dissimilarity matrix of operational taxonomic units (OTUs) present in the rumen, cecum, and colon at weaning 165

Figure 4.2 Stacked bar chart of microbial abundances at the phylum level, calculated as a percentage of total 16S rRNA reads within each group 166

Figure 4.3 Phylogenetic Trees of rumen, cecum, and colon microbiota, built from a multiple sequence alignment generated in QIIME 167

Abbreviations

3-NOP – 3-Nitroproxypropanol

AMP – Antimicrobial peptides

ANOVA – Analysis of variance

BAM – Binary alignment map

BCM – Bromochloromethane

BHBA – β-hydroxybutyrate

CASP – Caspase gene family

CCN – Cyclin gene family

CDK – Cyclin dependant kinase gene family

CH_4 – Methane

CLD – Claudin gene family

CO_2 – Carbon dioxide

DAFM – Irish Department of Agriculture, Food, and the Marine

DEF – Defensin gene family

DEG – Differentially expressed gene(s)

DGGE – Denaturing gradient gel electrophoresis

ECS – Elective caesarean section

FAO – Food and Agriculture Organisation of the United Nations

FCR – Feed conversion ratio

FDR – False discovery rate

FE – Feed efficiency

FKBP5 - FK506-binding protein 51 gene

GALT – Gut-associated lymphoid tissue

GH – Glycoside hydrolase

GHG – Greenhouse gas

GIT – Gastrointestinal tract

GO – Gene ontology

GR – Glucocorticoid receptor

GWP – Global warming potential

H_2 – Hydrogen

HPRA – Health Products Regulatory Authority

Ig – Immunoglobulin

IL – Interleukin family

IPA – Ingenuity pathway analysis

IPCC – United Nations Intergovernmental Panel on Climate Change

ITS – Internal transcribed spacer gene

LAP – Lingual antimicrobial peptide

LPS – Lipopolysaccharide

MALT – Mucosa-associated lymphoid tissue

MCR – Methyl coenzyme-M reductase

MCT – Monocarboxylic transporter gene family

N_2O – Nitrous oxide

NES – Normalised enrichment score

OCLD – Occludin gene

OTU – Operational taxonomic unit

P4HA3 - Prolyl 4-hydroxylase subunit A3

PCA – Principle component analysis

PCR – Polymerase chain reaction

PEG – Partial efficiency of growth

PERMANOVA – Permutational analysis of variance

PICRUSt – Phylogenetic investigation of communities by reconstruction of unobserved states

PRID – Progesterone-releasing intravaginal device

PRR – Pattern recognition receptor

QIIME – Quantitative insights into molecular ecology

qPCR – Quantitative polymerase chain reaction

RFI – Residual feed intake

RIM-DB – Rumen and intestinal methanogens database

RIN – RNA integrity

rRNA/DNA – ribosomal RNA/DNA gene

SB – Sodium butyrate

SCFA – Short chain fatty acid

SLC – Solute carrier gene family

SNP – Single nucleotide polymorphism

STAR – Spliced transcripts alignment to a reference

TAEC – Teagasc Animal Ethics Committee

TAP – Tracheal antimicrobial peptide

TJP – Tight junction protein

TLR – Toll-like receptor

TMM – Trimmed mean of m-values

VFA – Volatile fatty acids

Chapter 1

Literature Review

1.1. Introduction

Ruminants are characterised by the presence of a specialised pre-gastric fermentation chamber which has evolved over millennia to facilitate feed degradation via its diverse consortium of resident microorganisms (Mackie, 2002). This symbiotic relationship offers an environment conducive to microbial growth and activity and allows the ruminant to harvest the nutritional value of recalcitrant plant fibres (Mackie, 2002, Mao et al., 2015).

Domesticated ruminant production systems occupy around 30% of the global landmass, and use around 30% of cropping output as feed (Aschenbach et al., 2011). The 3.9 billion ruminants estimated to exist today are important in sustainable agricultural practices, as they can synthesise energy from low-quality forages for high-quality milk and meat production (Cammack et al., 2018). With the global population expected to grow to 9.15 billion by the year 2050, the issue of world-wide food security – "adequate access to safe and nutritious food for all people always" – has become increasingly topical (FAO, 2013). This rapid population expansion brings with it a sharp increase in projected demands for animal products, with meat consumption expected to increase by 75% in the next three decades (Duthie et al., 2017). Provision of adequate nutrition to this growing population is estimated to require a 70% increase in food production from 2007 levels in developed countries, and perhaps a doubling of output from developing nations (FAO, 2013).

Compounding this increased pressure on food production, concerns about the environmental footprint of ruminant production are also increasing. The livestock sector contributes around 14.5% of anthropogenic greenhouse gas (GHG) emissions annually, with enteric emissions from ruminants accounting for 39.1% of these (FAO, 2013, Veneman et al., 2015). Recent legislative agreements have mandated substantial reductions in global GHG emissions, including those derived from agricultural practices, and increased food outputs (e.g. Kyoto Protocol, Paris Agreement, Foodwise 2020, Food Harvest 2025). Methane (CH_4) is the most prominent GHG associated with ruminant production, synthesised in the rumen and lower gut by methanogenic archaea, and has a global warming potential (GWP) around 28 times greater than carbon dioxide (IPCC, 2014). Furthermore, the loss of gross dietary energy to the animal via enteric methanogenesis is estimated at 2-12%, and is therefore a major contributor to reduced

host feed energy utilisation efficiency (Johnson and Johnson, 1995). The economic and environmental concerns outlined here suggest that improving the efficiency of ruminant production is of paramount importance.

Studies of the rumen microbial community as an avenue to improve cattle production efficiency has been ongoing for many decades (Hungate, 1960). The advent of high throughput sequencing technologies in the last number of years has generated a large amount of data on the structure and function of the bovine rumen microbiota, across a range of hosts and environments (Henderson et al., 2015, McCann et al., 2014a). It has become apparent that the highly individualised and resilient nature of the adult rumen microbiome may preclude persistent manipulation in older animals (Weimer, 2015). In contrast, the rumen microbiome in early life appears far more dynamic and malleable to change, and therefore has emerged as a target for manipulations that may persist into adulthood (Yanez-Ruiz et al., 2015). However, little is known regarding the dynamics of the rumen microbiome in early life, and how patterns of microbial development may impact on animal productivity throughout life. Thus, improved understanding of the temporal dynamics of the rumen microbiome throughout life may facilitate opportunities to enhance animal performance via dietary intervention, or direct manipulation of the microbiota.

This literature review consists of four main sections. The first will detail the digestive structure and rumen microbial population and fermentation of cattle. The second section relates to contribution of the rumen microbiome to livestock production. The third focusses on the temporal patterns of physical and functional maturation of the rumen and its microbiota from, while the final section reviews methods for investigating the rumen microbiome.

1.2. The ruminant digestive tract

In ruminants, the pre-gastric digestive tract is divided into four chambers; the rumen, reticulum, omasum, and abomasum (Fig. 1.1). The reticulum is often regarded as the anterior sac of the rumen, and hence the term reticulorumen is often used in reference to both (Harfoot, 1978), representing around 70% of the total gastrointestinal tract (GIT) size in adult cattle (Warner et al., 1956). The rumen comprises the dorsal and ventral sacs, which are laterally divided by a series of pillar-like columns (Harfoot, 1978).

The rumen itself is characterised by the presence of a stratified squamous epithelium consisting of four layers (Fig. 1.2), which is also present in the omasum. The apical epithelial surface (the stratum corneum) is covered in small finger-like projections called papillae, which

increase the surface area available for nutrient and volatile fatty acid absorption (Steele et al., 2016). Over 75% of the volatile fatty acids (VFA) produced in the rumen are absorbed through the rumen epithelium, and less than 10% reach the small intestine (Harfoot, 1978). Additionally, this layer protects the host animal from potentially pathogenic rumen microorganisms, toxins and other harmful chemicals found in the lumen, preventing their unregulated movement into host circulatory systems (Chaucheyras-Durand and Fonty, 2002). Temperature, pH, buffering capacity, osmotic pressure, and redox potential all contribute to the maintenance of ruminal homeostasis (Castillo-Gonzales, et al., 2014), and are highly regulated within the rumen (Firkins and Yu, 2015, Aschenbach et al., 2011, Krause and Oetzel, 2006). Maintaining optimum biochemical conditions in the rumen is important, as perturbations in any of the above factors can have severe implications for animal health and production (Krause and Oetzel, 2006, Owens et al., 1998).

1.2.1 Development of the rumen during early life

At birth, the rumen is little more than an under-developed and non-functioning pouch. Rumen development is an important physiological milestone in the life-cycle of a ruminant (Jiao et al., 2015b), entailing cellular growth and differentiation, and a significant shift in nutrients being delivered to host peripheral tissues (Baldwin et al., 2004). Three important processes contribute to rumen development (Yanez-Ruiz et al., 2015); (i) rumen muscularisation, enlargement, and papillae growth (Reynolds et al., 2004), (ii) anaerobic microbial colonization (Fouts et al., 2012, Fonty et al., 1987), and (iii) the associated establishment of active microbial fermentation and enzymatic action (Rey et al., 2012, Faubladier et al., 2013). Proper development of the rumen at weaning is crucial in ensuring a smooth transition from milk-based to solid-based diets (Heinrichs, 2005). Inadequate rumen development can affect host nutrient utilisation (Baldwin et al. 2004), and complete maturation is required to facilitate the microbial digestion and subsequent absorption of feed components (Yanez-Ruiz et al., 2015). Therefore, the appropriate feeding and management regimen is crucial, as rumen development is crucial to for optimum calf growth and subsequent animal production in later life.

There are several phases of physical development of the rumen, from the non-functioning period in the first weeks of life when the animal operates as a nominal monogastric, through the transition from milk- to solid-based diets, and full rumination in the post-weaned animal (Wardrop and Coombe, 1960; Lane et al., 2002). Microbial colonisation, starter feed consumption, and fermentation and absorption processes are all crucial in triggering and

accelerating rumen development (Baldwin et al., Khan et al., 2011a). In the first weeks of life, the calf receives its energy requirements in the form of milk or milk replacer, which is routed directly to the abomasum by oesophageal groove action (Castro et al., 2016). Milk feeding does not directly stimulate rumen growth, as activation of the reticular groove shunts liquid feed directly to the abomasum (Black & Sharkey, 1970). This can be seen in the stagnation of rumen development in calves maintained solely on milk compared to those fed with grain or hay (Tamate et al., 1962). However, the amount of milk fed may indirectly influence rumen development in dairy calves. Traditionally, milk allowance was restricted to around 10% of body-weight daily, as higher milk intake causes supressed calf starter intake (Khan et al., 2011b). However, a body of evidence has emerged more recently showing that feeding elevated amounts of milk is beneficial for calf growth, and calves fed higher amounts of milk can still digest feed and grow sufficiently well despite reduced levels of solid feed intake (Liang et al., 2016, Bach et al., 2013). Feeding unrestricted amounts of milk replacer in early life, followed by gradual reduction, improved animal growth and rumen development (Schaff et al., 2017). When infused directly into the rumen, milk stimulated papillary growth, which was also observed following direct infusion of VFAs (Lane and Jesse, 1997, Tamate et al., 1962). This indicates that the production of VFAs, and thus the acquisition of an active microbial community, is essential for maturation of the rumen. Feeding calves solid feed should therefore stimulate rumen development via VFA production (Tamate et al., 1962).

The nature of solid diet offered (concentrate or forage) affects the pattern of VFA production and subsequently the rate of rumen development (Brownlee, 1956). Forages are usually a minor component of the pre-weaned diet, with cereal based calf starters widely used (Nocek and Kesler, 1984), though this varies across production systems. A recent study compared the provision of grain, forage, and no solid feed in conjunction with milk replacer feeding. The results showed that cereal-based calf starters resulted in rapid papillae development, while hay had little impact on papillary growth, and that the provision of solid feed had a major bearing on gene expression profiles in the rumen epithelium (Connor et al., 2013). Thus, it is important to note that simply offering low quality forage to growing calves may not be sufficient to propagate rumen development during the first weeks of life.

1.3. Rumen microbial diversity

The rumen microbiota comprises highly anaerobic bacteria, fungi, methanogenic archaea, ciliate protozoa, and viruses, which are central players in ruminant production. This microbial

consortium contains cellulolytic, hemicellulolytic, amylolytic, proteolytic, and biohydrogenating (lipolytic) species, exhibiting a high level of functional redundancy (Firkins and Yu, 2015, Hobson and Stewart, 1997). Thus, the rumen microbiota is well equipped to digest the varied diets of ruminant animals. The ruminal microorganisms are spatially organised into three fractions; free in the rumen fluid, attached to ingested feed particles, and adherent to the rumen wall – epimural (Stewart et al., 1988, Cheng et al., 1979). The prokaryotes which are directly associated with the eukaryotic members of the microbiota have also been proposed to constitute a fourth rumen fraction (Miron et al., 2001), but this is not consistently recognised in the literature. Studies comparing microbial communities across these fractions have found that the epimural microbiota differs significantly from that of the digesta, and may be more closely controlled by the host (Sadet et al., 2007, Malmuthuge et al., 2014). The ingested fibre, carbohydrates, protein, lipids and lignin are (except for lignin) hydrolysed first to monomers (e.g. glucose, amino acids) by the microbiota (Millen et al., 2016). Investigation of the temporal colonisation of ingested feed by the rumen microbiota showed divergent taxonomic and functional profiles among the primary and secondary colonisers, pointing to variation in their role(s) and/or substrate specificity (Wilkinson et al., 2018, Huws et al., 2016).

Unlike in monogastrics, ruminally-derived glucose is not available to the animal as a direct source of energy and is instead used by the microbiota to produce VFAs (Aschenbach et al., 2011, Aschenbach et al., 2010), so a certain amount enters the lower intestinal tract as bypass-starch. The rumen VFAs (mainly acetate, propionate, and butyrate), are absorbed and utilised as energy sources by the host animal (Jami et al., 2013). Acetate is primarily used for fat synthesis in adipose tissue and is also used as energy substrate by all extrahepatic tissues of the ruminant (Baldwin and Connor, 2017). Propionate produced in the rumen is largely used as a substrate for gluconeogenesis in the liver, which can account for 60% of the glucose used by the host animal (Baldwin and Connor, 2017, Harfoot, 1978, Purushe et al., 2010b). Butyrate is a significant energy source for the growth and maintenance of rumen epithelial cells (Donohoe et al., 2011). VFAs synthesised in the rumen can provide up to 70% of the host energy requirement (Bergman, 1990), and thus their production is essential to host performance. Metabolism of nitrogen-containing compounds (i.e. peptides) by the microbiota is also vital in the provision of microbial protein to the host animal for muscle and milk production (Bach et al, 2005). Other products of rumen microbial fermentation include carbon dioxide, methane, ammonia, and lactic acid (Mackie et al., 2001a). A schematic of major pathways of ruminal fermentation is presented in Fig. 1.2.

Many factors are postulated to influence microbial composition and activity in the rumen, and these are disseminated further in section 1.4.2 of this chapter. It is recognised that the rumen microbiota becomes more stable with age, and inter-animal variation decreases (Jami et al., 2013). Host genetics, age, diet, and geography (Hernandez-Sanabria et al., 2013, Jami et al., 2013, Henderson et al., 2015, Goodrich et al., 2016, Roehe et al., 2016) are among the determinants of rumen microbial composition and activity, and diet is the best studied to date. Interestingly, while diet has a major bearing on the digesta-associated microbiota, the epimural community is less affected, suggesting a stronger host influence on the epithelial-associated microbiota (Sadet-Bourgeteau et al., 2010, Sadet et al., 2007). Microbial composition in the rumen is also associated with variations in feed efficiency (Jami et al., 2014, Carberry et al., 2014b, Li and Guan, 2017), intensity of methane emissions (Kittelmann et al., 2014), health (Silberberg et al., 2013), and milk composition (Jami et al., 2014). More recently, heritability of certain groups of rumen bacteria in individuals has been investigated (Sasson et al., 2017), but the extent of the heritability of the rumen microbiota is not yet clear. Furthering our understanding of this complex microbial community throughout the life cycle of the animal will be critical in meeting future environmental and socioeconomic targets. The next sections will discuss in depth the microbial membership of the rumen and their functions.

1.3.1. Rumen bacteria

Bacteria are the most widely studied group of rumen microbes as they are the primary contributors to feed degradation and fermentation, thus underpinning the majority of VFA and microbial protein synthesis. The viable bacterial cell count has been estimated at 10^{11} cells / gram of rumen content (Mackie et al., 2001b), and bacteria can account for up to 95% of the rumen microbial community (Choudhury et al., 2015). The rumen bacteria are dominated by members of the *Firmicutes, Bacteroidetes* and *Proteobacteria* phyla, which can collectively account for >90% of bacterial abundance (McCabe et al., 2015, Henderson et al., 2015, Fouts et al., 2012). Bacteria in the rumen can be tightly or loosely attached to ingested feed particles, and digesta-associated bacteria are the most numerous among the four microbial fractions (McAllister et al., 1994). The liquid-based (planktonic) fraction are free in the rumen liquor and comprise around 30% of the rumen bacterial population (Millen et al, 2016). The digesta-associated bacteria ferment ingested feed or utilise the end-products of this fermentation and thus are dominated by fermentative species. The epimural bacteria, on the other hand, are often facultative anaerobes, producing urease and scavenging oxygen to assist in the maintenance of ruminal anaerobiosis (Liu et al., 2016).

The rumen bacteria can be further described based on their substrate preference. As outlined above, many rumen bacteria can metabolise several different substrates. While a range of plant components can be utilised by the rumen microbiota, many ruminal bacteria are not primary utilisers of plant biomass, and instead use as substrate the monomers or oligomers released from plant fibre by other members of the microbial consortia (Hobson and Stewart, 1997). Cellulose is a major constituent of forage and is the most abundant organic polymer on earth (Klemm et al., 2005). The primary cellulolytic bacteria of the rumen include *Fibrobacter succinogenes, Ruminococcus flavefaciens*, and *Ruminococcus albus* (Shi and Weimer, 1997). Hemicellulose comprises a significant proportion of polysaccharides in forage-heavy diets (Dehority, 1973) and is degraded by a range of bacteria including *Butyrivibrio fibrosolvens, Prevotella ruminocola, R. flavefaciens,* and *R. albus* (Zhou et al., 2015). Pectin is also present in forages, though to a lesser degree than cellulose and hemicellulose, and may be fermented by *B. fibrosolvens, P. ruminocola,* and *Lachnospira multiparus* among others.

The diet fed to ruminant livestock varies across production systems. While forage/pasture based diets are rich in fibre, high-energy grain-rich diets contain high amounts of starch, which can be fermented by a range of bacteria, including *P. ruminocola, Clostridium spp., B. fibrosolvens, Ruminobacter amylophilius* (Zhou et al., 2015). While fibres and starch generally are the predominant components of ruminant feeds, proteins are also degraded, usually by species of the *Prevotella* genus. *Prevotella* accounts for up to 70% of rumen bacterial abundance as identified by both enumeration and molecular techniques (van Gylswyk, 1990, Jami and Mizrahi, 2012). As well as peptides, *Prevotella* are also capable of metabolising starch and other simple sugars, producing succinate as the major fermentation product (Hobson and Stewart, 1997). The varied fermentative roles of *Prevotella* species mean they are usually the predominant rumen bacterium regardless of diet and other factors (Henderson et al., 2015).

While culture-based approaches and pioneer molecular techniques have identified between 200-400 distinct bacterial species within the rumen (Fouts et al., 2012) only a small fraction of the rumen bacterial community can be recovered using these methods. The application of deep sequencing technology has revealed that over 3,000 microbial species may inhabit the rumen (Denman and McSweeney, 2015), but many of these putative ruminal species have not been definitively identified. In a global census of rumen bacterial membership carried out in 2015, of 742 rumen content samples collected from 32 ruminant species across 35 countries, 30 dominant genus-level bacteria groups were found in over 90% of animals and represented 89.4% of all generated sequence data (Henderson et al., 2015). This suggests that current technologies

have probably defined the dominant groups of rumen bacteria. The same study also revealed the existence of a "core" group of rumen microbes, ubiquitous to ruminants worldwide, containing many of the notable ruminal species described above such as *Prevotella spp., Butyrivibrio spp.,* and *Ruminococcus spp.* (Henderson et al., 2015).

However, this core group also contained large amount of bacterial sequences that could not be classified below the family level (e.g. members of the *Lachnospiraceae* & *Ruminococcaceae*), indicative of the significant extent of uncharacterised diversity within the rumen. The high abundance of poorly characterised bacteria in the rumen, including the presence of putative species that do not have reliable phylogeny, particularly at the genus level (Kim et al., 2011, Creevey et al., 2014) highlights that whilst we may now detect the dominant ruminal bacteria, continued identification of novel members using both modern sequencing approaches and traditional culturing efforts will be required to fully characterise the composition and function of the rumen bacteriome. An example of such an initiative is the Hungate1000 Project (http://www.rmgnetwork.org/hungate1000.html), which aimed to provide a reference set of 1000 rumen-specific microbial genomes (Seshadri et al., 2018). While ultimately this project did not meet its initial goal, the novel rumen microbial genomes it produced have already shown their worth in facilitating significantly improved classification rates of rumen metagenomic sequences (Stewart et al., 2018).

It must also be noted that that among the thousands of bacterial species postulated to exist in the rumen, only 7 groups have been identified as core in ruminants globally, indicative of large variation between species and between individual animals. However, despite many years of research, the factors which contribute to large scale differences in bacterial diversity in the rumen, and how these differences impact on animal performance remains largely unknown.

1.3.2 Rumen archaea

Archaea, represented by the phylum *Euryarchaeota*, are methanogenic prokaryotes present in the rumen. Archaea account for 0.3-3.3% of the rumen microbial population, based on 16S rRNA gene analysis (Janssen and Kirs, 2008). These microorganisms scavenge hydrogen, formate, and methyl-containing compounds from the rumen environment to produce CH_4 gas. The rumen methanogens may be divided into three groups, according to the metabolic pathway by which they produce methane; (i) hydrogenotrophs which use H_2 as electron donors to reduce CO_2 to CH_4 (formate can also be used as an electron donor and may contribute to the production

of up to 18% of ruminal methane (Hungate, 1967) (ii) methylotrophs, which use methylamines or methanol, and (iii) acetoclastics (*Methanosarcinales*) which can use acetate and hydrogen to produce CH_4, making them the most metabolically diverse of the ruminal methanogens (Leahy et al., 2013).

Hydrogen is a by-product of normal rumen fermentation, and is a regulator of methane production in ruminants (Hegarty et al., 2007), whereby methanogens consume ruminal H_2 in the terminal step of carbohydrate fermentation (Deppenmeier, 2002). The volume of H_2 generated depends on the pathway of fermentation employed other members of the microbiota. For example, the conversion of a molecule of glucose to acetate will yield 8 hydrogen atoms, while the conversion of glucose to propionate consumes hydrogen (Deppenmeier, 2002). The build-up of H_2 in the rumen has been long thought to inhibit microbial metabolism and function (Sharp et al., 1998), though this has been questioned recently (Hristov et al., 2015). Hydrogenotrophic methanogenesis is the predominant pathway in the rumen, and is carried out mainly by *Methanobrevibacter* species, which typically account for over 90% of archaeal 16S rRNA reads (Hristov et al., 2012). This genus is divided into two subgroups; The SBGT clade contains *M. gottshalkii, M. thauri, M. Millerae, and M. smithii*, while *M. ruminantium, and M. ollayae* are contained in the RO clade (Janssen and Kirs, 2008, Kittelmann et al., 2013). Species of *Methanosphaera, Methanimicrococcus,* and *Methanobacterium* also utilise H_2 to produce CH_4 (Tapio et al., 2017). The less abundant methylotrophic methanogens include members of the *Methanosarcinales*, *Thermoplasmatales*, *Methanophaera,* and *Methanomassillicoccaceae* (Tapio et al., 2017, Janssen and Kirs, 2008, Poulsen et al., 2013).

Due to the environmental concerns outlined previously, the rumen archaea have been closely studied for their role in methanogenesis. Interestingly, it does not appear that the total abundance of rumen archaea is directly related to the intensity of methane emissions (Zhou et al., 2011, Danielsson et al., 2012). Rather, it seems that the expression of certain archaeal genes may be a more measurable predictor of rumen methanogenesis (Roehe et al., 2016), as the transcription of methanogenesis pathway genes within the rumen microbiome is greater in high-methane emitting animals (Shi et al., 2014). Methods of reducing methane emissions in livestock are discussed further in section 1.4.2 of this chapter.

1.3.3 Rumen protozoa

Protozoa were the first microorganisms described in the rumen, when Gruby & Delafond (1893) observed “animalcules” through their microscope. Despite many years of study, the role(s) of the protozoa within the rumen is still unclear (Newbold et al., 2015). Protozoa contribute to rumen function via digestion of structural and storage carbohydrates, oxygen scavenging, and regulate prokaryotic populations via predation and sequestration of carbohydrates (Hobson and Stewart, 1997). Some protozoa produce large amounts of H_2 during fermentation, and thus may contribute to CH_4 production via interspecies hydrogen transfer (Hobson and Stewart, 1997, Kittelmann et al., 2015, Gijzen et al., 1988).

Morphological studies have identified over 250 ciliate species in a range of ruminant hosts, represented by around 40 genera, and present at concentrations of 10^3 – 10^6 cells/ml of rumen fluid (Veira, 1986, Williams and Coleman, 1997). Rumen protozoa are currently assigned to two orders; the *Entodiniomorphids* and the *Vestibuliferida* (also referred to as the holotrichs) (Yohe et al., 2017). The *Entodiniomorphids* are smaller and highly abundant, and are dominated by the *Ophryoscolecidae* family, which contains more than 50% of the known rumen ciliates (Wright and Lynn, 1997). These ciliates are capable of degrading structural carbohydrates, starches, simple sugars and proteins, therefore providing an abundance of energy substrate and protein to the host (Williams and Coleman, 1992). *Entodinium spp.* are the most abundant protozoa in the rumen, and other common genera include *Polyplastron, Epidinium* and *Eudiplodinium* (Kittelmann et al., 2013, Carberry et al., 2012, Sylvester et al., 2004). Members of this order can absorb soluble compounds, but feed mainly by engulfment of feed particulate and the attached microbes, with bacteria as their main protein source (Williams and Coleman, 1997). They help stabilise fermentation in the rumen by storing starch grains in an amylopectin-like storage polysaccharide (Williams and Coleman, 1992).

The holotrich protozoa are less abundant, with *Isotricha*, *Dasytricha* and *Oligoiscotricha* being the most frequently occurring genera (Williams and Coleman, 1997). These ciliates primarily utilise easily soluble carbohydrates in the rumen, with specific sugar preference varying across genera (Williams, 1986). *Isotricha* and *Dasytricha* have a high abundance of storage polysaccharides in their endoplasm. They protect easily fermentable sugars from bacteria, and utilise them more slowly thus contributing to pH regulation in the rumen (Williams and Coleman, 1997, Dehority, 2003). Sequestration of carbohydrates by rumen ciliates may also confer a competitive advantage over the other microbial groups (Denton et al., 2015).

The importance of the protozoa in rumen microbial metabolism is still a point of debate. Protozoa are not essential for animal survival, and therefore defaunation (removal of protozoa by chemical or physical means) has been used to study their role in the rumen (Qin et al., 2012, Sahoo et al., 2005, Veira, 1986, Belanche et al., 2012). A recent meta-analysis examining the effects of defaunation in ruminants found that rumen organic matter digestibility and protein degradation were reduced in defaunated animals (Newbold et al., 2015). Defaunation also increases bacterial abundance in the rumen, likely due to niche and substrate availability in the absence of protozoa (Johnson & Coleman, 1992). Protozoa sequester protein in the rumen, as only about 50% of bacterial nitrogen gained from predation can be converted to protozoan protein for host utilisation, and are also capable of self-retaining in the rumen (Coleman, 1975, Abe et al., 1981). Their contribution to methanogenesis is also of interest with protozoa-associated methanogens estimated to generate up to 37% of total ruminal CH_4 (Finlay et al., 1994; Hegarty, 1999). *In vivo*¸ defaunation has reduced methane emissions by around 11% (Morgavi et al., 2012, Newbold et al., 2015). Presently there is no suitable farm-scale method for defaunation, and the associated effects can be inconsistent. Though not essential for host function, protozoa make important contributions to feed digestion and ruminal fermentation stability, but their role in methanogenesis and protein sequestration may be detrimental to the host.

1.3.4 Rumen fungi

The presence of anaerobic fungi in the rumen was first documented by Colin Orpin over 40 years ago. Fungi had been observed in the rumen as early as 1910, but were classified as being a type of flagellate protozoa, *Callimastix* (Liebetanz, 1910). Orpin's work showed that those microbes previously thought to be flagellate protozoa were in fact fungal zoospores (Orpin, 1975, Orpin, 1974). Even after Orpin's identification of the motile fungal zoospores (1975), acceptance of the presence of a fungal population in the rumen was slow, due to the long-held belief that all fungi were obligate aerobes. However, Orpin (1977) subsequently demonstrated the presence of chitin in the cell walls of the these "flagellates", which rubberstamped their place among the fungi.

Fungi are the least numerous microbial group in the rumen (around 10% of the microbial biomass, with variation according to diet and host) but are effective fibre degraders (Krause et al., 2013, Abecia et al., 2017). Fungi are among the initial colonisers of ingested feedstuff in ruminants, attracted via chemotaxis of sugar or phenolic constituents of plant fibre (Wubah and Kim, 1996, Theodorou MK, 2005), and harbour a vast repertoire of glycoside hydrolase (GH) enzymes including cellulases, hemicellulose, proteases, pectinases and amylases (Choudhury et

al., 2015). Degradation of plant cell walls is carried out both via rhizoidal invasion, growth and the complementary enzymatic secretion of hydrolytic enzymes (Orpin, 1977). Fungal metabolism of feed pentoses or hexoses results in the production of acetate, formate, lactate, ethanol, CO_2, and H_2 (Patra et al., 2017). The rich repertoire of fungal lignocellulosic enzymes detected within the rumen metagenome indicates the importance of these species in fibre degradation (Yousuf et al., 2013).

The anaerobic fungi differ from their aerobic relatives, and are characterised by the presence of a hydrogenosome in place of mitochondria, for energy production. Anaerobic fungi have their own distinct taxonomic clade, confined to the order *Neocallimastigales*, part of the phylum *Neocallimastimycota,* and containing 6 genera (Hibbett et al., 2007, Gruninger et al., 2014). However, as with the other microbial groups found in the rumen, modern high-throughput molecular analysis suggests that there is a large proportion of the rumen fungi that remain uncharacterised (Fouts et al., 2012). Furthermore, variation in rumen fungal composition across individuals appears to be greater than for the other microbial groups (Kittelmann et al., 2013, Kittelmann et al., 2012). Like the protozoa, the overall contribution of the fungi to feed digestion remains to be elucidated. Much diversity of the rumen fungi remains unknown and more studies are required to properly characterise the role of these eukaryotes in ruminal ecology and feed digestion.

1.3.5 Rumen viruses

The rumen virome is the most recent of the major rumen microbial sub-populations to be studied. Despite the existence of approximately 10 viral cells for each bacterium in a given ecosystem, the virome has been neglected in studies of the rumen microbiota to date, due mainly to the absence of suitable marker genes (Wallace et al., 2014) . Viruses are important regulators of microbial populations and facilitators of horizontal gene transfer (Duthie et al., 2017, Berg Miller et al., 2012). An abundant cohort of bacteriophages and archaeaphages live within the rumen, but their ecological role is poorly understood (Chaucheyras-Durand and Ossa, 2014). The first report of bacteriophage recovery from the rumen came in the 1960s (Duffield et al., 2012). The development of electron microscopes and subsequent analysis of rumen fluid confirmed the presence of a diverse virome in the rumen, dominated by members of the order *Caudovirales* (He et al., 2013).

The rapid development of deep genomic and metagenomic sequencing technologies in the last decade has revolutionised our understanding of the rumen phage community. A pioneer metagenome study examined the phage composition in the rumen of dairy cows using pyrosequencing (Berg Miller et al., 2012). Over 28,000 distinct viral genotypes were recovered, indicating large viral diversity in the rumen, in contrast to the 40 species postulated to exist using electron microscopy. Unsurprisingly, the most abundant bacteriophage and prophage genomes were associated with the major rumen bacterial groups; *Firmicutes, Bacteroidetes*, and *Proteobacteria*, but 78% of sequences recovered from the rumen did not match a previously described virus (Miller et al., 2012). A 2013 study examined the rumen virome of dairy cattle using shotgun metagenomic sequencing, and reported that though taxonomic divergence of the rumen virome was observed between animals housed together and those housed individually, the functional profile of the rumen virome is highly conserved between individuals, and different to that of the rumen bacteriome (Ross et al., 2013). The generation of more information by deep sequencing of the virome will aid such studies, allowing the proper elucidation of the roles played by the virome in the maintenance of rumen microbial balance, and its influence on ruminant nutrition. While a small number of studies have investigated the viral community of adult ruminants, there is no currently available information concerning the temporal dynamics of the rumen virome during early life.

1.3.6 Hindgut microbiota in ruminants

Though the rumen is the major site of microbial fermentation and nutrient absorption in cattle, a substantial amount of rumen undigested organic matter may pass to the hindgut regions (cecum, colon, rectum, Fig. 1.1) for digestion (Moss et al., 2000). As with the rumen, the hindgut regions contain a diverse microbial community (though protozoa are absent) capable of hydrolysing fibres, carbohydrates and peptides to produce CH_4, VFA and microbial proteins (Gressley et al., 2011). Hindgut fermentation is generally less effective than that of the rumen however, due to poor quality substrates which have already been partially metabolised by the ruminal and intestinal microbiota, and lower particle retention time in the hindgut than in the rumen (13h vs. 30h) (Vanhatalo and Ketoja, 1995, Yang et al., 2002). The contribution of the hindgut and its microbiota to ruminant production remains poorly understood. Up to 10% of ingested feed may be metabolised in the hindgut regions (Gressley et al., 2011), and the microbiota of the cecum and colon have been related to feed efficiency in steers (Myer et al., 2015c, Myer et al., 2015b). Moreover, the hindgut microbiota is speculated to play an elevated role in organic matter digestion during the pre-weaning period (Castro et al., 2016). Further research is needed

to accurately define the role(s) of the hindgut and its microorganisms in ruminant digestion and production.

1.4 Role of the rumen microbiome in animal production

1.4.1 The rumen microbiome and feed efficiency

The projected increases in food demands outlined in introduction of this chapter mean the efficiency of food production, both animal and crop derived, must be improved (Berry and Crowley, 2013). The term feed efficiency (FE) describes the efficacy at which the conversion of feed to useable product occurs, and it is a moderately heritable trait in cattle (Cammack et al., 2018, Berry and Crowley, 2013). Feed inputs account for up to 75% of variable costs in beef operations, and 40-60% of those in dairy systems (Finneran et al., 2010, Bach, A., 2012), and so improving feed efficiency is a means of increasing output while minimizing costs. Several measurements of FE have been used in cattle (e.g. feed conversion ratio (FCR) (Sherman et al., 2008) and partial efficiency of growth (PEG) (Lucila Sobrinho et al., 2011)), but residual feed intake (RFI) is the most commonly used today. First proposed in 1963, RFI is defined as the difference between actual and predicted feed intake of an animals for maintenance of body weight and for weight gain (Koch et al., 1963). Genetically independent of growth, animals may be classified as Low-RFI (efficient) or High-RFI (inefficient) (Alemu et al., 2017, Kong et al., 2016, Carberry et al., 2014b).

While a range of physiological processes contribute to divergence in FE within a population (Richardson and Herd, 2004) the fact that the conversion of ingested feedstuff to energy substrate (e.g. VFA) is dependent on the rumen microorganisms suggests that the rumen microbiome may play an important role in determining an animal's efficiency status. Several studies have shown associations between rumen microbial profiles and FE (Shabat et al., 2016, Guan et al., 2008, McCann et al., 2014b, Carberry et al., 2014a, Carberry et al., 2014b, Carberry et al., 2012, Li and Guan, 2017). In a landmark study, Guan and colleagues (Guan et al., 2008) demonstrated that rumen microbial ecology of efficient (L-RFI) cattle differed from that of their inefficient (H-RFI) counterparts, and there was also a greater similarity in microbial profiles among the efficient animals. More recently, the use of high throughput sequencing technologies demonstrated that efficient animals had lower rumen microbial diversity and richness, both in terms of microbial species and gene content (Shabat et al., 2016, Li and Guan, 2017), and metabolic profile (Roehe et al., 2016). This suggests that the rumen microbiome of efficient

animals contains less non-essential microbes, though it is unclear if this is a cause or result of the efficiency phenotype. Variation in VFA concentration according to RFI classification has also been reported, but these differences appear to be diet-dependant (Hernandez-Sanabria et al., 2012, Shabat et al., 2016, Guan et al., 2008).

A range of microbial groups, from phylum to species level, have been associated with FE in the literature, including associations between improved FE and the abundances of the bacterial *Lachnospiraceae* and *Veillonellaceae* families (Myer et al., 2015a, Li and Guan, 2017), and a number of archaeal taxa (Carberry et al., 2014a, Carberry et al., 2014b, Li and Guan, 2017). However, there are some inconsistencies in these reports; for instance, while the ruminal abundance of *Dialister* was associated with improved FE in steers (Myer et al., 2015a), species belonging to this genus were associated with reduced efficiency in lambs (Ellison et al., 2017). As the rumen microbiome is influenced by diet composition (Henderson et al., 2015), and FE classification is rarely consistent in individuals across diets (Durunna et al., 2011), associations between the rumen microbiota and FE may be driven, partially at least, by diet. However several studies have demonstrated diet-independent effects of FE on the rumen microbiota (Hernandez-Sanabria et al., 2012, Ellison et al., 2017, Carberry et al., 2012), indicating that a core group of microbes associated with variation in FE could be used to identify efficient animals irrespective of diet (Cammack et al., 2018). Furthermore, there is evidence that selection for improved FE may also contribute to reduction in ruminant methanogenesis, as discussed in the next section (Basarab et al., 2013, Shabat et al., 2016).

1.4.2 The rumen microbiome and methane production

Livestock industries are a significant source of environmentally harmful GHG, with carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O) the major GHG emitted from food and agriculture chains. The potent GWP of CH_4 means it is the most extensively studied GHG in terms of ruminant production emissions, and reducing levels of CH_4 formation in the rumen is desirable in terms of both improved animal productivity and environmental stability. The biochemical pathways underpinning ruminal methanogenesis have been described in section 1.3.2 of this chapter.

There are many factors which underly the rate of intensity of rumen methanogenesis. Dietary composition can have a major effect on the volume of measurable ruminal CH_4; high forage diets favour microbial acetate synthesis in the rumen, leading to increased H_2 and

consequentially more CH_4 production than under concentrate-rich diets, where starch is mainly metabolised to propionate (Wolin, 1960). While it may seem profitable to simply move away from feeding forages to cattle, reduced rumen pH under high-starch diets may contribute to imbalance of the microbial community and fermentation, and lead to subacute ruminal acidosis (Plaizier et al., 2008). Furthermore, given that the majority of global livestock rely on forage sources for growth, different strategies for reducing methane formation across a range of diets are needed. A variety of methods of reducing ruminal methane emissions have been investigated, and work either by directly targeting the methanogen community or attempting to reduce/redirect hydrogen flow in the rumen, thus providing less substrate for archaeal metabolism. These mitigation strategies have been comprehensively described elsewhere (Hristov et al., 2013, McAllister et al., 2015, Martin et al., 2010, Knapp et al., 2014, Kumar et al., 2014, Cammack et al., 2018, Pickering et al., 2015), and include dietary manipulation, plant lipid feeding, synthetic methanogen inhibitor supplementation, and genetic selection for low-emitting animals. Methanogens may also acquire H_2 via interspecies hydrogen transfer, particularly from protozoan populations, as some methanogens are symbiotically associated with protozoan cells (Janssen and Kirs, 2008). Consequentially, some studies have examined the significance of defaunation on methane production (Qin et al., 2012), finding that defaunation results in an average of 11% reduction in CH4 emissions (Newbold et al., 2015). However, as discussed in an earlier section, options for farm-scale defaunation are limited.

Arguably the most effective mitigation strategy demonstrated to date is basal dietary supplementation with 3-nitroproxypropanol (3-NOP). 3-NOP was developed in 2012 (Duval, 2012), and acts by inhibiting the methyl coenzyme-M reductase (MCR) enzyme in the terminal step of methanogenesis. Supplementation of 3-NOP has been shown to dramatically reduce ruminal CH_4 production in lactating dairy cows, and crucially does not have any adverse effect on milk yield (Hristov et al., 2015), though an increase in milk fat has been reported (Lopes et al., 2016). It has also proven an effective methane inhibitor in sheep (Martínez-Fernández et al., 2014) and beef cattle (Romero-Perez et al., 2015). Furthermore, there is no current evidence of microbial adaptation to the feed additive, as has been observed when other MCR inhibitors such as bromoethanesulfonate were added to the basal diet (Immig et al., 1996). Yet, with a large proportion of the world's domesticated ruminants existing in open pasture, the practicalities and economics of continued supplementation with 3-NOP (or any dietary additive) are unclear, and the compound has not yet been approved for commercial use. It should also be noted that total inhabitation of methanogen growth in the rumen is not necessary, as a 50% reduction in their growth rate appears sufficient to cause their washout from the rumen (Tapio et al., 2017).

A critical aspect of an effective methane abatement strategy (or indeed any intervention which aims to elicit a change in rumen microbial composition or function) is the persistence of such changes in the long term. Thus, in recent years, there has been much focus on the early life colonization of the rumen, in the hope that gaining insights into the patterns of microbial establishment may offer a "window of opportunity" for effective, persistent management or dietary interventions to permanently modify the rumen microbiota towards a more desirable composition. The current state of the art and knowledge gaps concerning this early life paradigm are presented in the following sections.

1.5 Early life – the "window of opportunity" for effective manipulation of the rumen microbiome?

While some of the strategies to manipulate rumen fermentation outlined in section 1.4 of this chapter have been successful in the short term, it has proven difficult to permanently modify the established microbiota of the mature rumen, which generally reverts to the original composition following the cessation of treatment/supplementation (Weimer, 2015, Weimer et al., 2010). This is less evident, however, in the first weeks of life, when the rumen community is highly dynamic and variable across individuals, as discussed below (Yanez-Ruiz et al., 2015). This has given rise to the notion of "microbial programming" of the rumen microbiota - dietary or management interventions in early life that will imprint a desirable and persistent microbial pattern on the rumen, before the microbiota becomes fully established – as a means of improving ruminant production (Yanez-Ruiz et al., 2015). There is evidence that such interventions during early life may have long-lasting effects on rumen microbial composition, but few long-term studies have been conducted to date (Yanez-Ruiz et al., 2010, Veneman et al., 2015, Krause et al., 2003). To effectively discern the optimal time for manipulation/intervention, the temporal sequence of rumen microbial colonisation, and the factors which influence it, must be fully defined. Accordingly, recent years have seen renewed interest in the patterns of microbial colonisation of the rumen during the first days and weeks of life (Jami et al., 2013, Li et al., 2012a, Jiao et al., 2015a). A number of studies using deep sequencing approaches have characterised microbial progression in rumen fluid recently (Malmuthuge et al., 2014, Rey et al., 2013, Jiao et al., 2015a, Jiao et al., 2015b), but there has not been a comprehensive study to determine microbial colonisation and succession across all ruminal niches. Early life experience has been shown to have a lasting effect on gut microbial communities in humans (Koenig et al., 2011), and feeding different diets prior weaning promoted divergent bacterial establishment in the rumen of bull calves (Eadie et al., 1959). The role of the birthing process in shaping this microbial community

also remains elusive. There is some, albeit conflicting, evidence concerning *in utero* microbial colonisation on the perinatal GI microbiota in humans (Mao et al., 2015), but it is unclear if the same may occur in ruminants, due to differences in placentome architecture (Steele et al., 2016). The following sections will discuss physical and microbial co-development of the rumen, the factors which influence it, and attempts to redirect rumen microbial metabolism via interventions in the first weeks of life.

1.5.1 Microbial colonisation of the pre-functional rumen

It has been commonly accepted for many years that the mammalian gastrointestinal tract is sterile at birth, devoid of microbial life (Escherich, 1885). Recently however, this long-held dogma has come under scrutiny as evidence of *in utero* colonisation of the human foetal GIT has come to light (Jimenez et al., 2008, Aagaard et al., 2014). Yet this remains a point of intense debate. In a recent critical review, Perez-Muñoz et al. (2017) examined all existing data relating to microbial colonisation of the mammalian GIT *in utero* and concluded, that based on currently available information, there is insufficient evidence to conclusively support the idea of GIT microbial colonisation of the developing foetus. Furthermore, there is at present no evidence of *in utero* colonisation of the ruminant GIT, and it is unknown if ruminant placentome structure would precludes the passage of any maternal microorganisms to the developing calf during gestation (Steele et al., 2016). Moreover, in a recent study, Malmuthuge and colleagues (2018) demonstrated the sterility of the foetal bovine GIT during the third trimester of pregnancy. Therefore, for the purposes of this review we accept the hypothesis of a sterile rumen prior to birth, which is rapidly colonised by a complex microbiota from the surrounding environment and other animals during or following delivery.

1.5.2 Early life rumen microbial dynamics

The dynamics of microbial establishment in the rumen were first scrutinised in the 1940's using microscopy (Pounden and Hibbs, 1948), but knowledge of the pattern of microbial development in the rumen was limited until the 1980s. Gerald Fonty performed a series of landmark studies using gnotobiotic lamb models (Fonty et al., 1987, Fonty et al., 1983a, Fonty et al., 1983b, Fonty et al., 1989), extensively characterising the establishment and development of the prokaryotic and eukaryotic populations in the first days and weeks of life of the young ruminant using culture-based approaches. These studies showed that microorganisms colonise the rumen in an ordered and sequential manner soon after birth, and that the major functional

groups of taxa including fibrolytic bacteria and methanogenic archaea, become established within the first week of life (Anderson et al., 1987). The initial colonisers of rumen are aerobes and facultative anaerobes (e.g. *Streptococcus* and *Lactobacillus* species (Fonty et al., 1987)). By the second day of life, anaerobic bacterial concentrations in the rumen of lambs reared with their mothers reached levels of 10^9 cells/ml, and strictly anaerobic bacteria predominated over facultative anaerobes to the order of 10-100-fold. However, this anaerobic population did not have the same characteristics as that of the adult ruminant, harbouring *Propionibacterium, Clostridium, Peptostreptococcus* and *Bifidobacterium* as dominant members. These genera are usually not major contributors to bacterial diversity in the adult rumen (Fonty et al., 1987). Fonty's work also demonstrated that cellulolytic bacteria important for microbial degradation in the adult rumen are already present within the first 48 hours of life (e.g. *Fibrobacter succinogenes*) (Fonty et al., 1987, Fonty et al., 1989). Methanogenic archaea were detected as early as day 2 using cultural methods, while a more recent study detected methanogens in the ovine rumen immediately after birth (Guzman et al., 2015). Fungal spores may be detected by day 8, dominated by *Neocallimastix frontalis*. However, anaerobic fungi could not be detected in the majority of lambs following the initiation of a high-energy grower diet at 3 weeks of age, showing the influence of diet on rumen microbial colonisation patterns (Fonty et al., 1987). The ciliate protozoa are the final microbial colonisers of the rumen, and their establishment seems to require the prior establishment of a complex microbiota (Fonty et al., 1988). A natural defaunation of the rumen occurs around weaning, but the rumen is re-occupied by protozoa around 3 months of age. It is not clear what induces this phenomenon, and protozoan establishment is considered a marker of maturation of the rumen microbiota (Belanche et al., 2012, Fonty et al., 1988).

In recent years several studies have applied modern molecular techniques to study the pattern of microbial progression in the rumen (Jiao et al., 2015a, Wang et al., 2016a, Jami et al., 2013). Bacterial life has been detected in the rumen immediately following birth using quantitative PCR (Guzman et al., 2015). While this was in some regards a landmark study, these findings were not replicated in a previous investigation (Rey et al., 2013), when bacterial amplicons could not be generated on day 1 of life. We must further note that DNA was used for the reactions, presenting the possibility that any amplification was of dead or contaminate nucleic acid. Further, while the authors of the 2015 study slaughtered new-born calves to obtain rumen samples, the latter relied on rumen tubing, which may be insufficient at such a young age. Yet, a diverse rumen fluid bacteriome was detected on the first day of life using pyrosequencing (Jami et al., 2013), with the same study also demonstrating the subsequent shift towards dominance of the bovine rumen by obligate anaerobes as observed by Fonty using gnotobiotic lamb models

(Jami et al., 2013). These discrepancies may be due to differential sampling technique and expertise or targeting of different hypervariable regions for 16S rRNA gene amplification. Culture based approaches showed early colonisation of known rumen bacteria in neonates (Fonty et al., 1987). Using qPCR, Jami and colleagues (2013) also quantified the abundance of important rumen biomass degraders in animals from day 1 to 2 years. The cellulolytic *Ruminococcus flavefaciens* was detected after 1 day of life, while important VFA producers like *Selenomonas ruminantium* and *Megasphaera elsdenii* were also present in the rumen in 1-day-old calves. The authors also reported the high abundance of facultative anaerobes (*Streptococcus bovis*) within 24h of birth, confirming earlier findings (Fonty et al., 1983a, Jami et al., 2013). These early colonisers are likely to function in scavenging the available oxygen in the rumen, thereby creating the anaerobic environment necessary for proper fermentation.

Consistent among the studies examining early life dynamics of the rumen microbiota is a high level of heterogeneity amongst individual animals, which decreases with age (Jami et al., 2013, Jami and Mizrahi, 2012, Malmuthuge et al., 2014). A study examining the taxonomic and functional profiles of rumen bacteria in 2-week and 6-week old calves, and 2-year old cows using amplicon and shotgun sequencing, found that the major types of rumen bacteria, including cellulolytic taxa, were present at 14 days of age, even in the absence of solid feed (Li et al., 2012b). The microbiota undergoes continued evolution through to adulthood. The major rumen bacterial groups, *Firmicutes, Bacteroidetes* and *Proteobacteria* are among the proto-colonisers of the rumen, but their proportion changes significantly as the animal ages (Jami et al., 2013, Jiao et al., 2015a, Jiao et al., 2015b), and 16S ribosomal RNA gene profiling of rumen fluid revealed only a small number of shared taxa between 2-week old and 2-year old dairy cows (Dill-McFarland et al., 2017). When calves were maintained on milk-only diets, *Prevotella* was replaced as the most abundant genera by *Bacteroides* on day 42, before regaining predominance in the rumen of adult cattle (Li et al., 2012b). Furthermore, of the 15 phyla detected in the rumen fluid of 14-day and 42-day old calves, four out of the five most abundant were significantly altered with age, indicating that rumen microbial composition is highly dynamic in early life (Li et al., 2012b).

Development of the rumen epimural community has also been shown to be age related. While the epimural community may only represent 1% of the total microbial biomass in the rumen, they are presumed to have important roles in oxygen removal (Stewart et al., 1988). *Proteobacteria* was the predominant bacterial phyla adhered to the rumen wall on the first day of life (Jiao et al., 2015a), agreeing with earlier observations using electron microscopy (Rieu et al., 1990). However, as the animal ages, the abundance of epimural *Proteobacteria* decreases, while

that of *Bacteroidetes* and *Firmicutes* increases, as also seen in the digesta-associated microbiota (Jiao et al., 2015b).

We must be cognisant that it is very difficult to separate "age" and "diet" into distinct factors during early life. Dietary composition and intake levels change substantially within the first weeks of life, and so a target of future studies should be to discern the extent to which diet influences microbial development in the rumen independently of calf age, and vice versa.

1.5.3 Functional achievement of the rumen microbiota in early life

As well as characterising the taxonomic composition of the early life rumen microbiota, several studies have also examined its function and activity, as proper microbial function is crucial in supplying sufficient VFA to support host growth and development. Proteolytic, ureolytic, xylanase, and amyltic enzymatic functions are established rapidly in the rumen following birth and appear to reach a peak between days 10 and 23 (Rey et al., 2012), although a subsequent study could not obtain a sample of sufficient quality from week old calves to determine microbial activity (Jiao et al., 2015b). Using unrestricted DNA shotgun sequencing of rumen fluid, over 8,000 protein families including important glycoside hydrolases have been identified in 2-week old calves (Li et al., 2012b). This study reported that while the taxonomic profile of the rumen fluid-associated microbiota is dynamic in early life, the functional profile is largely stable from 2 to 6 weeks. These studies, taken together and considered with the microbial colonisation process outlined above, suggest that there is a large potential for carbohydrate digestion present in the developing rumen, even in the absence of solid feed.

1.5.4 Factors influencing rumen microbial colonisation and development in early life

The composition and function of the rumen microbiota are sensitive to a range of factors (Henderson et al., 2015). The diets of ruminants can change many times throughout the life cycle of the animal, from the weaning transition (milk to solid feed) to high-grain finishing rations in the feedlot, to the standard dietary cycles of dairy cows. However, less is known of the factors and mechanisms which may exert control over microbial establishment and dynamics in the developing rumen. Contact with other animals appears to be a factor in microbial succession in the rumen. When neonatal lambs were separated from their dams within the first 24 hours of life and fed milk replacer, cellulolytic bacteria, fungi, or protozoa did not colonise the rumen (Fonty et al., 1983a). When lambs were reared in a flock with other adult ruminants, a more complex

microbiota was observed versus those raised only with their dam and establishment of cellulolytic bacteria in the rumen was slower (Fonty et al., 1987). Similar findings were reported recently in goat kids, where goats raised with their dam acquired a more complex rumen microbiota throughout the first month of life than their twin counterparts who were raised on artificial milk (Abecia et al., 2017). Therefore, it appears that specific maternal contact may be important in the initial acquisition of important rumen microbiota following birth.

However, while several factors may influence the colonisation process in the rumen, the introduction of solid feed appearing to drive the most evident change (Rey et al., 2013, Jami et al., 2013, Dill-McFarland et al., 2017). Introducing solid feed as part of an early weaning strategy in calves (3-week weaning) increased microbial abundance in the rumen versus calves weaned at six weeks of age (Anderson et al., 1987). In 3-week old calves offered milk replacer and calf starter, *Prevotella* and *Bacteroides* both accounted for ~15% of the 16S rRNA genes (Malmuthuge et al., 2014). However, when calves were offered only milk, there was a significant increase in the proportion of *Bacteroides* as the animal aged, with *Prevotella* found to be predominant in adult cattle fed a hay-based diet (Li et al., 2012b). Interestingly, when the microbiota of 6-month and 24-month old cattle fed the same diet was investigated, it was still found to differ significantly, indicating that even in older animals, microbial composition continues to change, independently of diet (Jami et al., 2013). The weaning transition is a stressful period for ruminants, and is associated with suppressed intakes and growth (Meale et al., 2017). While it is difficult to separate weaning and diet into distinct factors, several recent investigations have demonstrated that weaning age and strategy may influence microbial colonisation patterns in the rumen (Meale et al., 2016, Meale et al., 2017). In particular, a later weaning strategy may allow the microbiota to adapt better to the dietary change, potentially reducing the negative effects of early and/or abrupt weaning practices (Meale et al., 2017).

Given that certain conditions can favour differential establishment patterns of the rumen microbiota, there have been attempts to imprint a modification on microbial colonisation in this period. The impact of pre-weaning direct fed microbial (probiotic) supplementation on the establishment of the rumen microbiota has been explored. The addition of live *Saccharomyces cerevisae* cultures to feed accelerated the colonisation of cellulolytic bacteria and protozoa, and promoted microbial activity in early life (Chaucheyras-Durand and Fonty, 2002). However the long term impact of such interventions is unclear; when *Megasphaera elsdenii* was supplemented to 14 day old calves, no effect on rumen fermentation, rumen metabolism, or the abundance of ruminal *M. elsdenii* was detected on day 70 post-dosage suggesting microbial adaptation to the

supplement (Yohe et al., 2017). Supplementation of goat kids with the anti-methanogenic compound bromochloromethane (BCM), resulted in an alteration of the rumen methanogen community which persisted for up to three months (Abecia et al., 2013, Abecia et al., 2014a).

This suggests that differential management and feeding programs in early life may have long term implications on patterns of microbial colonisation in the developing rumen, and furthering our knowledge of rumen microbial kinetics in this period may facilitate the design of novel strategies to imprint a lasting effect on rumen microbial diversity.

1.6 Methods to study the rumen microbiome

As discussed in previous sections, the rumen microbiome is a complex community postulated to contain upwards of 2000 species (Firkins, 2010). Studies of the rumen microorganisms generally aim to answer one or more of the following questions; (i) "who are there?" (ii) "how many of them are there?" and (iii) "what are they doing?".

Early traditional microbiology and molecular biology techniques used to study this community lacked the sensitivity to accurately characterise the breadth of rumen microbial diversity. While today such approaches have been largely superseded by high throughput omics technologies, they continue to be used to study the rumen microbiota. The following sections will detail methods of microbial community analysis and how they pertain to the rumen microbiome.

1.6.1 Terminology

As the methods used to study the rumen microbiome have been developed and refined over the years, the terminology surrounding their use has also evolved. Today there remains incongruity over the correct nomenclature when referring to host-associated microbial communities. The term "microbiome" refers to the entire habitat in question; the resident microorganisms, their genomes, and the surrounding environment (Marchesi and Ravel, 2015). Therefore, this should not be used when referring purely to community membership, where "microbiota" is more suitable (e.g. when discussing results of a 16S rRNA amplicon survey). The term "microflora" is antiquated and is not widely used in reference to gut microbial ecology.

1.6.2 Classic cultivation-based methods

Investigations of anaerobic microbial communities in the early 20th century toyed with different approaches, but it was the development of the roll-tube technique by Robert Hungate in 1969 that allowed the first robust assessment of the microbiota of the rumen (Hungate, 1969). Hungate's research allowed the successful simulation of anaerobic conditions *in vitro* which facilitated major breakthroughs and progress in knowledge of the rumen microbiota, as anaerobic microorganisms could be isolated, and their preferred substrates and products examined and described in detail (McCann et al., 2014a, Hungate, 1950). This work laid the foundation for much fundamental knowledge of rumen microbiology, and his roll-tube method continues to be used today. There is still a considerable amount of research on the rumen microbiome that employs culture-based methods, aiming to improve cultivation techniques and characterise as many novel rumen microbes as possible (Miltko et al., 2015, Kenters et al., 2011, Nyonyo et al., 2014, Creevey et al., 2014, Fukuma et al., 2015, Kobayashi et al., 2008, Oh et al., 2017). This work is essential to definitive elucidation of the rumen microbial community, as the biochemical characteristics of novel rumen microorganisms can only be fully understood once they have been grown and observed *in vitro*.

However, such methods have allowed for the identification of less than 15% of the putative bacterial diversity in the rumen (Morgavi et al., 2013, E. Edwards et al., 2004). Additionally, there is no single culture medium that can be used to grow the vast extent of ruminal bacteria. To overcome these limitations, several molecular techniques were developed in the latter stages of the 20th century whereby microbial species could be identified based on their genetic content, rather than biochemical or phenotypic identification in culture.

1.6.3 Molecular methods to evaluate the rumen microbiome

As it became clear that most anaerobic microorganisms could not be characterised using traditional microbiological approaches, newer culture-independent methods were needed for robust investigations of microbial communities. Several novel molecular techniques were developed in late 20th century which distinguished microbial species based on their genetic content rather than biochemical or phenotypic characterisation in culture. Studies of bacterial and archaeal populations have utilised DNA (or RNA-based analysis via reverse transcription) sequencing of the 16S rRNA gene (Deusch et al., 2017, McGovern et al., 2017, McCabe et al., 2015), while the 18S rRNA and Internal Transcribed Spacer (ITS) genes have been used to study protozoan (Kittelmann et al., 2015) and fungal (Kittelmann et al., 2013) composition, respectively. There have been vast technological advances in the last decade which have revolutionised our

understanding of the rumen microbiome and its relationship with the host, and these will be detailed in the following sections.

1.6.4 Pioneer molecular techniques

Work in the 1970s and 1980s showed that bacterial composition of a community could be measured using the 16S ribosomal RNA (16S rRNA) gene (Woese, 1987, Woese et al., 1983, Woese and Fox, 1977). The 16S rRNA gene is around 1,550bp in length (Bouchet et al., 2008), is ubiquitous in bacteria and archaea, and is today regarded as the gold standard marker gene in prokaryotic ecology studies, as it is phylogenetically conserved across species and is thought to be only weakly affected by horizontal gene transfer (Sunil Kumar Sirohi, 2012, Woese et al., 1983). The gene contains nine hypervariable regions (V1-V9) which can be used to distinguish between bacterial/archaeal species, flanked by conserved regions (Chakravorty et al., 2007). PCR primers complementary to the conserved regions are used to amplify the interspersed hypervariable regions, allowing for rapid identification of individual bacterial species (e.g. for quantification using qPCR) or general bacterial and archaeal diversity using universal primers. The choice of variable region is important and should be habitat-specific, as this can have a significant bearing on results (Soergel et al., 2012). In studies examining methanogen populations in the rumen, type-2 chaperonins and the methyl co-reductase A (mcrA) genes have also been used as taxonomic markers (Ozutsumi et al., 2012, Chaban and Hill, 2012, McGovern et al., 2017). PCR amplicons from these marker genes can then be used in a variety of subsequent techniques to assess microbial composition in a sample.

A number of molecular techniques were developed to harness the discriminatory power of the 16S rRNA gene, most of which used PCR amplicons (Mullis and Faloona, 1987, Mullis et al., 1986) to broadly assess rumen microbial composition. Among them, denaturing gradient gel electrophoresis (DGGE) (Lukas et al., 2010, Petri et al., 2012, Sadet et al., 2007), termination restriction fragment length polymorphism (TRFLP) (Yanez-Ruiz et al., 2010, Castro-Carrera et al., 2014), fluorescence in-situ hybridisation (FISH) (Xia et al., 2014), and molecular cloning (Koike et al., 2003) have been used in investigations of the rumen microbiota. Among the most widely used, DGGE can identify microbial phylotypes based on their differential migration through a gel, and was initially used in the detection single point mutations (now usually referred to as single nucleotide polymorphisms – SNPs) in disease-associated genes (Fischer and Lerman, 1979). It was first applied to microbial community analysis in the early 1990s (Muyzer et al., 1993). Subsequently DGGE was employed in studies of the rumen microbiota (Kocherginskaya et al.,

2001), most notably being used in the first descriptions of the relationship between feed efficiency and rumen microbial composition and fermentation in beef cattle (Guan et al., 2008, Carberry et al., 2014b, Carberry et al., 2012).

Molecular cloning and subsequent sanger sequencing (Sanger et al., 1977) of 16S rRNA gene amplicons has also been widely used to study the rumen microbiome. Briefly, following isolation of DNA, PCR amplicons from the target group are cloned into a plasmid cloning vector. Following growth in culture medium, positive clones (i.e. containing the DNA sequences of interest) are randomly selected for plasmid DNA extraction and sequencing (Wright, 2005). Molecular cloning has been used to demonstrate the existence of divergent methanogen genotypes between efficient and inefficient cattle (Carberry et al., 2014b), and to investigate strain-level diversity of *Ruminococcus flavefaciens* in the rumen (Brulc et al., 2011), among other studies.

While technologies like DGGE and molecular cloning made important contributions to our knowledge of rumen microbial diversity, they are hampered by low throughput, poor resolution of taxonomic profiles, and have been rendered mostly obsolete by the advent of second and third-generation technologies discussed in the next section.

1.6.5 Modern technologies to study the rumen microbiome

Though the first generation of sequencing technologies, based on Sanger sequencing of PCR clones, could sequence the entire 16S rRNA gene, such approaches were severely limited by low throughput (Shendure and Ji, 2008). When it was determined that full-length sequences of the 16S rRNA were not necessary to resolve the composition of a bacterial community (Liu et al., 2007), the focus shifted towards the generation of large volumes of shorter reads. The last decade has seen significant development of high-throughput DNA- and RNA-sequencing platforms for studies of microbial communities. Today, hundreds of meta-barcoded samples may be sequenced simultaneously. This has reduced the need for cloning of individual genes or cultivation studies to identify members of a microbial consortia (Arnold et al., 2016).

Illumina and 454 Pyrosequencing technologies have been the main platforms of choice for evaluation of the mammalian gut microbiome in the last decade (Arrieta et al., 2014). The 454 Pyrosequencing platform, commercialised by Roche in 2005 (Margulies et al., 2005) was the first next generation sequences (NGS) system applied to studies of the rumen microbiome, used to show significant disparity between taxonomic and functional profiles of the liquid and fibre-

adherent fractions (Brulc et al., 2009). Subsequently, DNA Pyrosequencing of PCR amplicons has been widely employed in characterisation of the composition and potential function of the rumen microbiota (Castro-Carrera et al., 2014, Kim et al., 2014, Li et al., 2016, Fouts et al., 2012). However, the high cost and greater error rate of Pyrosequencing analysis led to this system being discontinued in 2016.

Illumina chemistry employs reversible terminators in combination with sequencing-by-synthesis chemistry on a glass slide, and allows a large number of reads to be generated relatively quickly, more cheaply, and with error rates a fraction of those found in 454 platforms (Li et al., 2014, Luo et al., 2012). Today, Illumina platforms are the systems of choice for sequence-based interrogation of the rumen microbial community. The Illumina MiSeq can produce ~550bp merged sequence reads via 2x300bp paired end sequencing (assuming a 50bp overlap), which compares favourably to read lengths of the Pyrosequencing system (Kim et al., 2017). Other platforms are available for large-scale analysis of microbial communities (e.g. Ion Torrent, PacBio), as well as the emerging Nanopore technology, but these have not been extensively used to study the rumen microbiome.

1.6.5.1 Amplicon Sequencing

Harnessing the discriminatory power of the 16S rRNA/18S rRNA/ITS genes as discussed above, amplicon sequencing remains the most widely used sequencing tool for investigation of the rumen microbiota today. In such analyses, barcoded amplicons of the desired hypervariable region are prepared from isolated DNA (or cDNA following reverse transcription of RNA) and can be multiplexed to allow the simultaneous sequencing of hundreds of biological samples. The reads generated in an amplicon sequencing project are typically first clustered into Operational Taxonomic Units (OTUs), usually using a similarity threshold of 97% (analogous to species level), which are then aligned against a reference database like Greengenes (DeSantis et al., 2006) for taxonomic classification. The inferred functional profile of a microbial community may also be assessed by amplicon sequencing, using tools like PICRUSt (Langille et al., 2013) and Tax4Fun (Asshauer et al., 2015). Indeed, a rumen-specific version of the PICRUSt tool, CowPi (Wilkinson et al., 2018), has recently been developed, and appears to outperform PICRUSt in functional inference, but has not yet been widely investigated for its accuracy. Such approaches are not particularly robust however, as they rely on inferred function based on known features of a taxon, rather than direct assessment using other methods discussed below. Yet they remain useful tools

to generate a broad picture of microbial function, which may then be investigated further using deep sequencing approaches.

Amplicon sequencing has been used to define the "core" rumen microbiota (Henderson et al., 2015), to investigate the heritability of rumen microbiome features (Sasson et al., 2017), and to characterise rumen composition and diversity in a wide range of ruminant hosts and under many different conditions (McGovern et al., 2017, Tapio et al., 2016, Li et al., 2016, McCabe et al., 2015, Myer et al., 2015a, Jami et al., 2013, Jami and Mizrahi, 2012). It has also been used to describe the relationship between the rumen microbiota and important production traits, such as feed efficiency and milk composition (McCann et al., 2014b, Jami et al., 2014). While amplicon sequencing provides a rapid and cheap "snapshot" of microbial diversity present within an ecosystem at a point in time, it is subject to several limitations. These include PCR bias (non-specific annealing, differential amplification specificity of taxonomic groups, artefact formation) poor resolution at the species level, and the fact that amplicon sequencing cannot account for marker gene copy number variation (Firkins and Yu, 2015, Poretsky et al., 2014). Thus, such approaches cannot be reliably interpreted quantitatively.

1.6.5.2 Metagenomic shotgun sequencing

As outlined above, amplicon sequencing has been a mainstay of microbial community analysis for much of the last decade but harbours some inherent limitations. To circumvent these issues, several other techniques have been used to study the rumen microbiota recently. Metagenomic shotgun sequencing can potentially catalogue all the microbial genes present in the rumen, by random sequencing of fragmented DNA. Prior to sequencing, total DNA extracts are randomly sheared (hence the name "shotgun"). These DNA fragments are then subjected to deep sequencing (e.g. on an Illumina HiSeq2500/4000), which can provide informative taxonomic and functional profiles using several analytical methods (Sunagawa et al., 2013), including the analysis of informative marker genes (e.g. the 16S rRNA gene) or contig assembly and subsequent alignment to a database of reference microbial genomes (Gupta et al., 2016). Several studies to date have employed shotgun sequencing in rumen microbiome investigations, including the first functional metagenomic assessment of the rumen microbiome in pre-ruminant calves (Li et al., 2012a), the identification of carbohydrate active enzymes in the adult rumen (Hess et al., 2011), characterisation of the taxonomic and functional profile of buffalo (Parmar et al., 2014), and in cattle with bloat (Pitta et al., 2016).

While metagenomic shotgun sequencing is advantageous over amplicon sequencing in that it is free of the PCR biases described above, it is costlier, and DNA-based methods may still assess only the functional potential of the microbial consortium, as opposed to active function revealed using RNA-based technologies. Another potential limitation of shotgun sequencing is contamination with host DNA, which will invariably be present to some degree in a sample and should be removed during the quality control stage (Li et al., 2018). While host DNA sequences in human faeces accounted for as much as 64% of total reads (Schmieder and Edwards, 2011), metagenomic studies of the rumen microbiota report that less than 1% of sequences are host derived, so this may not be a major obstacle in this instance (Shabat et al., 2016, Neves et al., 2018).

1.6.5.3 New frontiers in omics technologies to study the rumen

Amplicon and shotgun sequencing approaches are powerful tools in assessing microbial composition and functional potential in a habitat but are hampered by the inability to assess active function. To address the shortfalls in DNA-based investigative tools, metatranscriptomic (Li and Guan, 2017), metaproteomic (Snelling and Wallace, 2017), and metabolomic (Deusch et al., 2017) analytical methods have been developed and used to more accurately define the functional activity of the rumen microbial community.

Metatranscriptomic sequencing is similar in principle to metagenomic shotgun sequencing, but sequencing is performed on reverse transcribed cDNA rather than directly on fragmented gDNA, allowing the active portion of a microbial cohort (i.e. those producing RNA at the time of sample collection) to be assessed. Therefore, this may be a preferable way to assess the function of a microbiome versus metagenomic sequencing (Kim et al., 2017), where nucleic acid from dead or inactive microorganisms can also be sequenced. To date, only a small number of studies have used metatranscriptomic sequencing to study the rumen, providing a more complete picture of the active rumen microbial community of beef (Neves et al., 2017) and dairy (Comtet-Marre et al., 2017) cattle, and describing further the relationship between these active rumen microorganisms and feed efficiency (Li and Guan, 2017). The costs associated with high-throughput sequencing analysis have reduced in recent years, and a continuation of this trend should see technologies like shotgun metagenomics and metatranscriptomics more widely applied (van Dijk et al., 2014). Metatranscriptomic analysis may also be complicated by host contamination as described above and extracting RNA of sufficient integrity for sequencing is more challenging than with DNA. For a more in-depth discussion of the application and

challenges of metagenomic and metatranscriptomic sequencing in studies of the rumen microbiome, the reader is directed to a recent review (Li et al., 2018).

It is important to note that mRNA expression levels do not necessarily relate to protein production (Maier et al., 2009), and thus further advances in protein and metabolite detection techniques could offer a more accurate profile of microbial activity in the rumen. Metaproteomics aims to characterise the entire protein content of an environmental sample (Wilms, 2004), and has recently been used to survey the rumen microbiome of both dairy and beef cattle, and lambs (Snelling and Wallace, 2017). Metabolomics can quantify the biochemical profile of a microbial community and has also been used to study the rumen metabolite profile (Deusch et al., 2017, Deusch et al., 2015). These technologies remain in their infancy but continued technical and analytical advances are likely to see their use rise sharply in the coming years.

1.7 Knowledge gaps, hypotheses, and objectives

The rapid advances in omics technologies in the last decade has dramatically improved our knowledge of the composition and function of the rumen microorganisms, and the associated host mechanisms for nutrient uptake. Yet, there remains gaps in the literature concerning the ontogeny of the pioneer rumen communities, and how they may be affected by factors like host gender and local environment. Furthermore, the development of the rumen wall transcriptome in early life has not been well characterised, and further studying gene expression profiles in the calf rumen will aid in the elucidation of the biological mechanisms which contribute to rumen development. Furthering our knowledge of the microbial dynamics in the developing rumen, and how they relate to later-life production, will be critical in designing innovative strategies to improve nutrient utilisation and reduce wasteful processes like methanogenesis via interventions in early life.

The hypothesis for the current research project was that early life dietary and management regimens will contribute to microbial composition and diversity in the rumen and lower gut. The research presented in this thesis contributes to our understanding of the temporal dynamics of microbial establishment in bovine GIT in early life, and what factors contribute to this colonisation pattern. The specific objectives of this project were as follows:

1. Characterise the temporal development of the rumen microbiota throughout early life, and assess the impact of poorly studied factors like farm environment on this pattern of microbial colonisation.

2. Use RNA-sequencing to characterise the rumen wall transcriptome from birth to post weaning.
3. Assess whether the elevated intestinal development and calf growth observed in calves supplemented with butyrate in early life was accompanied by changes in the microbial composition and fermentation in the rumen and lower gut.

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1.9 Figures

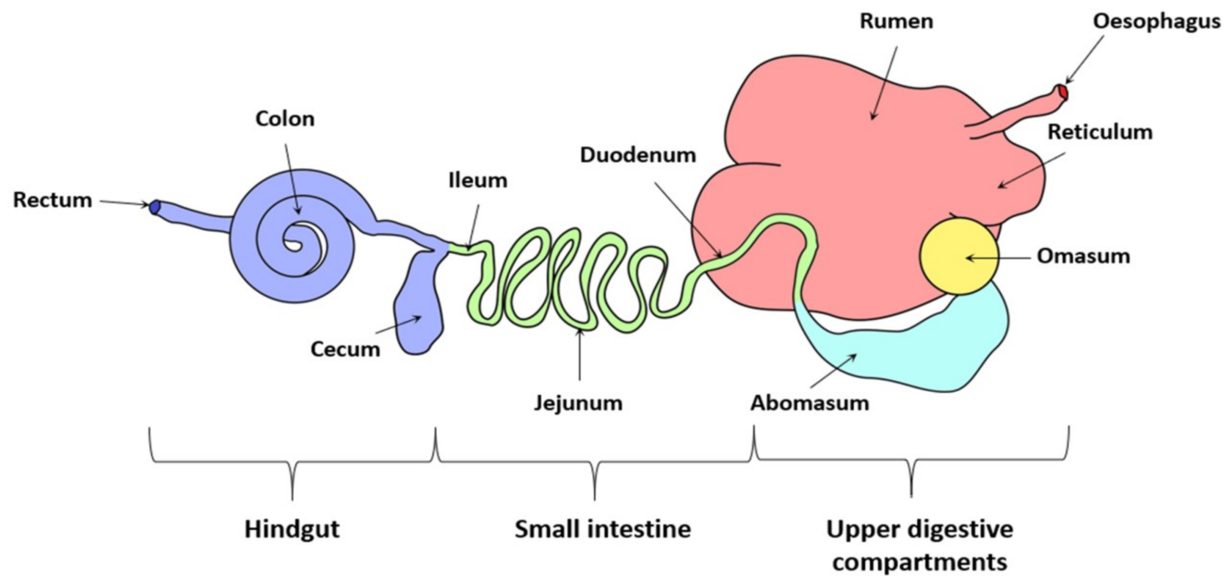

Figure 1.1: Schematic representation of the gastrointestinal tract of ruminant animals.

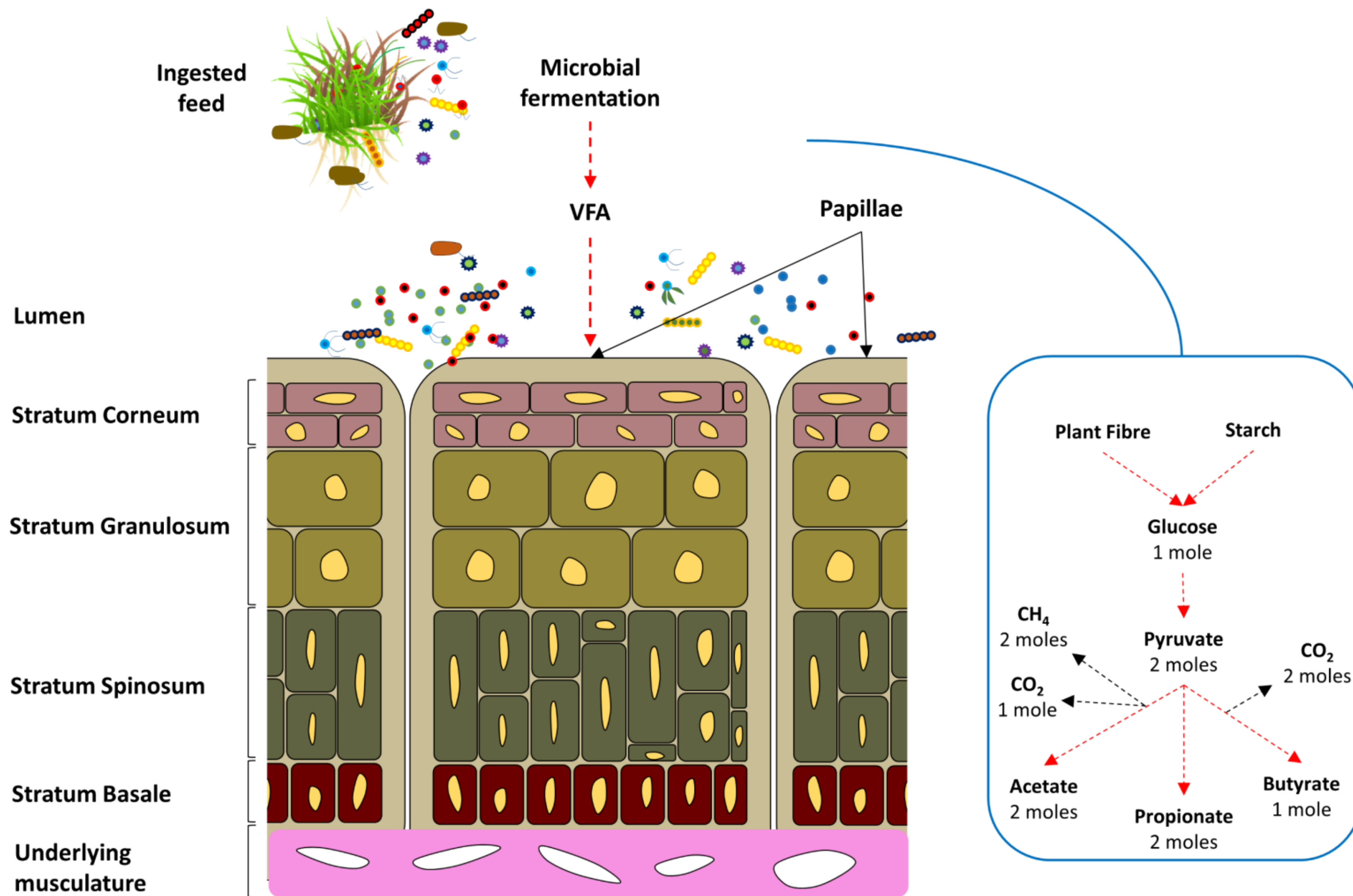

Figure 1.2: Diagram of rumen epithelial structure and major microbial fermentation pathways and products.

Chapter 2

Diet, age, and farm environment influence rumen microbial establishment patterns during early life

2.1 Introduction

The symbiotic rumen microbiota is a diverse community of prokaryotic and eukaryotic microorganisms, contributing to host production via the bioconversion of ingested feed to VFAs and other nutrients. Furthermore, it is associated with a plethora of important economic traits in cattle, including feed efficiency (Guan et al., 2008, Li and Guan, 2017, Sasson et al., 2017, Shabat et al., 2016) and milk composition (Jami et al., 2014). However, methanogenesis performed by the rumen archaea limits host energy harvesting efficiency by 2-12% and contributes around 18% of agriculturally derived GHG emissions annually (FAO, 2013). Thus, improving the efficiency of animal protein production systems whilst ameliorating their climatic impact has become a priority to meet the requirements of a rising global population (FAO, 2013).

The rumen microbiota differs at a compositional and functional level between beef cattle of high and low feed efficiency (Guan et al., 2008, Li and Guan, 2017, Carberry et al., 2012, Carberry et al., 2014a, Carberry et al., 2014b), and thus redirection of the rumen microbiota to improve its digestion capacity has been investigated as a method of enhancing host feed efficiency and host productivity (Zhou et al., 2018, Denman and McSweeney, 2015, McAllister and Newbold, 2008, Wang et al., 2017, Yanez-Ruiz et al., 2015, Yang et al., 2018). However, the settled and resilient nature of the adult rumen microbiota makes it refractory to permanent change (Weimer, 2015, Weimer et al., 2010). However, recent work indicates that preweaned ruminants may harbour a more heterogenous microbiota than that evident in adulthood (Jami et al., 2013, Abecia et al., 2017, Yanez-Ruiz et al., 2015, Abecia et al., 2014b), which is more amenable to persistent manipulation via dietary or management interventions. This has led to renewed interest in the composition and function of the rumen microbiota during early life.

Microbial life has been detected in the rumen as early as 20 minutes post-partum, though many of the taxa present were not usual members of the rumen consortium (Guzman et al., 2015). Several additional studies have investigated temporal dynamics of the calf rumen microbiota during the first weeks of life (Fonty et al., 1983a, Fonty et al., 1987, Fonty et al., 1989, Stewart et al., 1988, Malmuthuge et al., 2014, Rey et al., 2013). However, despite these significant efforts,

substantial gaps and limitations remain in our knowledge of microbial establishment patterns during early life. For instance, much current understanding is drawn from studies using small ruminants such as sheep and goats (Abecia et al., 2014b, Martínez-Fernández et al., 2014, Yanez-Ruiz et al., 2015, Abecia et al., 2013, Yanez-Ruiz et al., 2010, Martinez-Fernandez et al., 2015), which may not be applicable to beef or dairy calves due to physiological and metabolic differences. Furthermore, studies performed in young cattle typically use dairy bull calves (Malmuthuge et al., 2014, Rey et al., 2013, Jami et al., 2013), and it is unclear if rumen microbial colonization of beef calves is that of dairy calves due to their varied rearing managements (isolation from dam in dairy production vs. raised with dam in beef systems). Finally, most studies exploring microbial dynamics in the developing rumen to date have relied exclusively on transoesophageal sampling of the fluid fraction, which may not be sufficient to describe the total rumen microbiota (Cammack et al., 2018).

In this study, we examined the dynamics of prokaryotic succession in the rumen solid and fluid phase digesta of calves raised on two farms during early life and postweaning using DNA amplicon sequencing of the 16S rRNA gene, with a view to further characterise patterns of microbial development in the rumen and to assess microbial changes that occur with normal dietary transitions in beef calves during early life.

2.2 Materials and methods

2.2.1 Ethical statement

All experimental procedures described herein were approved and carried out under certification from the Teagasc Animal Ethics Committee (TAEC) and the Irish Health Products Regulatory Association (HPRA; certification number AE19132).

2.2.2 Experimental animal trial

Animal management protocols have previously been described (Surlis et al., 2017). Ninety-three commercially purchased Aberdeen Angus crossbred (all had an Aberdeen Angus sire) heifers were obtained for this experimental trial and housed together at Teagasc Mellows Campus, Athenry, Co. Galway, Ireland (F1). Oestrous cycles were synchronised using a standard 7-day PRID protocol, and each heifer was then artificially inseminated with semen from a single pedigree Aberdeen Angus bull, selected for ease of calving (EBI Five Star Rating; Portauns Mike), resulting in 66 viable pregnancies divided into four calving replicates. Foetal sex was determined at 100 days of gestation. In the third trimester of gestation, heifers in replicates 3 and 4 were

transferred to a second research farm (DAFM Longtown Research Facility, Clane, Co. Kildare, Ireland, F2) for calving. Heifers were housed indoors for eight weeks prior to the projected date of calving and had *ad libitum* access to a medium energy diet (concentrates plus grass silage). Experimental design and calf management is detailed in Fig. 2.1. One week prior to calving, heifers were blocked by foetal sex into one of seven groups based on their day of slaughter: euthanised immediately following birth (vaginally delivered (NAT) n = 11, delivered via elective caesarean section (ECS, n = 10)); vaginally delivered and euthanised on D7 of life (D7 – n = 8); D14 (n = 9); D21 (n = 9); D28 (n = 10); D96 (n = 9). To facilitate prompt sample collection, heifers not assigned to the ECS group received a 2ml injection of prostaglandin (Estrumate™, Merck) 48 hours prior to projected calving date to induce parturition. Caesarean sections were carried out by a veterinary surgeon, following a standard veterinary protocol. Calves assigned to both D0 treatments were delivered onto sterilised plastic and euthanised within 5 minutes of delivery via an intravenous injection of pentobarbital sodium (Dolethal™ (Vetoquinol, France), or Euthatal (Boehringer, UK), 1ml/1.4kg of live weight). Death was verified by the absence of a corneal reflex and heartbeat. Calves assigned to subsequent groups (D7-D96) were allowed to suckle their dam for 48 hours post-partum and were housed individually thereafter. Calves were offered 5L of milk replacer daily in one feeding (Blossom™, Volac, UK), housed on clean straw, and had access to clean drinking water. Calves assigned to the D14-D96 treatment groups were offered milk replacer with calf starter (Suckler Mate™; DOC Feeds, Ireland) at a rate of 300g/d from D7-14, 500g/d between days 14-21, 700g/d between days 21-28, 1kg/day from D21 until weaning, and on an *ad libitum* basis thereafter. Calves were weaned around D56 of life, when they had consumed at least 1kg of calf starter/day for three consecutive days. Calves were subsequently offered calf starter, hay, and water *ad libitum* for the remainder of the experimental period until D96.

Calves were euthanised after morning feeding on the dates described above. The gastrointestinal tract was quickly exteriorised, and a sample of rumen digesta was collected and passed through four layers of cheesecloth to separate the solid and liquid fractions. These were then collected separately in sterile 50ml tubes, snap-frozen on liquid nitrogen, and stored at -80°C pending molecular analysis. There was insufficient content in the new-born rumen (both D0 groups) to facilitate sample collection, and so the interior wall of the rumen was swabbed using a sterile polystyrene swab to collect the small amount of fluid present, and the entire swab was immediately snap-frozen within a sterile collection tube. All samples were collected and frozen within 25 minutes of verification of death.

2.2.3 DNA isolation

Frozen digesta samples were ground to a fine powder under liquid nitrogen using a chilled pestle and mortar prior to DNA extraction. Genomic DNA was then isolated from approximately 250mg of ground rumen solid/liquid digesta using the repeated bead beating with column purification (RBB+C) method as previously described (Yu, 2004). DNA quantity was assessed by two consecutive readings on a Nanodrop1000 (Thermo-Fisher, CA, USA) and purity was verified via visualisation in a 1% agarose gel. DNA was isolated from swabs in a similar manner; prior to bead beating, the swab was submerged in 1000µl of sterile phosphate-buffered saline (PBS) for 5 minutes, and vigorously vortexed for ~5 minutes to ensure removal of microbial cells. The swab was then removed, and the supernatant was used in the standard RBB+C protocol as described above.

2.2.4 16S rRNA gene amplicon library preparation and sequencing

Amplicon libraries targeting the V4 hypervariable region of the bacterial 16S rRNA gene were prepared from purified DNA extracts using the primers 515F (5'-GTGCCAGCMGCCGCGGTAA) and 806R (5' - GGACTACHVHHHTWTCTAAT) (Caporaso et al., 2011). Cycle conditions were as follows: initial denaturation at 95°C for 3 minutes, followed by 25 cycles of 95°C for 30 seconds, annealing at 55°C for 30 seconds, elongation at 72°C for 30 seconds, followed by a final elongation step of 72°C for 5 minutes. The primers 915aF (5' AGGAATTGGCGGGGGAGCAC and 1386R (5'- GCGGTGTGTGCAAGGAGC) (Jeyanathan et al., 2011) were used to amplify the V6-V8 portion of the archaeal 16S rRNA gene, using the same conditions described above. It was not possible to produce a bacterial or archaeal amplicon from the rumen swabs or tissue of new-born calves (ECS or NAT), and thus these samples were excluded from further analysis in the present study. Amplicon libraries were indexed with Illumina Nextera indices using an 8 cycle PCR with the same conditions as above and purified using AMPure beads (Illumina, USA). Bacterial libraries underwent 2x250bp paired end sequencing on an Illumina MiSeq (TrinSeq, Dublin, Ireland), while 2x300bp paired end sequencing was employed for the archaeal libraries using the same platform at a commercial sequencing laboratory (Genome Quebec, QC, Canada).

2.2.5 Bioinformatic analysis

Demultiplexed paired-end 16S rRNA gene sequences were merged and quality-filtered using the BBTools suite (Bushnell, 2015), with a minimum per-base phred score cut-off of 25, and were analysed using tools implemented in the QIIME wrapper (v.1.9). Chimeric reads were

removed using USEARCH (v.6.1) (Edgar, 2010). Both bacterial and archaeal taxonomic classification used the open reference OTU picking strategy implemented in QIIME. Bacterial sequences were first clustered into Operational Taxonomic Units (OTU) at a 97% similarity threshold using UCLUST (Edgar,2010). A representative of each bacterial OTU was aligned against the Greengenes database (v.13_5) for microbial classification. Archaeal sequences were clustered into OTUs at 99% similarity using UCLUST. A representative sequence from each OTU was then aligned to the Rumen and Intestinal Methanogens Database (RIM-DB) (Seedorf et al., 2014) using BLAST (Altschul et al., 1990) with a maximum e-value of 0.001 for species assignment. BIOM table files created in QIIME were exported to R (R Team, 2008) for all downstream statistical analyses and visualisation.

2.2.6 Statistical analysis

Alpha diversity indices (OTU Richness, Simpson's Index of Diversity) were calculated based on rarefied OTU tables in R using the Phyloseq (McMurdie and Holmes, 2013) package, and plotted with ggplot2 (Wickham, 2016). Significant differences in α-diversity indices across groups were determined using a non-parametric Kruskall-Wallis test and Dunn's post-hoc test (Dunn, 1964), with $P<0.05$ declared as significant. Non-metric multidimensional scaling (nMDS) analysis was performed and plotted in R using Bray Curtis dissimilarity matrices. Permutational Multivariate Analysis of Variance (PERMANOVA) tests in Vegan (Dixon, 2003) were used to assess the effect of time, location, and rumen digesta fraction on the bacterial and archaeal community structure. Mean sample divergence (β-dispersion) across farms and age group was also performed using Vegan, and significant differences were determined using Analysis of Variance (ANOVA) and Tukey's Honest Test for multiple comparisons, with $P<0.05$ declared as significant.

Differential abundance analysis of microbial taxa at the phylum and genus levels was carried out against a negative binomial distribution implemented as the Wald test in DeSeq2 (Love et al., 2014). To determine the longitudinal development of the rumen microbiota, age was included as a fixed effect and the model controlled for farm and calf gender. The model design was adjusted appropriately to test for differences according to rumen fraction and farm environment at each time-point, while controlling for variation in other factors. For taxonomic comparisons, raw *P*-values were adjusted into FDR and statistical significance was declared at FDR-adjusted *P*-value <0.05, with trends occurring at FDR-adjusted $P<0.1$. Only taxa

contributing at least 0.01% of sequences in one age category were deemed detected, and further divided into minor (<0.1%) and major (≥0.1%) for ease of description.

2.3 Results

2.3.1 Sequence data information

Paired end DNA amplicon sequencing of the partial 16S rRNA genes yielded 9,349,786 (mean of 108,718 ± 60,742 reads per sample) and 3,394,846 (41,912 ± 9,105) high quality bacterial and archaeal sequences, respectively. Sequencing depth for both archaeal and bacterial communities was sufficient as demonstrated by Goods Coverage rate > 97% for all samples, and inspection of rarefaction curves based on bacterial OTU/archaeal species richness (Appendix A).

2.3.2 Rumen microbial diversity and structure during early life

2.3.2.1 α-Diversity

There was no significant effect of rumen fraction on bacterial or archaeal α-diversity during early life ($P>0.05$). Alpha-diversity indices of the rumen bacterial community remained stable for the first four weeks of life, but OTU Richness and Simpson Diversity values were higher in weaned (D96) animals compared to all pre-weaned calves (Fig. 2.2, $P<0.05$). When compared across farms, F1 animals had a richer rumen archaeome than F2 animals (Fig. 2.3, $P<0.05$). Opposite trends were evident for the bacteria, with F2 animals having significantly higher OTU Richness and Simpson Diversity values (Fig. 2.3, $P<0.05$, Fig. 2.3)

2.3.2.2 β-Diversity

NMDS plots (Fig. 2.4, Fig. 2.5) and PERMANOVA tests (Table 2.1) of rumen liquid and solid digesta microbiota showed no differences in bacterial or archaeal community structure between the two fractions ($P>0.05$). There were significant effects of age ($P<0.05$, Fig. 2.4), and farm ($P<0.05$, Fig. 2.5) on both bacterial and archaeal communities. NMDS plots of both solid and fluid fractions exhibited similar temporal patterns. Bacterial profiles of 7-day old calves clustered together, and those of 14-day old calves formed a relatively distinct cluster for both fluid and solid fractions. The D21 and D28 samples also clustered closely, while those of D96 old calves formed another distinct cluster (Fig. 2.4a). Less obvious temporal clustering was evident for the archaeal communities (Fig. 2.4b), but there was a more pronounced farm effect in both liquid and solid fractions compared to the bacteria (Fig. 2.5a). Group divergence (β-dispersion, i.e. mean distance to the centroid) based on the Bray-Curtis dissimilarity was used to assess if these

dissimilarities were attributable to differences in community dispersion. There was no significant difference in dispersion of bacterial communities based on age or farm ($P>0.05$), but the archaeal community of calves raised on F1 had greater inter-animal variation than those raised on F2 ($P<0.05$, Fig. 2.5d). There was a limited age effect on the archaeal community β -dispersion, with D28 animals' rumen archaeome being significantly more dissimilar to each other than that of D14 calves ($P<0.05$).

2.3.3 Microbial composition in the rumen digesta during early life

Twenty bacterial phyla were present between both fractions in the bovine rumen digesta during early life. The most abundant phyla across all samples were *Firmicutes* (41.98%; mean proportion of total bacterial 16S rRNA reads for all solid and liquid fraction samples), *Bacteroidetes* (36.49%), and *Proteobacteria* (8.79%). *Actinobacteria* (6.33%), *Fibrobacteres* (1.25%), *Spirochaetes* (1.13%) and *Cyanobacteria* (1.03%) were also prominent members of the rumen bacteriome. Other less abundant phyla included *Fusobacteria* (0.81%), *Tenericutes* (0.42%), *Synergistes* (0.25%), *Planctomycetes* and *Elusimicrobia* (both 0.12%). Abundances of detected rumen phyla are presented in Fig. 2.6. Details of the remaining eight rumen phyla are presented in Appendix B.

Prevotella was the most abundant bacterial genus (22.49%), followed by unclassified *Lachnospiraceae* (4.66%), unclassified *Clostridiales* (4.55%), and *Bacteroides* (4.00%). Abundances of the most prominent rumen bacteria are presented in Figs. 2.7 and 2.8, and details of all detected genera are presented in Appendix C. The methanogenic archaea were dominated by *Methanobrevibacter spp.,* including the predominant *Mbb. gottshalkii* clade (28.30%), *Mbb. ruminantium* (25.98%), *Mbb. smithii* (19.89%), *Mbb. wolinii* (6.75%), and *Mbb. boviskoreani* (4.89%). Members of the *Methanosphaera* genus and *Methanomassilicoccaceae* family were also detected, and details of their abundances are presented in Fig. 2.8. Both bacterial and archaeal communities were significantly influenced by diet and/or calf age, as well as farm environment during early life, and the following sections will outline these changes.

2.3.4 Effect of rumen fraction on microbial composition during early life

While β-diversity analyses described above did not show broad scale microbial differences between the solid and liquid fractions, we investigated if there were compositional differences at the phylum or and genus levels throughout early life. Only taxa that contributed at least 0.1% in either rumen fraction within a time point are described here. Clear taxonomic divergence between

rumen fractions was only evident on D7. The relative abundances of *Fusobacterium* (4.48% vs 2.53%), *Veillonella* (3.87% vs. 1.01%), *Fibrobacter* (1.44% vs. 0.85%), *Odoribacter* (1.68% vs. 0.06%), *Succiniclasticum* (1.56% vs. 1.22%) and unclassified *o.Bacteriodales* (2.07% vs. 1.50%) were higher in the solid fraction compared to those in the fluid (*P*<0.05). Conversely, the relative abundances of *Porphyromonas* (3.51% vs. 4.62%), unclassified *f.Lachnospiraceae* (2.37%vs. 2.96%), unclassified *f.Alcaligenacae* (1.35% vs. 2.31%), *Comamonas* (1.35% vs 3.55%) and *Parabacteroides* (1.52% vs 0.51%) were higher in the rumen fluid (*P*<0.05) than in the solid. The only significant difference observed at subsequent time points was the increased abundance of putative [*Prevotella*] genus in the rumen fluid of 28-day old calves compared to that of the solid fraction (*P*<0.05). Archaeal community composition was similar across fractions, with no significant differences found (*P*>0.05). Details of all differentially abundant taxa according to digesta fraction are presented in Table 2.2.

2.3.5 Temporal development of the rumen microbiota during early life

Temporally adjacent contrasts (i.e. D14 vs D7, D21 vs D14, D28 vs D21, and D96 vs D28) of microbial relative abundances were performed using DeSeq2 to assess the development of both rumen solid and liquid fraction microbiota during early life, and its response to early life dietary change and calf development (age).

2.3.5.1 Rumen liquid digesta

Sixteen bacterial phyla were detected (≥0.01% of total abundance in at least one age group) in the rumen fluid during early life. *Bacteroidetes, Proteobacteria, Firmicutes* and *Actinobacteria* were consistently the most abundant phyla in all age groups, except on D96 when *Cyanobacteria* (4.50%) were more abundant than *Actinobacteria* (4.27%) (Fig. 2.6). The abundance of *Firmicutes* increased significantly by D14 (19.05% - 51.74%) and remained the most abundant taxa for the remainder of the experimental period. *Elusimicrobia* was also more abundant on D14 (ND – 0.50%), while *Proteobacteria* (19.75% - 4.01%), *Fusobacteria* (2.57% - 0.20%), and *Bacteroides* (51.33% - 31.41%) had significantly reduced abundance by D14 compared to D7 (*P*<0.05). *Proteobacteria* (4.01% - 10.61%), *Cyanobacteria* (0.06% - 1.59%), and *Planctomycetes* (ND – 0.12%) was of greater abundance on D21 compared to D14 (*P*<0.05). The relative abundances of *Verucomicrobia* (2.206% - 0.43%), *Elusimicrobia* (0.50% - 0.03%), and *Fusobacteria* (0.20% - 0.03%) all reduced within the same timeframe (*P*<0.05). There were no significant differences in bacterial composition at the phylum level between 28- and 96-day old

calves. Abundance of *Cyanobacteria* was higher on day 96 (4.50%) compared to day 28 (1.03%), while *TM7* (recently reclassified as *Saccharibacteria* (He et al., 2015) (ND – 0.41%), *Elusimicrobia* (0.11% - 0.28%), *WPS-2* (ND – 0.18%) and *Planctomycetes* (0.10% - 0.12%) had increased abundances in the same period (*P*<0.05).

To better understand the biological implications of these changes, we investigated microbial dynamics during early life at the genus level. In the rumen liquid digesta, 99 genera were above the detectable limit (≥ 0.01% in one age group). For ease of description, only taxa with abundance ≥ 0.1% in at least one time point within a temporally adjacent contrast are presented here. Fourteen genera had greater abundance on D14 than D7 (*P*<0.05) including *Prevotella* (9.85% - 22.200%), *Catenibacterium* (0.01% - 6.30%), *Roseburia* (0.01% - 4.30%), *Bifidobacterium* (0.01% - 5.16%), *Lachnospira* (0.03% - 2.33%), *Megasphaera* (0.01% - 5.60%), *Acidaminococcus* (0.01% - 0.57%), *f.Coriobacteriaceae* (0.08% - 1.27%), *[Eubacterium]* (0.01% - 0.24%), *Lactobacillus* (0.05% - 1.01%), *Mitsuokella* (ND – 0.14%), *Psudo. Eubacterium* (0.01% - 0.35%), *Succinivibrio* (0.05% - 0.72%), *f.Lachnospiraceae* (2.96% - 7.86%) and *f. Succinivibrionaceae* (0.02% - 0.23%). Twenty-two bacterial genera had significantly lower abundance on D14 versus D7 (*P*<0.05), including *Bacteroides* (13.23% - 4.53%), *f.Neisseriaceae* (4.01% - 0.80%), *Actinomycetes* (2.33% - 0.68%), *Oscillospira* (1.04% - 0.53%), *Butyricimonas* (1.37% - 0.30%), *Porphyromonas* (4.62% - 0.27%), *Gallibacterium* (5.30% - 0.20%), *Fusobacterium* (2.53% - 0.19%), *Bibersteinia* (0.57% - 0.08%), *Corynebacterium* (0.15% - 0.04%), *Acinetobacter* (0.46% - 0.04%), *Moraxella* (0.34% - 0.04%), *Peptostreptococcus* (0.42% - 0.03%), *Haemophilus* (0.26% - 0.03%), *f.[Weeksellaceae]* (0.16% - 0.03%), *Kingella* (0.21% - 0.02%) and *Peptococcus* (0.13% - 0.01%). Although they were present in rumen on D7, *Parvimonas*, *f.Pasteurellaceae*, *c.Alphaproteobacteria*, *Filifactor,* and *Paludibacter* were not detected in the rumen fluid on D14 of life (*P*<0.05). *Methanobrevibacter ruminantium* (35.35%) was the most abundant archaeal species in the rumen fluid on D7, followed by *Mbb. smithii* (30.98%)*, Mbb gottshalkii* (26.58%) and *Mbb. wolinii* (2.54%). *Mbb boviskoreani, Methanosphaera sp. A4* and *Methanosphaera* sp. ISO3-F5 were minor (<0.1%) contributors to archaeal composition on D7, and there were no significant shifts in the methanogen community between days 7 and 14.

Seven bacterial genera had significantly higher abundances on D21 compared to D14, including *Butyrivibrio* (0.21% - 3.81%), *f.Veillonellaceae* (0.49% - 1.96%), *o.YS2* (ND - 1.58%), *Anaerostipes* (ND - 0.82%), *S24-7* (0.01% - 0.46%), *Sharpea* (0.01% - 0.31%), and *Pirellulaceae* (ND - 0.12%) (*P*<0.05), while 17 taxa had reduced abundances on D21, including

f.Lachnospiraceae (7.86% - 2.96%), *Bacteroides* (4.53% - 1.78%), *Oscillospira* (0.53% - 0.37%), *Parabacteroides* (0.93% - 0.33%), *Phascolarctobacterium* (0.57% - 0.29%), *Veillonella* (3.02% - 0.27%), *Anaerovibrio* (0.76% - 0.22%), putative *[Ruminococcus]* (0.79% - 0.14%), *Lactobacillus* (1.01% - 0.14%), *Pseudobutyrivibrio* (0.20% - 0.11%), *f.Succinivibrionaceae* (0.23% - 0.09%), *Streptococcus* (4.38% - 0.07%), *Actinomyces* (0.68% - 0.07%), *Gallibacterium* (0.20% - 0.02%), *Porphyromonas* (0.27% - 0.02%), *Fusobacterium* (0.19% - 0.01%), and *Akkermansia* (2.206% - ND) ($P<0.05$). Three methanogenic species belonging to the *Methanomassillicoccaceae* were only detected from D21 onward; and the abundances of *Candidatus Methanomethylophilus alvus* (7.06% of archaeal 16S rRNA gene sequences on D21), *Group 9 sp. CH1270* (9.10%), *Group 9 sp. MpT1* (0.13%). *Methanosphaera sp. A4* also increased significantly (ND – 8.48%) in this period ($P<0.05$). Additionally, the abundances of three bacterial genera changed significantly between days 21 and 28 with *Ruminobacter* (2.23% - ND) and *Dialister* (1.80% - 1.41%) having lower abundances on D28, while the abundance of *Phascolarctobacterium* increased (0.29% - 0.30%) ($P<0.05$). For archaea, *Candidatus Methanomethylophilus alvus* was not detected, while *Methanomassillicoccaceae Group 12 sp. ISO4-H5* emerged (5.41% on D28), as did *Group 10 sp.* (9.73%) ($P<0.05$) on D28.

Between days 28 and 96, *Mbb. boviskoreani* had significantly higher abundance in the weaned calves (D96) compared to the 28-day-old animals (0.05% - 22.203%), while *Candidatus Methanomethylophilus alvus* increased from <0.01% - 0.49% on D96 ($P<0.05$). *Methanomassillicoccaceae Group 9 sp. CH1270* had lower abundance in weaned calves (0.80% - 0.02%) ($P<0.05$). The abundances of twenty bacterial genera were higher on D96 compared to D28, including *o.Clostridiales* (2.90% - 8.71%), *f.Ruminococcaceae* (1.80% - 5.79%), *o.Bacteriodales* (1.83% - 5.70%), *f.Succinivibrionaceae* (0.14% - 4.79%), *o.RF39* (0.17% -1.87%), *Treponema* (0.54% - 1.52%), *Shuttleworthia* (0.02% - 1.18%), *Coprococcus* (0.02% - 1.03%), *YRC22* (0.06% - 1.00%), *o.RF32* (0.02% - 0.87%), *f.F16* (ND – 0.41%), *f.RF16* (ND – 0.32%), *f.Elusimicrobiaceae* (ND – 0.25%), *Pseudobutyrivibrio* (0.02% - 0.25%), *f.Christensenellaceae* (ND – 0.23%), *p.WPS-2* (ND – 0.18%), *c.Alphaproteobacteria* (0.01% - 0.18%), *Ruminobacter* (ND – 0.15%), *f.Pirellulaceae* (0.11% - 0.12%), and *o.ML615J-28* (ND – 0.11%). Fifteen bacterial genera had lower abundances in postweaning, including *Dialister* (1.41% - 0.33%), *Catenibacterium* (5.82% - 0.37%), *Bacteroides* (1.20% - 0.11%), *Succinivibrio* (9.45% - 1.25%), *Oscillospira* (0.32% - 0.11%), *Roseburia* (2.23% - 0.53%), *Pseudo. Eubacterium* (0.23% - 0.15%), *Collinsella* (0.21% - ND), *Parabacteroides* (0.29% - 0.01%), *Phascolarctobacterium* (0.30% - ND), *Blautia* (0.42% - 0.04%), *Corynebacterium* (0.23% - 0.02%), *Campylobacter* (0.23% - 0.04%), *Anaerostipes* (0.29% - 0.09%), and *Lactobacillus* (0.13% - 0.02%) ($P<0.05$).

2.3.5.2 Rumen solid digesta

Broadly reflecting the characteristics of the rumen liquid digesta, among the 20 bacterial phyla detected in the rumen solid fraction, *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, and *Proteobacteria* were consistently the most abundant, though their relative proportions changed significantly with age (Fig. 2.6). Methanogenic archaea were represented exclusively by the *Euryarchaeota* phylum. *Firmicutes* became the most abundant phylum by D14 (27.95% - 50.98%; $P<0.05$) and did not change significantly thereafter. The relative abundance of *Actinobacteria* increased between days 7 and 14 (5.43% - 9.54%; $P<0.05$), while that of *Proteobacteria* decreased in the same timeframe (11.86% - 2.205%; $P<0.05$). The abundance of *Fusobacteria* tended ($P<0.1$) to be lower on D14 compared to D7 (4.47% - 0.82%) and decreased significantly again on D21 (0.04%; $P<0.05$). The abundance of *Cyanobacteria* was higher on D21 (1.17%) versus D14 (0.16%; $P<0.05$). No phyla changed in abundance between days 21 and 28, but *Cyanobacteria* (0.65% - 0.98%) was of higher abundance on D28 compared to D96 ($P<0.05$).

Temporal changes in bacterial proportions in the rumen solid digesta resembled those observed among the liquid microbiota. The abundance of thirty-seven bacterial genera changed significantly between days 7 and 14, corresponding to the introduction of calf starter. Of these, fourteen increased ($P<0.05$) including *Megasphaera* (ND (not detected) - 8.31%), *Lachnospira* (0.02% - 2.49%), *Acidaminococcus* (ND - 2.62%), *Roseburia* (ND - 4.00%), *Bifidobacterium* (0.01% - 5.90%), *Mitsuokella* (ND - 0.17%), *Catenibacterium* (ND – 4.14%), *[Eubacterium]* (ND – 0.24%), *Psuedoramibacter_Eubacterium* (0.01%- 0.29%), *f.Coriobacteriaceae* (f = family, unclassified at the genus level; 0.06% - 2.37%), *Faecalibacterium* (ND – 0.77%), *Succinivibrio* (0.03% - 0.37%), *o.RF32* (o = order, ND – 0.14%), and *Blautia* (0.21% - 1.50%). The proportion of *Prevotella* tended ($P<0.1$) to be higher on D14 (9.90 – 23.67%), and it was the most abundant genus at all subsequent ages. Conversely, the abundances of eleven major genera (≥ 0.1% total abundance) declined between days 7 and 14 ($P<0.05$). *Bacteroides* was predominant genus on D7 but its abundance reduced from 13.60% to 4.68% on D14, and the abundances of *Veillonella* (3.87% - 3.57%), *o.Clostridiales* (5.26% - 1.35%), *Fusobacterium* (4.48% - 0.81%), *Oscillospira* (0.93% - 0.53%), *Succiniclasticum* (1.56% - 0.48%), *Porphyromonas* (3.51% - 0.45%), *f.Erysipelotrichaceae* (0.55% - 0.28%), *Actinomyces* (4.68% - 0.21%), *Clostridium* (0.24% - 0.19%), and *f.Mogibacteriaceae* (0.78% - 0.18%) also declined. The abundances of the remaining 11 genera, *Coprococcus, Peptostreptococcus, Adlercreutzia*, *Comamonas*, *Acinetobacter*, *Peptococcus*, *Parvimonas*, *Helcococcus*, *Paludibacter, Filifactor* and *Desulfotomaculum* reduced

(*P*<0.05) on D14 to such a degree that they contributed < 0.1% total bacterial abundance (Fig. 2.9).

The abundances of 22 genera changed significantly between days 14 and 21. Of these, 9 had higher abundances on D21 (*P*<0.05) including *Anaerostipes* (ND – 0.43%), *f. Pirellulaceae* (ND – 0.42%), *o.YS2* (ND – 1.08%), *f. S24-7* (proposed reclassification *Candidatius Homeothermaceae* (Ormerod et al., 2016); 0.01% - 1.11%), *f. RFP12* (ND – 0.33%), *Butyrivibrio* (0.41% - 6.74%), *Sharpea* (ND – 0.23%), *Succiniclastium* (0.48% - 3.25%), and *o.Clostridiales* (1.35% - 4.92%). The abundances of *Bacteroides* (4.68% - 0.50%), *Veillonella* (3.57% - 0.45%), and *Oscillospira* (0.53% - 0.20%) were lower on D21 (*P*<0.05) than on D14. Finally, the abundances of *Meganomonas, Akkermansia, Parabacteroides, f.Erysipelotrichaceae, Fusobacterium, [Ruminococcus], f.Alcaligenaceae, Porphyromonas, Dorea,* and *Actinomyces* reduced significantly (*P*<0.05) on D21 compared to D14 and were minor taxa (<0.1% total bacterial abundance) on D21. As at the phylum level, no bacterial genera or archaeal species differed significantly in abundance between days 21 and 28.

Between days 28 and 96, which included the weaning transition around day 56, abundances of seventeen bacterial genera changed significantly, as well as several minor taxa which are detailed in Appendix C. Nine had higher abundance in the rumen solid fraction of weaned calves (*P*<0.05) including *Coprococcus* (0.06% - 0.98%), *f.F16* (ND – 0.19%), *Atopobium* (ND – 0.13%), *Shuttleworthia* (ND – 1.11%), *f.Succinivibrionaceae* (0.08% - 4.21%), *f.Christensenellaceae* (0.01% - 0.32%), *f.Ruminococcaceae* (2.04% - 5.33%), *o.RF32* (0.01% - 0.20%) and *Sharpea* (0.16% - 1.64%). Conversely, *Bacteroides* (0.36% - 0.02%), *Campylobacter* (0.29% - 0.01%), *Corynebacterium* (0.1% - 0.01%), *Succinivibrio* (5.49% - 0.85%), *Catenibacterium* (4.75% - 0.58%), *Blautia* (0.39% - 0.11%), *Clostridium* (0.70% - 0.06%) and *Roseburia* (1.40% - 0.34%) were the eight bacterial taxa that had significantly lower abundance in the solid fraction between days 28-96, while the abundances of archaeal species were unchanged. Temporal dynamics of selected bacterial groups in the rumen digesta are presented in Figures 2.7-2.12, and are further discussed below.

2.3.6 Influence of farm on microbial community composition in the rumen during early life

As we noted substantial difference in taxonomic composition according to the farm on which calves were raised (Figs. 2.5, 2.8), further analysis was performed to ascertain what taxa

were contributing to this dissimilarity. Based on their demonstrated similarity, and to increase statistical power, solid and liquid taxonomic profiles were combined for differential abundance analysis. To more robustly assess if diet is confounded with the farm effect, the analysis was performed with the animals grouped according to diet - MR (milk replacer only, D7 calves), MS (milk replacer plus calf starter, days 14, 21, 28), and HS (hay and starter, D96 calves), based on their similar taxonomic profile (Fig. 2.2). Seventeen genera were significantly influenced by farm in both MR and MS groups ($P<0.05$), while nine and four genera were common to both MS and HS, and MR and HS groups, respectively. We identified six "core" bacterial genera that were significantly affected by farm during early life across all dietary groups: *f.RFP12* (from *p.Verrucomicrobia*), *Atopobium*, *Dialister*, *Pseduoramibacter_Eubacterium*, *Shuttleworthia*, and *o.ML615J-28* (*p.Tenericutes*) ($P<0.05$, Fig. 2.13). The response to farm was not consistent for each bacterial taxon. For example, *Shuttleworthia* was more abundant on F1 in both MR and MS groups, but more abundant on F2 in the HS animals ($P<0.05$), but the other five genera had a similar response throughout the experimental period with *Dialister* being more abundant on F2, while the remaining taxa were more abundant on F1 ($P<0.05$).

The abundances of seven archaeal species were significantly influenced by farm. *Mbb. smithii, Methanosphaera sp. A4, Mbb. boviskoreani* and the *Methanomassillicoccaceae* members *Candidatus Methanomethylphilus alvus*, *Group 10 sp.,* and *Group 9 sp. CH1270* were more abundant on F1 than F2 ($P<0.05$), while *Mbb. ruminantium* was more abundant on F2 ($P<0.05$). When animals were grouped by diet as described above, only *Mbb. smithii* was affected by farm in each group, being consistently more abundant on F2 ($P<0.05$). *Mbb. ruminantium* was more abundant on F1 in both MR and MS groups ($P<0.05$). Poorly defined species belonging to the *Methanomassillicoccaceae* were more abundant on F1 in both HS and MS groups ($P<0.05$). Additionally, *Methanosphaera stadtmanae* was more abundant on F1 in HS animals ($P<0.05$). Details of bacterial and archaeal taxa significantly affected by farm environment are presented in Fig. 2.13.

2.4 Discussion

There is much interest currently in the design of novel dietary interventions during the early life to impart a permanent footprint on longitudinal development of the rumen microbiota, as a potentially effective avenue to improve lifelong production efficiency while mitigating farm-level contributions to climate change phenomena (Yanez-Ruiz et al., 2015). However, to define the optimum window of opportunity for such interventions, the precise sequence of rumen

colonisation must first be established. Here we used DNA amplicon sequencing of bacterial and archaeal 16S rRNA genes to demonstrate an ordered microbial establishment pattern in the rumen fluid and solid fractions, which is influenced by both diet and calf age. We also demonstrate a substantial effect of farm environment on the early life rumen microbiota, which may have important implications in attempts to manipulate microbial metabolism in the developing rumen.

Little is known regarding the source of the initial rumen microbial inoculum, and to date only two studies have reported the presence of microbial life in the rumen of new-born calves (Guzman et al., 2015, Malmuthuge, 2016). In this study, we could not isolate or amplify DNA from swabs collected from the neonatal rumen of calves delivered either naturally or via ECS. This is unlikely to be due to procedural deficiencies, as several swab-specific DNA isolation protocols were tested (QIAmp Cador Pathogen Mini Kit, Qiagen; modified RBB+C protocol as described in the methods section) using human and bovine buccal swabs and with swabs of a mature rumen, all of which yielded DNA of sufficient quantity to produce bacterial 16S rRNA gene amplicons (data not shown). Therefore, we concluded that any microorganisms present in the rumen at birth are, using these technologies, at undetectable levels. It is also possible that the microbiota present at birth are VBNC (viable but non-culturable), and the use of more sensitive techniques may allow them to be characterised. In contradicting the studies listed above, this suggests further research is needed to confirm the presence of microbial life in the new-born rumen. A diverse hindgut microbiota is present in dairy calves who did not receive milk or colostrum within 30 minutes of birth (Song et al., 2018), but activation of the reticular groove during birth may prevent transfer of maternal uterine fluids and microbiota to the rumen. A recent study showed longitudinal differences from 24h through the first 6 months of life in rumen microbial composition of calves delivered naturally versus via ECS (Cunningham et al., 2018), though the authors noted that the influence of calf age outweighed that of birth process. Further studies are required to validate if the rumen is indeed sterile at birth.

Divergent microbial populations in the rumen solid and liquid fractions have been extensively reported, as reviewed elsewhere (Cammack et al., 2018). However, there was only a minor fraction-wise difference observed in the present study. This discrepancy may be due to the method of sample collection and age (and thus level rumen maturity) of the animals. Previous studies have centrifuged rumen fluid to further reduce cross contamination with particulate matter (Guan et al., 2008), but here we directly ground the frozen fluid and proceeded to DNA isolation. A recent study in dairy calves collected solid and liquid-phase digesta using a protocol comparable to ours, and the authors reported high similarity between fractions (Dill-McFarland

et al., 2018). The only substantial difference according to fraction here was on D7. While calves had not received solid feed by this point, there was poorly digested straw particulate in the rumen of D7 calves. The rumen harbours the species and genes necessary for plant matter degradation even before the provision of solid food (Li et al., 2012b), so it is likely that limited fermentation was taking place by D7 in the present study, supported by the increased abundance of the fibrolytic *Fibrobacter* in the solid fraction compared to the liquid. Many of the taxa which presented as differentially abundant are not noted members of the mature rumen (e.g. *Fusobacterium, Veillonella, Odoribacter*), and that the difference in rumen fractions did not persist beyond D7 suggests that it may be due simply to the inconsistent and transient nature of the microbiota in the first days of life, as previously reported (Jami et al., 2013). The majority of data showing differences between rumen digesta fractions is based on work in adult animals (Cammack et al., 2018), and further studies spanning the entire life cycle of the animal are needed to conclude if and how the rumen fractions may differ in microbial composition. Nonetheless, that the D7 rumen exhibited increased variation across fractions indicates that the first week of life may be a key period to take advantage of the unsettled rumen microbiota.

Based on our results, microbial colonisation in the rumen during early life occurred in three main phases, which could be stratified by diet; (i) milk replacer only (D7), (ii) milk replacer with calf starter (days 14, 21, 28) and (iii) calf starter plus hay (D96, weaned). Significant differences in composition were also evident between days 14 and 28 when calves were consuming the same diet, indicating that calf developmental or growth stage (age) is also a determinant of the rumen microbiota during early life. We also note that intakes were not recorded in this trial, though increasing amounts of calf starter were offered weekly to D28. This could have influenced microbial composition, but bacterial α-diversity did not change with diet or age prior to weaning, when the D96 animals had a richer bacteriome than all preweaned calves (Fig. 2.2a). As well as increased age, this likely reflects the changing nutrient source for the microbiota after weaning, when both forages and concentrates were offered to the calves. In contrast to bacteria, archaeal diversity was more stable throughout, and the increases in richness during preweaning appeared to be driven by farm environment as discussed below.

Corresponding to previous investigations of the calf rumen microbiota, *Bacteroidetes*, *Firmicutes* and *Proteobacteria*, and *Actinobacteria* were among the most abundant bacterial genera during early life, and *Methanobrevibacter* species were the dominant archaeon (Tapio et al., 2017). Among them, *Firmicutes* become predominant by D14 (Fig. 2.6), which is different than previous studies reported *Bacteroidetes* as the dominant microbial phyla of calves fed milk

replacer exclusively or in combination with calf starter (Rey et al., 2013, Jami et al., 2013, Li et al., 2012b), as well as in weaned calves (Meale et al., 2016). It has been reported that proportional increases of *Firmicutes* are associated with forage intake (Jami et al., 2013, Li et al., 2012a, de Menezes et al., 2011) and weaning (Meale et al., 2017), even when it is not the predominant phylum. As described above, there was evidence of early consumption of straw bedding in the calves of our study, and we speculate thus that even choice of bedding may contribute to early life microbial composition in the rumen. *Bacteroidetes* abundance did not change significantly throughout the experimental period in our study, suggesting its stabilisation even within the first week of life.

Milk consumption is unlikely to have a direct influence on the rumen microbial community, as liquid feed is shunted directly to the abomasum (Castro et al., 2016). Nonetheless, 7-day old calves had a microbiota of comparable diversity and richness to the other preweaning animals, indicating that even within the first week, a complex microbiota colonises the rumen, though this may have been exacerbated by straw consumption from the first days of life. Corresponding to previous reports, *Bacteroides* was the predominant genus in D7 calves (received milk replacer only; Fig. 2.10a). Calf starter intake is negatively correlated with *Bacteroides* abundance (Meale et al., 2017), and when calves were maintained exclusively on a milk-based diet, *Bacteroides* were the dominant taxa (Li et al., 2012b). However, it was also reported to be the predominant genus in 3-week old bull dairy calves offered both milk replacer and calf starter (Malmuthuge et al., 2014). This discrepancy may be attributed to divergent targeting of 16S rRNA variable regions, as well as differences between dairy and beef calves. There were also relatively high numbers of aerobic and pathogenic genera in the 7-day old calves (e.g. *Comomonas*, *Campylobacter*), which are not usually present in a mature rumen. These significantly reduced abundance following allocation of calf starter were barely detectable in the older animals (Fig. 2.9), indicating solid feed consumption is important for the establishment of prominent rumen bacteria. Regardless, on D7 of life the rumen contained many of the major bacterial groups found in the mature animal (Fig. 2.7) (Hobson and Stewart, 1997, Henderson et al., 2015), while *Methanobrevibacter spp.* along with *Methanosphaera* (Fig. 2.8) accounted for the majority (98.42%) of archaeal reads. This agrees with previous observations (Fonty et al., 1983a, Rey et al., 2013, Jami et al., 2013) that a complex microbiota colonises the proto-rumen of young calves, even before the provision of solid feed.

Among the observed microbial taxa, we noted significant increases of several important carbohydrate utilising and VFA producing microbial groups between days 7 and 28. VFA (mainly

acetate, propionate, and butyrate) biosynthesis by the microbiota is critical for physical and morphological development of the rumen (Heinrichs et al., 2005). This was broadly characterised by a migration toward a more mature microbial assembly, with *Prevotella* becoming the predominant bacterial genus by D14 (Fig. 2.7a). *Prevotella* is routinely reported as the most abundant bacterial genus in the adult rumen (Mao et al., 2015, Henderson et al., 2015, McCabe et al., 2015), and its establishment in early life was associated with elevated (>100g/d) levels of calf starter consumption (Rey et al., 2013), in support of our findings. We observed significantly lower abundance of *CF231* (*f. Paraprevotellaceae*) by D14, agreeing with a previously demonstrated inverse relationship between starter intake and ruminal *CF231* abundance in lambs (Wang et al., 2016c).

In addition, allocation of calf starter caused the establishment of several prominent butyrate producers and the functional interdependence between different microbial groups was also evident. Butyrate is the primary energy source for rumen epithelial cells, and as such is vital for rumen development during early life (Baldwin et al., 2004). *Megasphaera* species produce butyrate usually via lactate metabolism, and their increased abundance between days 7 and 14 occurred in tandem with significant enrichment in the proportions of the lactate producer *Bifidobacterium* (Hobson and Stewart, 1997), as previously reported (Trovatelli and Matteuzzi, 1976) (Fig. 2.10b, 2.10c). Both *Megasphaera* and *Bifidobacterium* seemed to establish within the niche for lactate utilisation among the microbiota at this point, displaying only numeric changes in abundance while lactate utilisers (e.g. *Veillonella*) significantly decreased in proportion with increasing age (Fig. 2.10b, 2.10c). Thus, it appears that microbial mechanisms for lactate production and utilisation in the rumen become established soon after solid feed allocation. *Butyrivibrio spp.* are usually the predominant butyrate-producing species of the adult rumen (Hobson and Stewart, 1997, Henderson et al., 2015), but their abundance only increased significantly in both fractions between days 14-21, suggesting that higher rates of starter allocation (500g/d vs. 300g/d) offered between days 14 and 21 were required for *Butyrivibrio* establishment. This increase in more prominent starch utilisers (*Butyrivibrio, Succinivibrio*) taxa during early life co-occurred with declines in others like *Streptococcus* and *Lactobacillus* (Fig. 2.11). *Succinivibrio* produce succinate, a propionate precursor, and while VFAs were not measured in this study, it is likely that *Succinivibrio* contribute to propionate production and thus host development in early life, as previously postulated (Meale et al., 2016). However, this is speculative, and requires validation by measurement of VFA profiles.

While many of the major rumen bacteria showed diet- and age-related changes in the first weeks of life, the archaeal community was more stable, with the dominant *Methanobrevibacter* species remaining unchanged throughout the preweaning phase. Archaeal richness increased by D21, suggesting that establishment of a mature-like bacterial assembly may be required for the diversification of the ruminal archaeome. The *Methanomassilicoccaceae* species were not detected prior to D21. These methylotrophs are typically less abundant than the *Methanobrevibacter spp.* (Tapio et al., 2017), and likely required increased fermentation rates by rumen bacteria for substrate availability. However as discussed below, the rumen archaea appeared to be more sensitive to farm environment than the bacteria. Overall, both bacterial and archaeal communities had settled by the third week of life, as evidenced by the lack of change between days 21 and 28, suggesting that this may represent the limit of any "window of opportunity" for interventions to take advantage of the heterogenous nature of the developing rumen microbiota. While there were small differences in microbial composition in the rumen fluid, the impact of these small changes on rumen fermentation is likely negligible due to the redundant and pleiotropic nature of the rumen microbiota (Weimer, 2015).

The weaning transition is a time of significant physiological and metabolic change and in ruminants and encompasses a substantial change in composition and function of the rumen microbiome (Meale et al., 2016, Meale et al., 2017) driven by dietary alteration, and physical maturation of the calf. The microbial changes accompanying the weaning transition have been comprehensively described recently (Dill-McFarland et al., 2018, Meale et al., 2016, Meale et al., 2017). However, while we observed higher bacterial richness and evenness in the post-weaning rumen, Meale et al. (2016) reported decreased microbial richness and evenness in rumen fluid collected from calves one week following weaning versus fluid collected from milk-fed calves. This discrepancy may be due to elevated stresses around weaning (Enríquez et al., 2011), which can reduce intakes and hence substrate for bacterial action (Eckert et al., 2015), and may not have been a factor at D96 in this present study.

The most prominent changes between days 28 and 96 concerned minor taxa or those with poor phylogenetic resolution, making it difficult to relate biological function to these taxonomic shifts. For instance, while *Ruminococcaceae* contains noted ruminal fibre degraders (Hobson and Stewart, 1997), it also contains species capable of starch hydrolysis (Klieve et al., 2007), and has previously been associated with a high-energy diet in beef steers (Li and Guan, 2017). We cannot conclude whether the observed increase of this taxa following weaning and forage allocation is driven by the fibre- or starch-utilising species, or a combination of both. However, it is notable

that the abundances of other important ruminal fibre degraders (e.g. *Fibrobacter, Ruminococcus*) remained stable between pre- and postweaning (Fig. 2.12), whilst those of starch utilisers like *Succinivibrio* and *Catenibacterium* significantly decreased, a reversal of their earlier increase following calf starter allocation on D7 (Fig. 2.11). Therefore, it is possible that the major starch-utilising members of the preweaning rumen microbiota were outcompeted for substrate by members of the *Ruminococcaceae* but elucidating the precise nature of this transition will require further investigation at lower taxonomic (i.e. species/strain) levels.

In addition to characterising the temporal sequence of prokaryotic succession in the rumen during early life, this study also highlights a substantial impact of the farm environment on the developing rumen microbiota (Fig. 2.13). F2 calves, as well as being more similar to one another (Fig. 2.5), were dominated by the *Mbb. gottschalkii* and *Ruminantium* clades and harboured a significantly less diverse archaeome in their rumen than the F1 animals (Fig. 2.3). On the other hand, *Mbb. smithii*, a major human archaeon which is not usually prevalent in the rumen, was highly abundant in the F1 animals (Fig. 2.8) and was the sole species to display a consistent response to farm throughout the experiment. *Mbb. smithii* can produce CH_4 via hydrogenotrophic and formate-utilising pathways, making it more metabolically flexible to available substrates (Samuel et al., 2007). While methane was not recorded in this study, this large shift in methanogenic composition may have implications in strategies to reduce enteric methanogenesis and must be investigated further. F1 animals also contained more *Methanomassilicoccaceae* taxa following calf starter allocation, as well as higher *Mbb. wolinii* and *Mbb. boviskoreani* postweaning. Together, this indicates that farm environment had a substantial impact on the development of the archaeal communities during early life. The rumen methanogens are often the target of efforts to manipulate rumen microbial composition and/or function (Abecia et al., 2017, Abecia et al., 2014b, Abecia et al., 2013), and our findings indicate that the effectiveness of such strategies may be significantly influenced by the farm or facility where the calves are raised.

Interestingly, the bacteria displayed an opposite response to farm, being more diverse on F2 (Fig. 2.3). At the genus level, *Dialister* was consistently the most strongly influenced by farm, and was more abundant on F1. *Dialister* is associated with feed efficiency (Myer et al., 2015a) and methane formation (Roehe et al., 2016), suggesting it might play an important role in rumen microbial function. While *Dialister* was not a major (<1%) member of the rumen bacteriome in the present study, the relationship between the presence of this taxa and calf performance should be investigated further. Moreover, other more prominent taxa (e.g. *Fibrobacter, Succinivibrio*)

were affected by farm at multiple time-points, showing that any farm effect is not restricted to minor or transient taxa. A limited number of studies have reported a farm effect on the bovine gut microbiota (Weese and Jelinski, 2017, Indugu et al., 2017). It is difficult to speculate as to the reason for the farm-wise differences observed here. All calves were raised in a comparable manner, fed the same diet, and were of good health status throughout the experimental period. The only major difference was that F2 calves were housed in the same barn as their dams, though without direct contact after 48 hours. Maternal interaction is a determinant of the rumen microbiota during early life (Abecia et al., 2017), and it may be that even the presence of mature ruminants in the same barn influenced colonisation dynamics in this study. This may have been more of an indirect effect (e.g. contribution to a divergent air microbiome by the mature animals, which may have colonised calf feed). More work is needed to confirm these findings however, as this study was not designed to explicitly test for a farm effect.

2.5 Conclusion

To summarise the temporal changes in microbial composition observed during early life, allocating increasing amounts of calf starter from D7, possibly aided by forage consumption in the first week of life, led to the establishment of a settled microbial community in the rumen by D21 which resembled that of the mature animal. The community stabilised by week three, suggesting that manipulation to take advantage of the heterogenous rumen microbiome should take place prior to D21 of life. This is among the first surveys of the temporal dynamics of the rumen microbiota in beef calves, and is, to the best of our knowledge, the first study to report a substantial farm effect on the dynamics of microbial colonisation, a finding that must be considered in future attempts to redirect microbial establishment patterns in young ruminants.

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2.7 Tables and figures

Table 2.1: PERMANOVA results based on Bray-Curtis dissimilarity matrices of rarefied OTU count tables for bacterial and archaeal communities during early life.

	Bacteria			**Archaea**		
Factor	**R2[1]**	**F[2]**	**P-value**	**R^2**	**F**	**P-value**
Age	0.24	7.03	<0.01	0.15	4.46	<0.01
Location	0.07	8.18	<0.01	0.18	21.53	<0.01
Fraction	0.01	0.93	0.52	0.00	0.37	0.88
Age*Farm	0.09	2.73	<0.01	0.07	2.06	0.01
Age*Fraction	0.01	0.41	1.00	0.01	0.33	1.00
Farm*Fraction	0.01	0.63	0.94	0.00	0.18	0.98

[1]Percentage of total variation in the model explained by each factor.
[2]Test statistic. Larger values represent larger dispersions from the mean.

Table 2.2: Abundances of taxa which were differentially abundant according to rumen fraction during early life. Archaea were unaffected by fraction at any timepoint and so are not presented.

Genus	Solid Digesta %	Liquid Digesta %	P-value
Day 7			
Fusobacterium	4.48%[1]	2.43%	0.025[2]
Veillonella	3.87%	1.01%	0.049
Porphyromonas	3.51%	4.62%	0.001
f. Lachnospiraceae	2.27%	2.86%	<0.001
o. Bacteroidales	2.07%	1.50%	0.001
Odoribacter	1.68%	0.06%	0.006
Succiniclasticum	1.56%	1.22%	0.001
Fibrobacter	1.44%	0.85%	<0.001
f. Alcaligenaceae	1.35%	2.21%	0.001
Comamonas	1.35%	3.55%	<0.001
Parvimonas	0.63%	0.28%	<0.001
Parabacteroides	0.51%	1.52%	0.003
Treponema	0.50%	0.18%	0.011
f. Veillonellaceae	0.49%	0.42%	<0.001
Butyrivibrio	0.33%	0.16%	<0.001
f. [Paraprevotellaceae]	0.24%	0.17%	0.007
Pseudobutyrivibrio	0.19%	0.07%	0.008
Mogibacterium	0.17%	0.09%	0.030
f. [Weeksellaceae]	0.15%	0.16%	0.041
Collinsella	0.15%	0.06%	0.043
Anaerovibrio	0.14%	0.11%	0.001
Lactobacillus	0.12%	0.05%	0.001
Filifactor	0.10%	0.18%	0.025
p-75-a5	0.09%	0.12%	0.006
c. Alphaproteobacteria	0.02%	0.12%	0.003
Paludibacter	0.00%	1.17%	<0.001
Day 14	No significant differences		
Dy 21	No significant differences		
Day 28			
[Prevotella]	0.32%	1.66%	0.026
Day 96	No significant differences		

[1]Percentage of total bacterial 16S rRNA reads at the relevant timepoint.
[2]FDR-corrected *P*-value generated using DeSeq2.

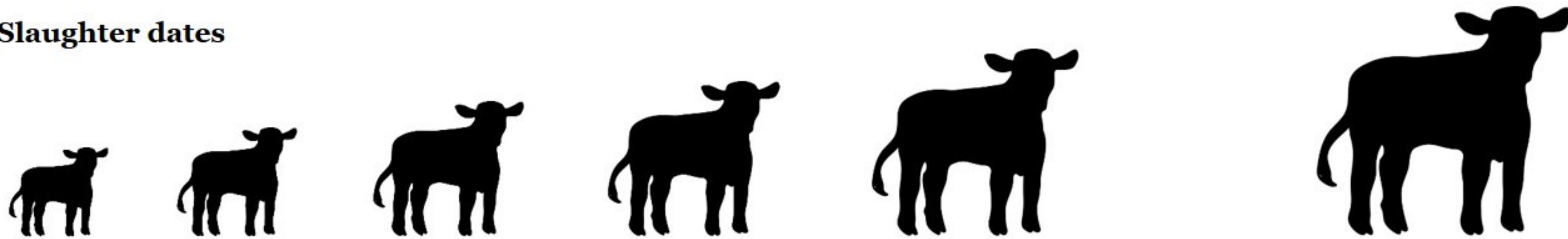
(a) **Feeding management**

Birth - 48h pp.
Colostrum

D3 – D56 (weaning)
Milk replacer + calf starter (D7 onward)

D56 – D96
Hay + calf starter

(b) **Slaughter dates**



Newborn
ECS: n = 10
NAT: n = 11

D7
n = 8

D14
n = 9

D21
n = 9

D28
n = 10

D96
n = 9

Figure 2.1: Experimental animal trial design and calf management. Calves raised on different farms received the same diet and were housed in the same manner.

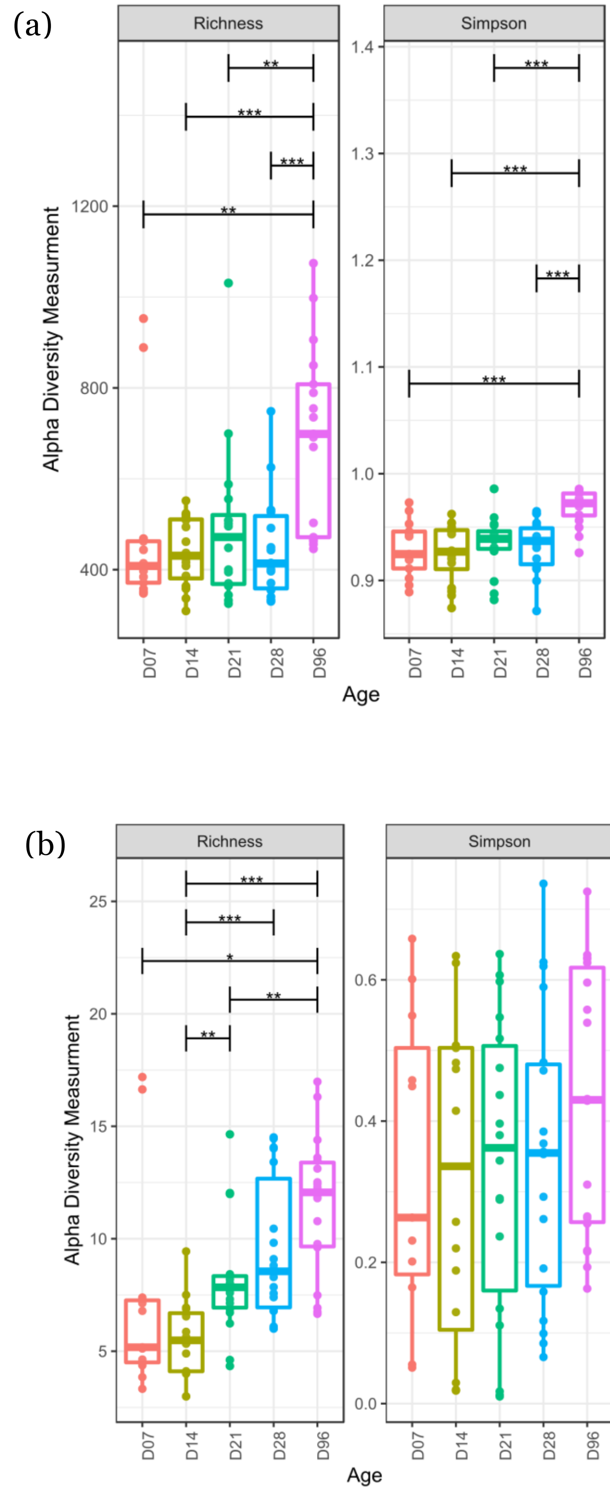
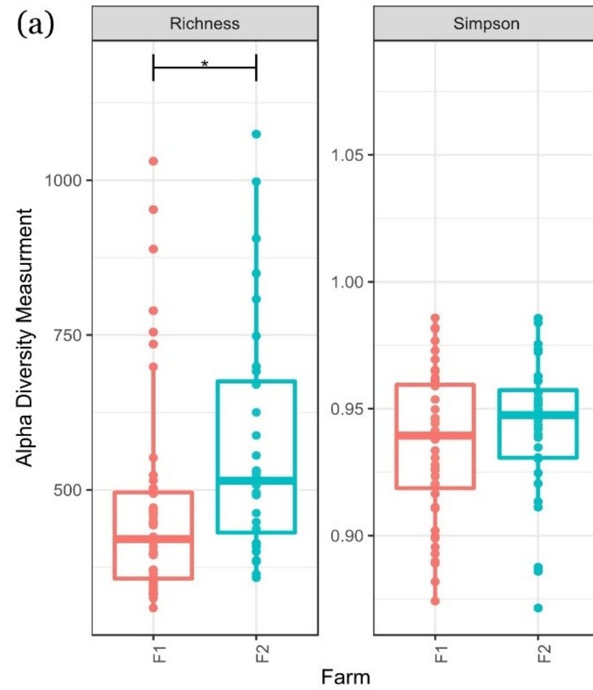


Figure 2.2: Temporal dynamics of (a) bacterial and (b) archaeal alpha diversity metrics during early life. * denotes significant differences ($P<0.05$).



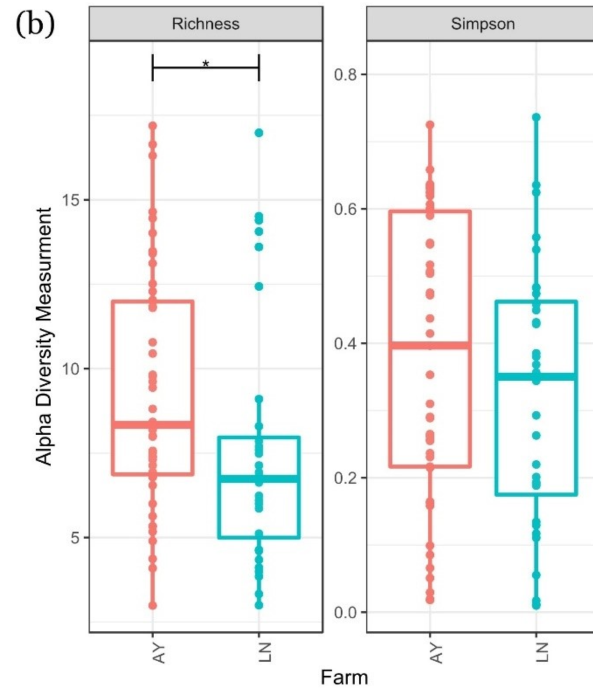


Figure 2.3: Effect of farm environment on alpha diversity indices of (a) bacterial and (b) archaeal communities during early life. * denotes significantly different values ($P<0.05$).

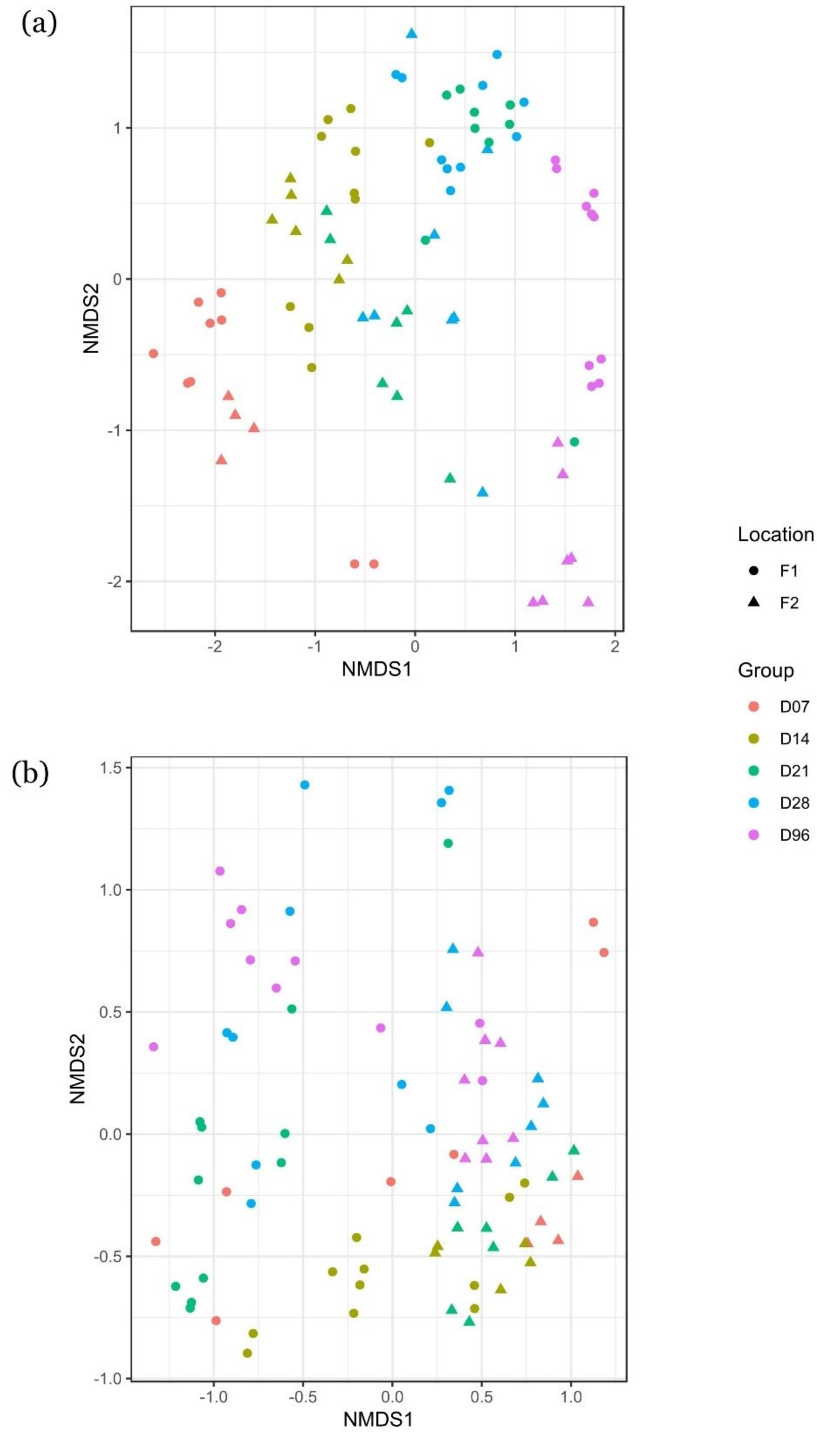


Figure 2.4: nMDS plots of (a) bacterial and (b) archaeal communities of the rumen solid and liquid digesta. Plots are based on Bray-Curtis dissimilarity matrices.

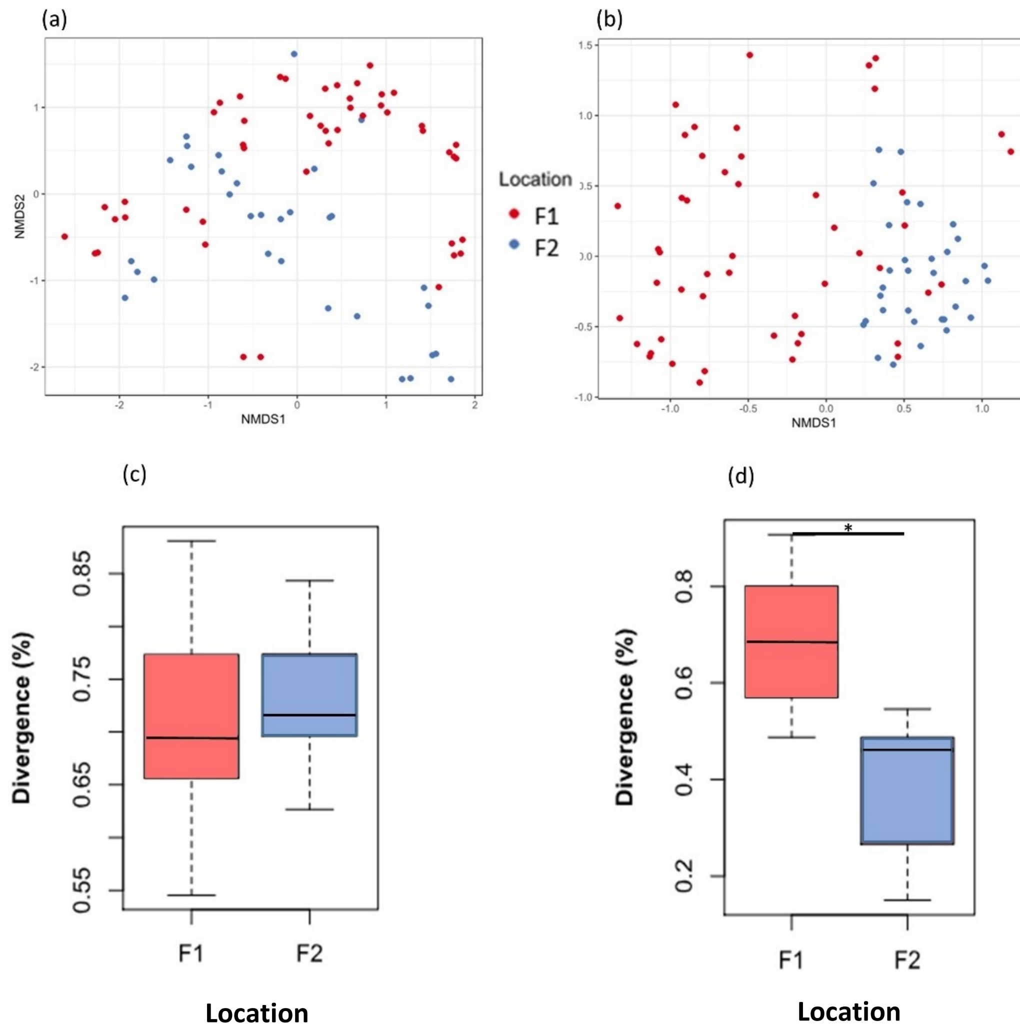


Figure 2.5. The effect of farm location on (a) bacterial and (b) archaeal communities during early life. Figures (c) & (d) depict the divergence (β-dispersion) of bacterial and archaeal communities according to farm.

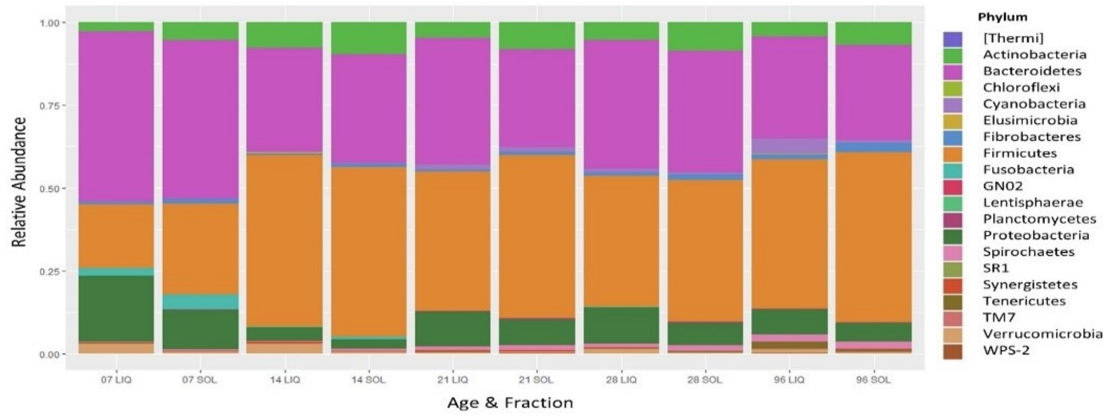
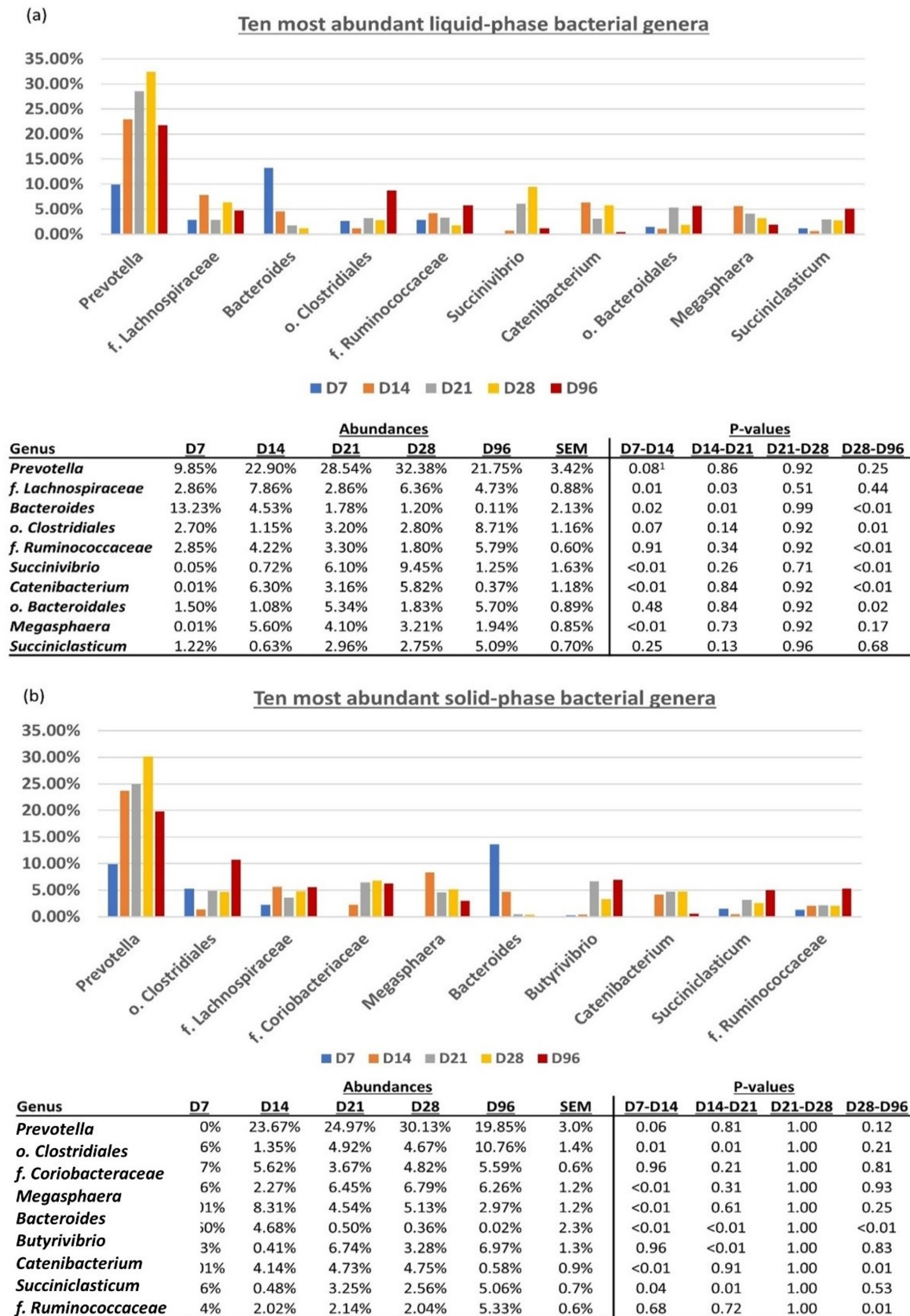


Figure 2.6: Stacked barchart of bacterial phyla composition in the rumen solid and liquid digesta during early life



	Abundances						P-values			
Genus	**D7**	**D14**	**D21**	**D28**	**D96**	**SEM**	**D7-D14**	**D14-D21**	**D21-D28**	**D28-D96**
Prevotella	9.85%	22.90%	28.54%	32.38%	21.75%	3.42%	0.08[1]	0.86	0.92	0.25
f. Lachnospiraceae	2.86%	7.86%	2.86%	6.36%	4.73%	0.88%	0.01	0.03	0.51	0.44
Bacteroides	13.23%	4.53%	1.78%	1.20%	0.11%	2.13%	0.02	0.01	0.99	<0.01
o. Clostridiales	2.70%	1.15%	3.20%	2.80%	8.71%	1.16%	0.07	0.14	0.92	0.01
f. Ruminococcaceae	2.85%	4.22%	3.30%	1.80%	5.79%	0.60%	0.91	0.34	0.92	<0.01
Succinivibrio	0.05%	0.72%	6.10%	9.45%	1.25%	1.63%	<0.01	0.26	0.71	<0.01
Catenibacterium	0.01%	6.30%	3.16%	5.82%	0.37%	1.18%	<0.01	0.84	0.92	<0.01
o. Bacteroidales	1.50%	1.08%	5.34%	1.83%	5.70%	0.89%	0.48	0.84	0.92	0.02
Megasphaera	0.01%	5.60%	4.10%	3.21%	1.94%	0.85%	<0.01	0.73	0.92	0.17
Succiniclasticum	1.22%	0.63%	2.96%	2.75%	5.09%	0.70%	0.25	0.13	0.96	0.68

	Abundances						P-values			
Genus	**D7**	**D14**	**D21**	**D28**	**D96**	**SEM**	**D7-D14**	**D14-D21**	**D21-D28**	**D28-D96**
Prevotella	0%	23.67%	24.97%	30.13%	19.85%	3.0%	0.06	0.81	1.00	0.12
o. Clostridiales	6%	1.35%	4.92%	4.67%	10.76%	1.4%	0.01	0.01	1.00	0.21
	7%	5.62%	3.67%	4.82%	5.59%	0.6%	0.96	0.21	1.00	0.81
f. Coriobacteraceae	6%	2.27%	6.45%	6.79%	6.26%	1.2%	<0.01	0.31	1.00	0.93
Megasphaera	)1%	8.31%	4.54%	5.13%	2.97%	1.2%	<0.01	0.61	1.00	0.25
Bacteroides	;0%	4.68%	0.50%	0.36%	0.02%	2.3%	<0.01	<0.01	1.00	<0.01
Butyrivibrio	3%	0.41%	6.74%	3.28%	6.97%	1.3%	0.96	<0.01	1.00	0.83
Catenibacterium	)1%	4.14%	4.73%	4.75%	0.58%	0.9%	<0.01	0.91	1.00	0.01
Succiniclasticum	6%	0.48%	3.25%	2.56%	5.06%	0.7%	0.04	0.01	1.00	0.53
f. Ruminococcaceae	4%	2.02%	2.14%	2.04%	5.33%	0.6%	0.68	0.72	1.00	0.01

Figure 2.7: Details of the 10 most abundant bacterial taxa detected in (a) rumen liquid and (b) rumen solid digesta during early life. Values in the accompanying tables are group mean relative abundances and FDR-corrected *P*-values generated by temporally adjacent contrasts in DeSeq2.

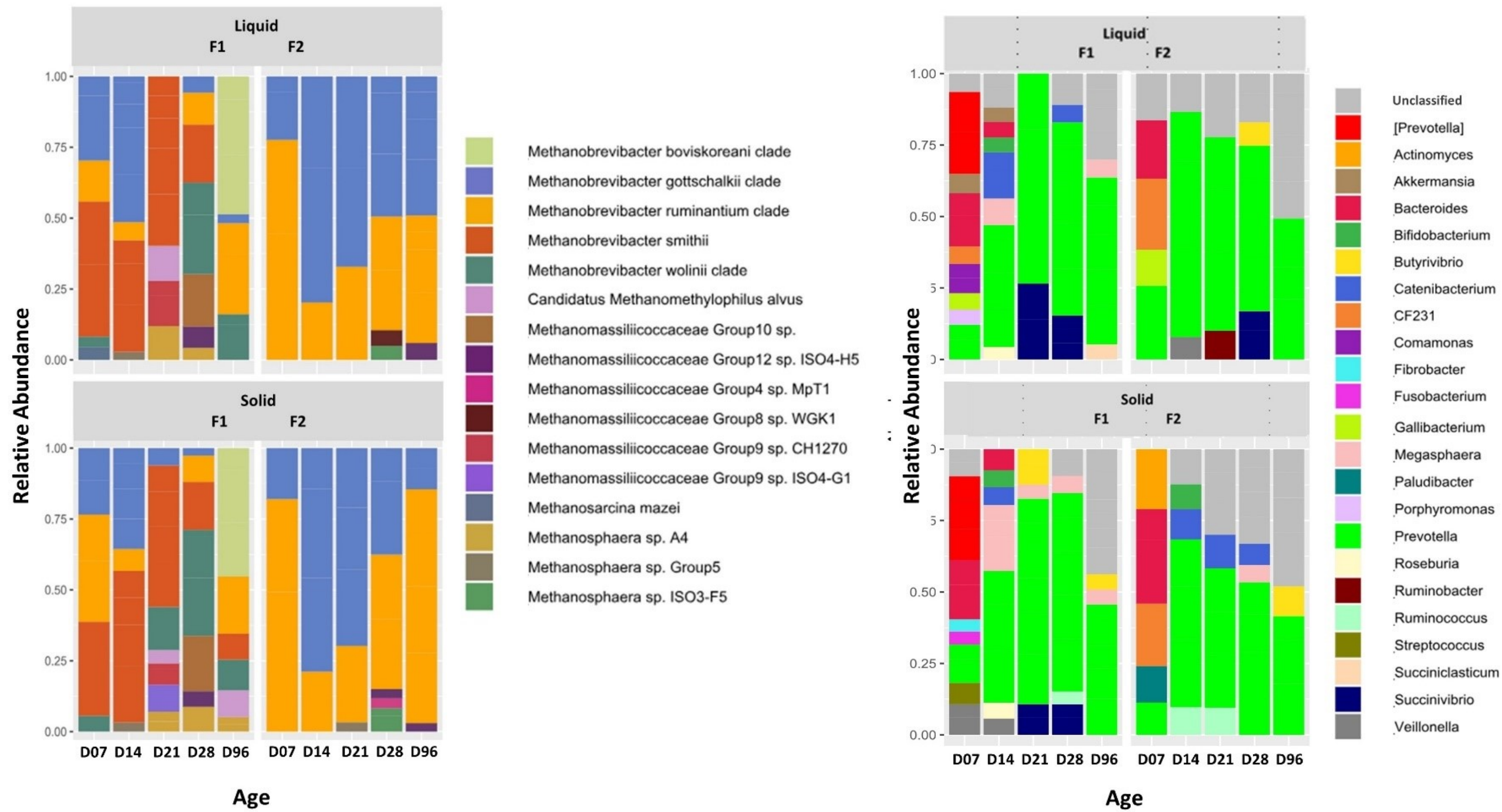
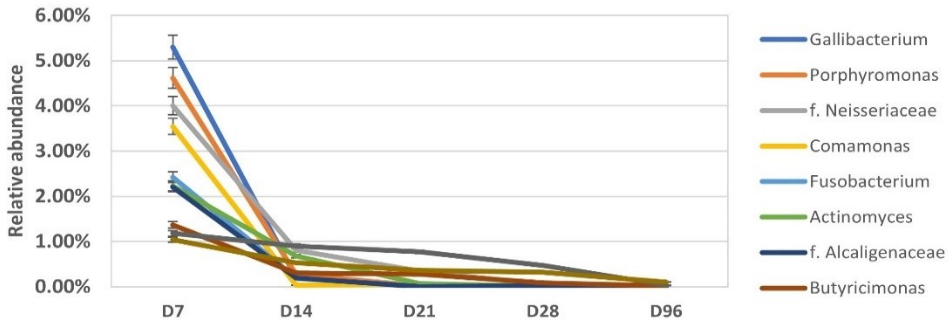
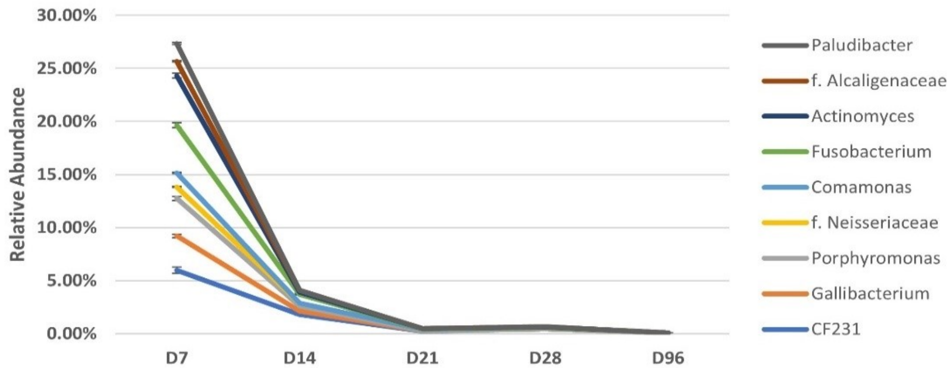


Figure 2.8: Barcharts depicting relative abundances of archaeal species and predominant bacterial genera across age, digesta fraction and farm location (F1 vs. F2) during early life.

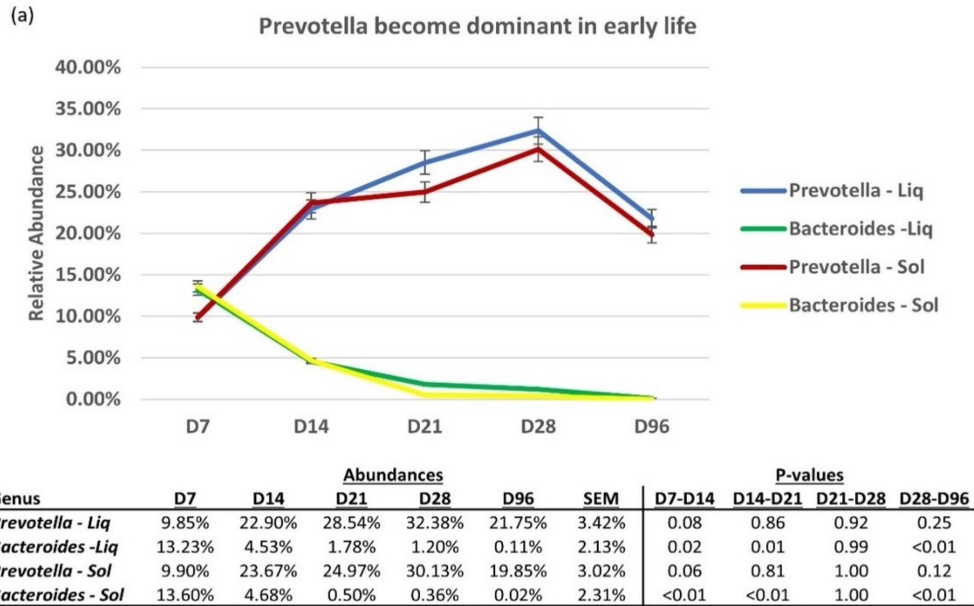


Genus	Abundances						P-values			
	D7	D14	D21	D28	D96	SEM	D7-D14	D14-D21	D21-D28	D28-D96
Gallibacterium	5.30%	0.20%	0.02%	0.01%	<0.01%	0.94%	<0.01	0.00	0.82	0.01
Porphyromonas	4.62%	0.27%	0.02%	0.02%	<0.01%	0.81%	<0.01	0.01	0.99	<0.01
f. Neisseriaceae	4.01%	0.80%	0.35%	0.05%	0.01%	0.67%	0.02	0.58	0.78	0.02
Comamonas	3.55%	0.04%	0.05%	0.02%	<0.01%	0.63%	NA	NA	NA	NA
Fusobacterium	2.43%	0.19%	0.01%	0.01%	<0.01%	0.43%	<0.01	<0.01	0.92	0.07
Actinomyces	2.23%	0.68%	0.07%	0.01%	<0.01%	0.38%	<0.01	0.01	0.70	0.12
f. Alcaligenaceae	2.21%	0.20%	<0.01%	0.02%	<0.01%	0.39%	NA	NA	NA	NA
Butyricimonas	1.37%	0.30%	0.28%	0.08%	0.02%	0.22%	<0.01	0.58	0.85	0.02
Campylobacter	1.18%	0.90%	0.77%	0.47%	0.04%	0.17%	0.85	0.89	0.94	<0.01
Oscillospira	1.04%	0.53%	0.37%	0.32%	0.11%	0.14%	<0.01	0.02	0.92	0.01

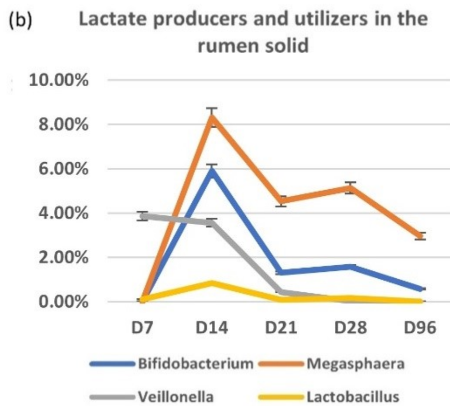


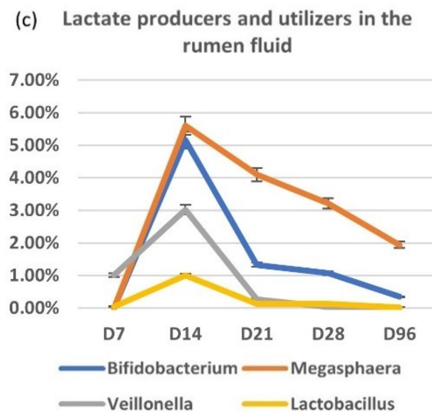
Genus	Abundances						P-values			
	D7	D14	D21	D28	D96	SEM	D7-D14	D14-D21	D21-D28	D28-D96
CF231	5.98%	1.81%	0.24%	0.44%	0.12%	1.0%	NA	NA	NA	NA
Gallibacterium	3.25%	0.32%	0.02%	0.01%	<0.01%	0.6%	NA	NA	NA	NA
Porphyromonas	3.51%	0.45%	0.02%	0.01%	<0.01%	0.6%	<0.01	0.01	1.00	<0.01
f. Neisseriaceae	1.08%	0.27%	0.13%	0.11%	<0.01%	0.2%	NA	NA	NA	NA
Comamonas	1.35%	0.03%	0.02%	0.01%	<0.01%	0.2%	<0.01	0.80	1.00	0.05
Fusobacterium	4.48%	0.81%	0.02%	0.01%	<0.01%	0.8%	<0.01	<0.01	1.00	0.01
Actinomyces	4.68%	0.21%	0.05%	0.02%	<0.01%	0.8%	<0.01	0.04	1.00	0.04
f. Alcaligenaceae	1.35%	0.19%	0.00%	0.04%	<0.01%	0.2%	0.26	<0.01	0.45	<0.01
Paludibacter	1.68%	0.00%	0.00%	0.00%	<0.01%	0.3%	<0.01	<0.01	<0.01	<0.01

Figure 2.9: Line charts showing temporal dynamics of selected transient taxa in (a) the rumen liquid and (b) the rumen solid digesta during early life. Values in the accompanying tables are group mean relative abundances and FDR-corrected P-values generated by temporally adjacent contrasts in DeSeq2.



Genus	Abundances D7	D14	D21	D28	D96	SEM	P-values D7-D14	D14-D21	D21-D28	D28-D96
Prevotella - Liq	9.85%	22.90%	28.54%	32.38%	21.75%	3.42%	0.08	0.86	0.92	0.25
Bacteroides -Liq	13.23%	4.53%	1.78%	1.20%	0.11%	2.13%	0.02	0.01	0.99	<0.01
Prevotella - Sol	9.90%	23.67%	24.97%	30.13%	19.85%	3.02%	0.06	0.81	1.00	0.12
Bacteroides - Sol	13.60%	4.68%	0.50%	0.36%	0.02%	2.31%	<0.01	<0.01	1.00	<0.01





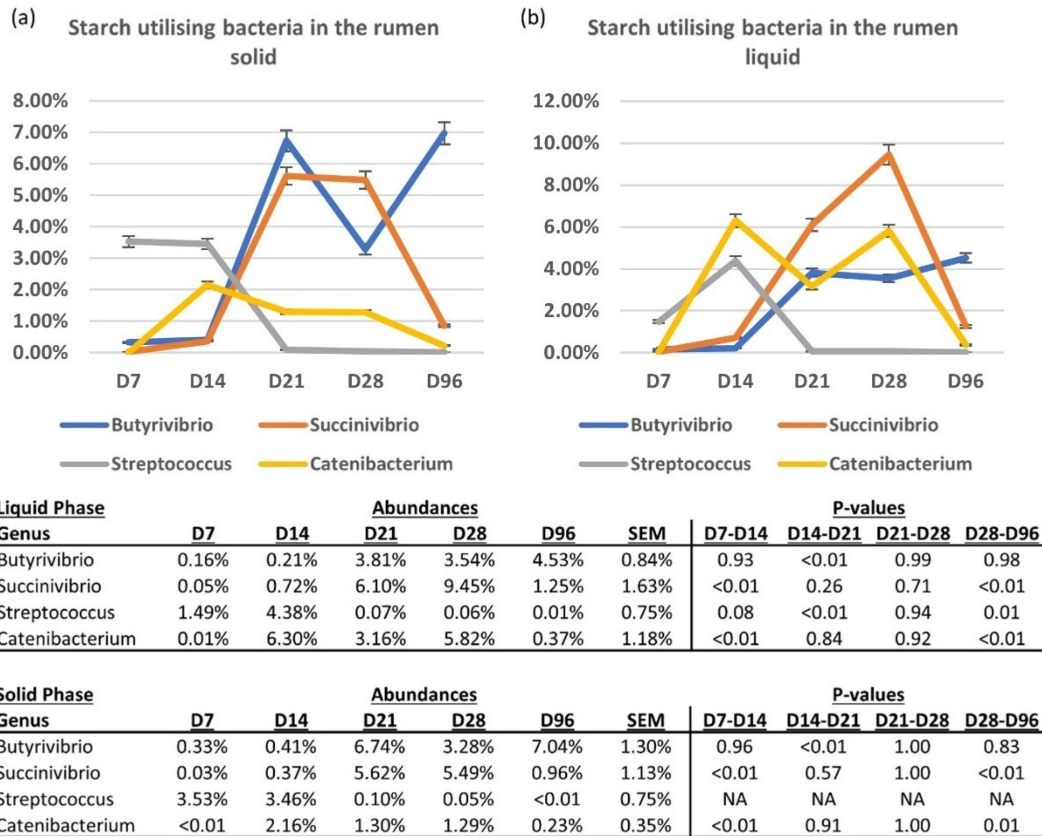
Liquid Phase

Genus	Abundances D7	D14	D21	D28	D96	SEM	P-values D7-D14	D14-D21	D21-D28	D28-D96
Bifidobacterium	0.01%	5.16%	1.33%	1.07%	0.35%	0.83%	<0.01	0.19	0.94	0.57
Megasphaera	0.01%	5.60%	4.10%	3.21%	1.94%	0.85%	<0.01	0.73	0.92	0.17
Veillonella	1.01%	3.02%	0.27%	0.04%	<0.01%	0.51%	0.59	<0.01	0.47	0.03
Lactobacillus	0.05%	1.01%	0.14%	0.13%	0.02%	0.17%	<0.01	0.01	0.96	0.03

Solid Phase

Genus	Abundances D7	D14	D21	D28	D96	SEM	P-values D7-D14	D14-D21	D21-D28	D28-D96
Bifidobacterium	0.01%	5.90%	1.31%	1.58%	0.62%	0.93%	<0.01	0.06	1.00	0.15
Megasphaera	<0.01%	8.31%	4.54%	5.13%	3.03%	1.22%	<0.01	0.61	1.00	0.25
Veillonella	3.87%	3.57%	0.45%	0.04%	<0.01%	0.78%	<0.01	<0.01	1.00	0.01
Lactobacillus	0.12%	0.84%	0.10%	0.17%	<0.01%	0.13%	NA	NA	NA	NA

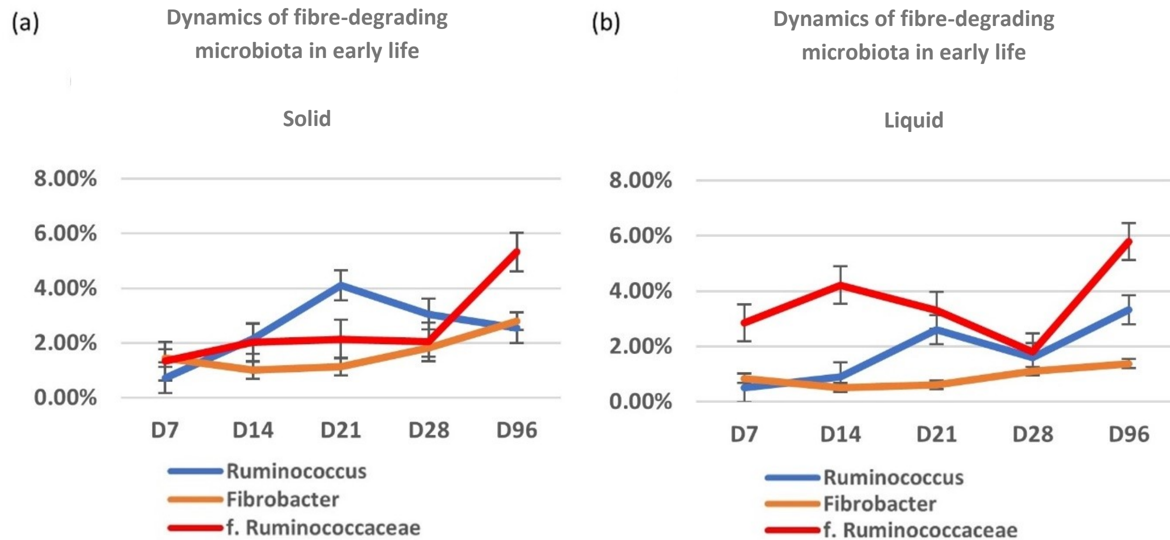
Figure 2.10: Line charts depicting the predominance of *Prevotella* and the decline of *Bacteroides* from day 7 onward, and the dynamics of lactate producing and utilising bacteria in the rumen (a) solid and (b) liquid digesta during early life. Values in the accompanying tables are group mean relative abundances and FDR-corrected *P*-values generated by temporally adjacent contrasts in DeSeq2.



Liquid Phase			Abundances					P-values		
Genus	D7	D14	D21	D28	D96	SEM	D7-D14	D14-D21	D21-D28	D28-D96
Butyrivibrio	0.16%	0.21%	3.81%	3.54%	4.53%	0.84%	0.93	<0.01	0.99	0.98
Succinivibrio	0.05%	0.72%	6.10%	9.45%	1.25%	1.63%	<0.01	0.26	0.71	<0.01
Streptococcus	1.49%	4.38%	0.07%	0.06%	0.01%	0.75%	0.08	<0.01	0.94	0.01
Catenibacterium	0.01%	6.30%	3.16%	5.82%	0.37%	1.18%	<0.01	0.84	0.92	<0.01

Solid Phase			Abundances					P-values		
Genus	D7	D14	D21	D28	D96	SEM	D7-D14	D14-D21	D21-D28	D28-D96
Butyrivibrio	0.33%	0.41%	6.74%	3.28%	7.04%	1.30%	0.96	<0.01	1.00	0.83
Succinivibrio	0.03%	0.37%	5.62%	5.49%	0.96%	1.13%	<0.01	0.57	1.00	<0.01
Streptococcus	3.53%	3.46%	0.10%	0.05%	<0.01	0.75%	NA	NA	NA	NA
Catenibacterium	<0.01	2.16%	1.30%	1.29%	0.23%	0.35%	<0.01	0.91	1.00	0.01

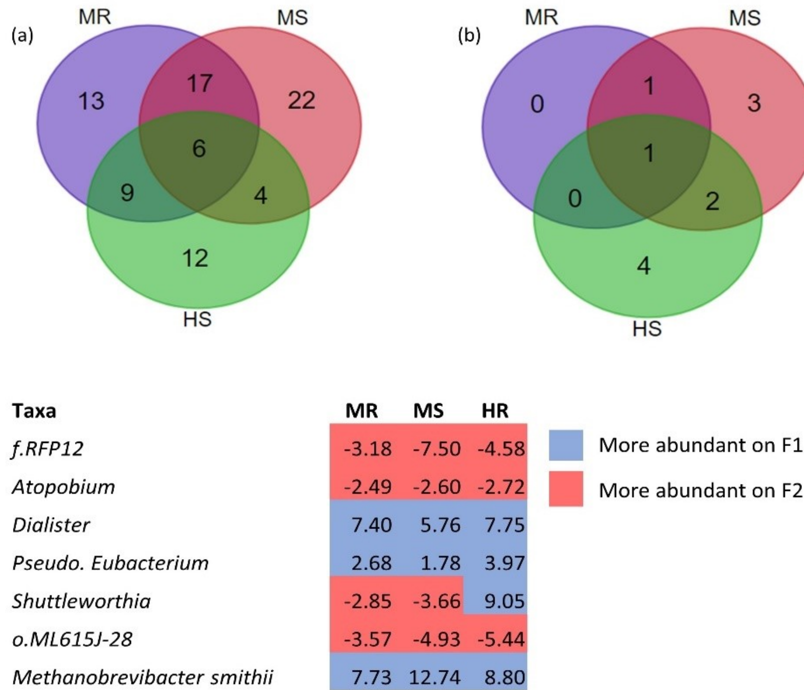
Figure 2.11: Line charts depicting temporal dynamics of selected VFA producers and starch utilising groups during early life in (a) the rumen solid and (b) the rumen liquid digesta. Values in the accompanying tables are group mean relative abundances and FDR-corrected *P*-values generated by temporally adjacent contrasts in DeSeq2.



Liquid Phase			Abundances				P-values			
Genus	D7	D14	D21	D28	D96	SEM	D7-D14	D14-D21	D21-D28	D28-D96
Ruminococcus	0.50%	0.91%	2.60%	1.60%	3.33%	0.47%	0.82	0.37	0.92	0.15
Fibrobacter	0.85%	0.52%	0.61%	1.10%	1.38%	0.14%	0.59	0.78	0.86	0.75
f. Ruminococcaceae	2.85%	4.22%	3.30%	1.80%	5.79%	0.60%	0.91	0.34	0.92	0.00

Solid Phase			Abundances				P-values			
Genus	D7	D14	D21	D28	D96	SEM	D7-D14	D14-D21	D21-D28	D28-D96
Ruminococcus	0.73%	2.15%	4.10%	3.06%	2.6%	0.50%	NA	NA	NA	NA
Fibrobacter	1.44%	1.02%	1.14%	1.81%	2.8%	0.29%	0.68	0.88	1.00	0.58
f. Ruminococcaceae	1.34%	2.02%	2.14%	2.04%	5.3%	0.63%	0.68	0.72	1.00	0.01

Figure 2.12: Line charts depicting temporal dynamics of selected fibrolytic taxa during early life and postweaning in (a) the rumen solid and (b) the rumen liquid digesta. Values in the accompanying tables are group mean relative abundances and FDR-corrected *P*-values generated by temporally adjacent contrasts in DeSeq2.



Taxa	MR	MS	HR
f.RFP12	-3.18	-7.50	-4.58
Atopobium	-2.49	-2.60	-2.72
Dialister	7.40	5.76	7.75
Pseudo. Eubacterium	2.68	1.78	3.97
Shuttleworthia	-2.85	-3.66	9.05
o.ML615J-28	-3.57	-4.93	-5.44
Methanobrevibacter smithii	7.73	12.74	8.80

Figure 2.13: Visual representation of taxa which were affected by farm during early life. Values in the Venn diagram denote the numbers of unique or shared taxa among MR (D7), MS (D14-28), and HS (D96) groups that were significantly differentially abundant across farm environments. The table describes the 7 "core" taxa affected by farm at each timepoint, and the values represent the $\log_2$ fold-change in taxon abundance between farms, calculated in DeSeq2.

Chapter 3

Temporal dynamics of the rumen transcriptome of beef calves from birth to post-weaning

3.1 Introduction

Ruminants have the unique ability to convert human indigestible grains and forages into protein-rich meat and dairy products. Volatile fatty acids (VFA) produced by rumen microbial fermentation (mainly acetate, propionate, and butyrate) provide up to 70% of host energy requirements (Bergman, 1990). The rumen itself has evolved to facilitate the absorption and transport of dietary nutrients, and to protect the host from the potentially pathogenic microbial inhabitants of the lumen. Rumen development entails growth and differentiation of the rumen epithelium, resulting in a substantial change in hepatic and lower-intestinal nutrient profiles, and consequentially those of the peripheral tissues (Baldwin et al., 2004). Barrier function of the rumen epithelium is a key facet of the host immune system, responsible for maintaining concentration gradients necessary for ionic absorption, and preventing the translocation of pathogenic bacteria (e.g. *Fusobacterium*) and their components (e.g. lipopolysaccharide (LPS)) into the circulatory system (Penner et al., 2011)

At birth, the rumen is essentially non-functional, lacking the characteristic papillae of adults, and proper development and function of the rumen prior to weaning is critical to general health and lifelong productivity of young ruminants (Baldwin et al., 2004, Khan et al., 2011a). Solid feed consumption during early life stimulates rumen development via increased microbial VFA biosynthesis (Drackley et al., 2008). Therefore, an integrated understanding of host and microbial co-development in the rumen is important to gain complete understanding rumen development and function during early life. Interest in the ontogeny of the rumen microbiota during early life has increased greatly in recent years (Jami et al., 2013, Rey et al., 2014), but understanding of how colonisation by a diverse microbiota contributes to the physical maturation of the rumen organ remains limited.

In chapter 2, the temporal colonisation patterns of the rumen bacteria and archaea during early life were discussed. However, despite its obvious importance, knowledge of the molecular mechanisms underpinning rumen physical development and nutrient absorption in calves during early life is limited. Studies to date have largely focused on the weaning transition (Nishihara et

al., 2018) and gene expression changes in response to a nutritional insult or disorder like feed restriction or acidosis (Steele et al., 2011a, Penner et al., 2011, Steele et al., 2011b, Keogh et al., 2017). Moreover, most studies performed in the first weeks of life have used small ruminants like sheep and goats, which may not be comparable to cattle due to physiological and management differences between large and small ruminants (Jiao et al., 2015b, Wang et al., 2016b). Furthermore, despite being among the most common surgical procedures in cattle, it is unknown if caesarean section (CS) delivery can influence rumen development.

Considering these knowledge gaps, our objective was to characterise the transcriptional mechanisms governing rumen development during early life in terms of (i) physical development, (ii) VFA and nutrient absorption, (iii) the establishment of host-microbial homeostasis, and (iv) to assess variation due to mode of delivery at birth.

3.2 Materials and methods

3.2.1 Ethical statement

All experimental animal procedures described herein were carried out at Teagasc Mellows Campus (Athenry, Co. Galway, Ireland – F1) and DAFM Longtown (Clane, Co. Kildare, Ireland – F2) under licence from the Irish Health Products Regulatory Authority (Licence no.: AE19142), and the Teagasc Animal Ethics Committee (TAEC). All individuals were further licenced to perform euthanasia of calves, and to carry all procedures described in this Chapter3.2.3 .

3.2.2 Experimental animal model

Data presented in this chapter were obtained using a subset of the same animal model as detailed in Chapter 2. Briefly, 66 artificially inseminated Aberdeen Angus x Charolais heifers were blocked by foetal sex and randomly allocated into one of seven experimental groups based on the date of calf slaughter as detailed in Chapter 2: NB.NAT, NB.CS (ECS), D7, D14, D28, and D96. Calves not assigned to the NB groups remained with their dam for 48h to facilitate adequate colostrum consumption. Calves were penned individually and offered milk replacer (13.5% solids when reconstituted to 5L) daily in one morning feeding via teeted bucket. Calves were bedded on straw and allocated calf starter from day 7 onward, with weekly increases in volume until day 28 as detailed in Chapter 2. Between days 28 and weaning on day 56, calves received 1kg of calf starter daily. Following weaning, calves in the D96 group had *ad libitum* access to grass hay and calf starter until slaughter.

3.2.3 Rumen tissue sampling

On the day of slaughter as described above, calves were euthanised via an intravenous overdose of pentobarbital sodium (Dolethal™, Vetoquinol, UK, or Euthatal™, Boehringer Ingelheim, UK: 1ml/1.4kg live weight). Calves assigned to both NB treatments were delivered onto sterilised plastic and euthanised within 5 minutes of delivery. The absence of both a heartbeat and a corneal reflex was used to verify death. Following euthanasia, the gastrointestinal tract was quickly exteriorised, and a 1cm^2 portion of the rumen wall was collected from the ventral sac. The tissue was washed in sterile phosphate buffered saline and immediately snap frozen in liquid nitrogen. All tissues were processed and frozen within 25 minutes of death and were subsequently stored at -80°C pending molecular analysis.

3.2.4 RNA isolation

Frozen rumen tissue samples were ground to a fine powder under constant liquid nitrogen using a pestle and mortar previously chilled in a -80°C freezer. Total RNA was extracted from ~80mg of ground tissue using the mirVana RNA extraction kit (Ambion, Austin, TX), following the manufacturer's instructions. RNA quality and quantity were assessed by 2 consecutive readings on a NanoDrop1000 (Thermo-Fisher, DE) and a Qubit 3.0 fluorometer (Invitrogen, CA) and assessed for integrity on an Agilent TapeStation™ (Agilent, CA). RNA integrity (RIN) numbers (cut-off of RIN ≥ 7) along with RNA quality and quantity values were used to select 8 samples from each treatment group for sequencing.

3.2.5 RNA-seq library construction and sequencing

RNA-seq libraries were constructed from 100ng of RNA using the Illumina TruSeq Stranded mRNA Sample Preparation kit, with mRNA enrichment (San Diego, CA) according to the manufacturer's instructions. Briefly, following RNA fragmentation, cDNA was synthesised via reverse transcription. The resulting double-stranded cDNA underwent end-repair and 3' adenylation, followed by sequence adapter ligation. Libraries were amplified via 15 cycles of PCR (98C for 30s, followed by 15 cycles of: 98°C for 10 seconds, 60°C for 30 seconds, and 72°C for 30s, with a final elongation at 72°C for 5 minutes. Library quality and quality were validated using a Qubit fluorometer 3.0 (Invitrogen, CA) and further assessed using the Agilent 2200 TapeStation™ (Agilent, CA). Indexed libraries were pooled and sequenced in four lanes on an

Illumina HiSeq2500 platform at the Genome Quebec Innovation Centre (McGill University, Quebec, Canada), to generate high quality 2x100 base pair paired-end sequences.

3.2.6 Bioinformatic and statistical analysis

Visual quality inspection of raw sequence libraries was performed using the FASTQC tool (Andrews, 2010). All reads were subjected to quality filtering and trimming using the BBTools suite (Bushnell, 2015), with a q-score cut-off of 25. The STAR short read aligner (Dobin et al., 2013) was used to map the reads to the bovine genome (UMD 3.1, Ensembl v.83.31). The resulting BAM files were used as input for the featureCounts tool (Liao et al., 2014) to count the number of reads per gene, using Ensembl (v. 83.31) bovine gene IDs. The final gene count table was exported for downstream analysis, and bovine gene symbols corresponding to the Ensembl ID were downloaded from Biomart (Durinck S, 2005) in RStudio (Team., 2008)

Differential gene expression analysis was performed using the edgeR (Robinson et al., 2010) package from Bioconductor in R. Prior to analysis, the gene count data was filtered to retain only those genes with a counts-per-million (CPM) value > 1 (corresponding to 12 reads) in at least 50% of the samples. Count data was corrected for differential library size using the TMM method implemented in edgeR. To identify differentially expressed genes (DEG), several temporally adjacent and biologically relevant contrasts (1. NB.NAT v NB.CS , 2. D7 vs. NB.NAT, 3. D14 vs. NB.NAT, 4. D28 vs. NB.NAT, 5. D96 vs. NB.NAT, 6. D14 vs. D7, 7. D28 vs. D14, 8. D96 vs. D28 and 9. D28 vs. D7) were performed using a Quasi-likelihood F-test under a generalised linear model in edgeR, which included age, farm, and calf gender. Using stringent parameters, only genes that displayed an absolute Log_2Fold-change > 2 with an FDR-corrected P-value < 0.001 were considered significantly differentially expressed between groups.

Volcano plots of differentially expressed genes were prepared in edgeR. Hierarchical clustering and Principal Component Analysis (PCA) plots were prepared using DeSeq2 (Love et al., 2014). Ingenuity Pathway Analysis (IPA, Qiagen, CA) was used for the canonical pathway analysis of DE genes, with pathways considered enriched at $P<0.05$, and activated/supressed at |z-score| ≥ 2. Predicted molecular, cellular, and physiological functions of the DEG were also predicted by IPA. Gene Set Enrichment Analysis was performed using the Fast Gene Set Enrichment Analysis (FGSEA) R package in R (Sergushichev, 2016), focussing on Gene ontology (GO) categories Biological Processes, Cellular Component, and Molecular Functions. Significance was declared at $P<0.05$, |NES (normalised enrichment score, indicating activation or suppression

of the term)| ≥ 1.5, and only terms containing at least five DEG were considered of biological relevance.

3.3 Results & Discussion

RNA sequencing of rumen tissue collected from 48 Aberdeen Angus calves generated an average of 22,438,172 ± 663,636 reads per sample. After quality filtering and normalisation, 13,154/24,616 genes were declared as expressed (CPM > 1 in at least 50% of the animals). We first investigated the number of shared and uniquely expressed genes among all timepoints. A total of 12,635 core genes (those expressed at all timepoints) were identified (Fig. 3.1a). New-born animals had the highest number of unique genes with 1,983, and 20 unique genes were identified on day 7, 8 genes on day 14, 21 genes on day 28 and 1 genes was unique to the 96-day old calves.

The DEG identified in each temporal comparison in edgeR are presented in Table 3.1, and graphically in Fig. 3.3. Two genes were differentially expressed between naturally delivered and ECS calves at birth ($P<0.05$). The most apparent divergence in gene expression profiles was when new-born animals were compared to older calves, but temporal changes in the rumen wall transcriptome during the milk-feeding period were also evident. Between days 7 (calves fed exclusively with milk replacer) and day 14 (milk replacer plus calf starter), 25 genes were differentially expressed (13 upregulated vs. 12 downregulated, $P < 0.05$), while two genes were differentially expressed between days 14 and 28 (both downregulated, $P<0.05$, Fig. 3.2). Because of the relatively small temporally adjacent differences during the milk-feeding period, gene expression profiles were also compared between days 7 and 28, with 103 DEG identified (42 upregulated vs. 63 downregulated; $P<0.05$). There were 213 DEG between days 28 and 96 (21 upregulated vs. 192 downregulated), indicating a substantial shift in the transcriptome profile with increasing age and changing diet across weaning.

Hierarchical clustering and PCA based on the normalised count data was used to broadly compare the rumen transcriptome profile at different time-points during early life (Fig. 3.2). The new-born calves clustered closely together, regardless of delivery method. The transcriptome profiles on days 7, 14, and 28 were generally similar, but sub-clustering corresponding to calf age was also evident, indicating some differences in the transcriptome profile in this period. The 96-day old calves form their own distinct cluster, though two animals from day 28 clustered closely to the day 96 animals in both the PCA and dendrogram.

GO term enrichment analysis, IPA canonical pathway analysis, and IPA molecular and cellular function analysis was used to investigate the biological roles of the DEG. The top 20 GO terms, and IPA-predicted canonical pathways, and molecular functions are presented in Tables 3.2, 3.3, and 3.4 respectively. Histomorphogical analysis of rumen tissue cross sections collected throughout early life was carried out in a sister study (Lyons et al, unpublished data). The following sections will detail the temporal changes in the rumen transcriptome, and their functional implications for rumen development in the young calf.

3.3.1 Mechanisms underpinning physical development of the rumen

Rumen maturation entails both physical enlargement and papillary development. Allocation of calf starter early in life is recommended to promote early rumen development (Meale et al., 2017), via stimulation of microbial VFA synthesis (Laarman et al., 2012, Sun et al., 2018), and previous studies have shown substantial improvements in rumen development following solid feed consumption (Naeem et al., 2014, Lesmeister and Heinrichs, 2004). Initial histomorphological evaluation of rumen wall samples collected from calves throughout early life showed clear development of the rumen following calf starter consumption on day 7, with papillary growth evident within the first week of life (Lyons et al., data not shown).

Cellular proliferation and apoptosis are two important biological mechanisms governing the cell life cycle and thus critical for rumen development. However, most studies to date examining the cell cycle dynamics in the rumen have focussed on its response to a nutritional insult (e.g. high grain feeding) in mature animals (Penner et al., 2011), and there is only limited information available in calves (Naeem et al., 2014, Naeem et al., 2012). Therefore, focus was directed to genes and pathways related to tissue development, to understand the molecular mechanisms underlying these improvements. Indicative of physical growth of the rumen, the GO terms “Tissue Development” and “Epithelium development” were enriched on day 28 of life compared to new-borns ($P<0.05$, Table 3.2). Moreover, the top IPA molecular and physiological functions predicted to be enriched by IPA throughout early life included “Cellular Development”, “Cell Cycle”, “Tissue Morphology” and “Cellular Assembly and Organisation” ($P<0.05$, Table 3.4). The “Cyclins and Cell Cycle Regulation” canonical pathway was also enriched in IPA ($P<0.05$) on days 28 and 96, compared to new-borns.

Cyclins and cyclin-dependant kinases are key molecules involved in cell proliferation (Norbury and Nurse, 1992), and in goat kids, early life growth of the rumen epithelium was

associated with accelerated cell cycle processes, regulated by these molecules (Liu et al., 2013). Five DEG enriched on day 28 (vs. new-borns) were involved in the "Cyclins and Cell Cycle Regulation" pathway in the present study: Cyclin A2 (*CCNA2*), *CCNB1, CCNB2,* Cyclin-dependant kinase 1 (*CDK1*), and *E2F8* (a transcription factor) ($P<0.05$). *CDK* proteins only possess kinase activity when they are complexed with a cyclin, and different CKD-Cyclin complexes have specific roles at various points in the cell cycle. The CDK1-Cylin B complex triggers mitosis (M phase of the cell cycle) in mammalian cells (Lindqvist et al., 2009), and is therefore critical for correct cell division. Cyclin-A (*CCNA*) forms a complex with *CDK2* to initiate DNA replication in the S phase of the cell cycle, but can also complex with *CDK1* to reinitiate the M phase (Plopper et al., 2013). Therefore, the increased expression of *CCNB1, CCNB2*, *CCNA2*, and *CDK1* genes in the present study from day 7 onward, compared to new-borns (Fig. 3.4), indicates elevated mitotic activity underpins the morphological development of the rumen during early life in beef calves.

In lambs, the upregulation of cyclin A (*CCNA*), cyclin D (*CCND1*), and *CDK2* genes was observed in animals fed starter and milk replacer compared to those raised exclusively on milk replacer, and the expression of these genes was positively correlated to rumen papillae development (Sun et al., 2018). Moreover, enhanced expression of cyclin and CDK genes in the rumen following grain consumption has also been reported in goat kids (Gui and Shen, 2016, Sun et al., 2018). In the present study, calves had access to calf starter in increasing quantities from day 7 onward, but the expression of cyclin and *CDK* genes only changed significantly in the week after birth and remained stable between days 7-96 (Fig. 3.4). This indicates that in beef calves, the activity of cyclin and CDK genes was not influenced by either starter allocation or the amount of starter offered. Histomorphological analysis showed significant papillary development as early as day 7 (Lyons et al., data not shown), and as discussed in Chapter 2, there was evidence that calves had been consuming straw bedding prior to day 7. Therefore, the consumption of even small amounts of forage during the first days of life might be sufficient to kickstart rumen fermentation, VFA production, and subsequent epithelial development.

Rumen papillae development increases the surface area for VFA and nutrient absorption and is key for animal growth. Papillae elongation, driven by butyrate metabolism in the rumen epithelium, is also thought to be mediated by activation of mitosis as described above, and an inhibition of cell death via apoptosis (Mentschel et al., 2001). Cellular apoptosis is coordinated by cysteine-aspartase-specific proteases (caspases), divided into initiators and executioners (Plopper et al., 2013). Decreased expression of Caspase 3 and 8 (*CASP3, CASP8*) genes was associated with

accelerated cell cycle activity and enhanced rumen morphological development in starter fed lambs (Sun et al., 2018), suggesting that apoptosis may be supressed to allow for rapid rumen tissue development during early life. Based on these findings, the expression of caspase genes in the rumen tissue during early life were investigated. Seven caspase genes were detected in the present study, but their expression did not change significantly until day 96, when the expression of Caspase 4 (*CASP4*) decreased compared to the new-borns ($P<0.05$, Table 3.1).

Sun and colleagues (2018) reported concordant acceleration of the cell cycle with depression in apoptosis-associated gene expression as discussed above, concluding that starter feeding enhanced cell proliferation while reducing apoptosis in the rumen. However, while elevated expression of cell cycle genes in the first weeks of life were observed in the present study, it appears that apoptosis was not inhibited until at least day 96. Previous work has demonstrated that butyrate enhances mitosis in rumen tissue *in vivo*, while inhibiting apoptosis (Mentschel et al., 2001). In this study, calves had access to 1kg of calf starter daily from day 28-56 and had *ad libitum* access post weaning. It may be that a certain threshold of starter consumption (and thus butyrate production) is required to suppress the apoptotic process. Thus, we speculate that while apoptosis does not appear to be inhibited in the first month of life, elevated starter feeding after day 28 may contribute to its downregulation, contributing to further ruminal development. This is supported by morphological data from the same animals, which showed papillae development was accelerated after day 28 (Lyons et al., data not shown).

Taken together, these data indicate that by day 28 of life, genes and pathways critical for cellular proliferation were established in the rumen tissue. The cell cycle genes showed trends of increase as early as day 7 and their expression remained stable throughout the rest of the experimental period (Fig. 3.4), so we cannot conclude if this activation is due to dietary grain consumption or age. In constrast to previous findings, this did not appear to be accompanied by a suppression of apoptosis, as caspase gene expression did not change until post-weaning. High levels (≥1kg/day) of concentrate feeding might be required to propagate rumen development in beef calves, and this should be noted in further investigations.

3.3.2 Transcriptional dynamics of genes and pathways underpinning VFA absorption in the rumen

VFA produced by microbial carbohydrate fermentation are the principle energy source for ruminants (Bergman, 1990), with butyrate the preferred energy source of epithelial cells (Bedford

and Gong, 2017). Moreover, VFA absorption across the rumen wall contributes to rumen stability via pH regulation (Penner, 2014). Apical uptake of VFA may occur via passive diffusion of un-dissociated VFA, or active transport of dissociated VFA mediated by host transporter genes (den Besten et al., 2013). In the rumen, the majority of VFA are in the dissociated state (Penner et al., 2011) but the mechanisms underpinning their active transport across the apical membrane and subsequent transfer to the portal circulatory system remain poorly understood. To this end, the activity of genes and pathways involved in VFA metabolism were investigated in the present study, and were found to increase following allocation of calf starter on D7. GO term analysis of the 25 DEG identified between days 7 and 14 showed enrichment of 9 functional terms, including "Lipid Metabolic Process", "Monocarboxylic Acid Metabolic Process", and "Monocarboxylic Acid Binding", "Cellular Response To Oxygen Containing Compound", "Inorganic Ion Transmembrane Transport", "Response to Organic Cyclic Compound", and "Passive Transmembrane Transporter Activity" ($P<0.05$, Table 3.2). Moreover, the top molecular functions assigned to the DEG in IPA following calf starter allocation on day 7 included "Lipid Metabolism", "Molecular Transport", and "Small Molecule Biochemistry" ($P<0.05$, Table 3.3).

Several candidate genes belonging to the SLC (Solute Carrier) family have been proposed as mediators of VFA transport in the rumen (Stumpff, 2018) with monocarboxylic transporters (MCT – SLC16 family) thought to play a primary role (Connor et al., 2010). Among the 14 MCT genes known to be expressed in the bovine GIT (Kirat et al., 2013), MCT1 and MCT4 are believed to be involved in ruminal VFA transport (Connor et al., 2010). Higher transcriptional abundance of *MCT1* (*SLC16A1*) was observed on day 28, and again on day 96 compared to the new-borns ($P<0.05$, Fig. 3.5), indicative of elevated rates of VFA transport. This is consistent with the findings of Laarman and colleagues in dairy calves (2012) but disagrees with those of Sun et al, (2018) who reported decreased *MCT1* expression with increased calf starter consumption in lambs. This inconsistency may point to divergent mechanisms for VFA absorption between bovine and ovine animals, which requires verification. Previous studies have speculated that *MCT4*, located on the luminal side of the rumen epithelium, is key to ruminal VFA absorption (Connor et al., 2010, Kirat et al., 2007). However, the expression of *MCT4* did not change throughout early life in the present study ($P>0.05$), suggesting that it may not be a major player in VFA absorption during early life, and that other transporter genes may facilitate transfer of VFA from the lumen to the basolateral membrane. To this end, we observed increased expression of another transporter-encoding gene, *DRA* (SLC26A3), on D14 vs D7, and again on D28 vs D7 ($P<0.05$, Fig. 3.5), corresponding to allocation of increasing amounts of calf starter as well as the expression

pattern of *MCT1*. Like *MCT4,* this gene is expressed on the apical (luminal) epithelium and has been previously proposed as a candidate protein mediating rumen VFA transport across the apical membrane (Penner et al., 2011, Stumpff, 2018, Schlau et al., 2012), but this has not yet been confirmed. This gene was also involved in several of the GO terms related to metabolism and transport listed above which were significantly enriched during early life ($P<0.05$, Table 3.2), indicating its metabolic importance in the developing rumen. It is plausible that *DRA* may have a prominent role in the initial take-up of VFA across the apical epithelium, and this warrants further study.

Following absorption, acetate and propionate are mostly transferred to the portal circulation system in their native form, but up to 90% of ruminal butyrate is metabolised to ketone bodies in the rumen wall (mainly β-hydroxybutyrate (BHBA)), where it is used as an energy source for ruminal epithelial cells (Penner et al., 2011). The rumen is the principle source of ketone body synthesis in the animal, providing a significant amount of energy substrate to the peripheral tissues (Penner et al., 2011, Aschenbach et al., 2011). Corresponding to increased allocation of calf starter (calves received 700g/d in the week up to day 28 of life, and had *ad libitum* access post weaning), the "Ketogenesis" pathway was enriched at days 28 and 96, compared to the new-born calves ($P<0.05$). The expression of key genes involved in this pathway (*BDH1, ACAT1, HMGCL,* and *HMGCS2*) increased numerically following calf starter allocation on day 7, and their expression levels were all higher on day 28 ($P<0.05$, Fig. 3.6) compared to new-borns. The respective roles of these genes in BHBA production in the rumen epithelium have been extensively summarised elsewhere (Steele et al., 2011a). It is also suggested that ruminal VFA are alternatively metabolised in the rumen for cholesterol synthesis (Steele et al., 2011b), but this has not been widely investigated in calves. The "Superpathway of Cholesterol Synthesis" was also enriched on days 28 and 96 compared to new-borns ($P<0.05$), indicating that VFA metabolism may proceed via cholesterol synthesis during early life. A recent study in lambs showed upregulation of genes involved in ketogenesis concomitant with downregulation of those involved in cholesterol synthesis in the rumen epithelium following starter consumption (Sun et al., 2018). Previous studies (Steele et al., 2011b, Sun et al., 2018) have reported the *HMGCS1* gene as a key regulator of VFA metabolism to cholesterol in the rumen epithelium, but we did not observe differential expression of this gene in our dataset ($P>0.05$). However, several other genes associated with this pathway in IPA (*DHCR7*, *NSDHL*, *IDI1*) were among the genes upregulated following calf starter allocation in the present study ($P<0.05$). These data indicate that by day 28, the rumen wall expresses the genes and pathways necessary to obtain energy for growth and development, and

that this may occur via both ketogenic and cholesterol synthesis pathways. The further enrichment of genes involved in the "Ketogenesis" pathway on day 96 ($P<0.05$, Table 3.3, Fig. 3.6), suggests that the rate of ketogenesis increases further across weaning. However, due to the limitations of our experiment, we cannot conclude if this is due to the dietary change after weaning, increased calf age, or, as is more likely, a combination of both. Nonetheless, these data provide valuable fundamental knowledge concerning molecular control VFA metabolism during early life in beef calves and will be valuable in development of optimum calf management strategies.

3.3.3 Genes and pathways involved in the host immune response are enriched in the rumen during early life

Microbial stability within an ecosystem is important in maintaining microbial function under changing conditions (Loreau et al., 2001), and perturbation of host-microbial homeostasis may lead to impaired microbial function, thereby affecting host health. The rumen lacks the organised lymphoid tissues (MALT and GALT) present in the lower gut (Yanez-Ruiz et al., 2015) and has been somewhat neglected in studies of the GIT immune system in cattle to date, with its major protective function presumed to be as a physical barrier. However, as recently reviewed (Yanez-Ruiz et al., 2015), immune homeostasis in the rumen is mediated through a number of mechanisms beyond barrier function, including secretory immunoglobulin (IgA, IgG) supply from saliva (Fouhse et al., 2017), and the activity of various pattern-recognition receptors (e.g. toll-like receptors (TLRs), and other antimicrobial peptides (e.g. defensins) (Malmuthuge et al., 2013, Malmuthuge and Guan, 2017). These mechanisms remain poorly understood in the rumen during the early life period, when they may be amenable to manipulation for microbial programming. Though our initial objective was to examine molecular control of rumen development and nutrient metabolism during early life, investigation of the DEG and associated functional annotations showed substantial enrichment of host immune function following birth. Therefore, we expanded our analysis to include genes, functions, and pathways involved in the enriched host immune response from birth.

Feeding elevated quantities of grain during early life is recommended to improve rumen development and calf growth (Drackley, 2008). However, there is evidence that such dietary programs severely disrupt the integrity of the rumen epithelial barrier (Liu et al., 2013). Tight junction proteins (TJP) including claudins and occludin control rumen permeability (Penner et

al., 2011), and previous work has reported a decline in their expression following starter consumption in calves (Malmuthuge et al., 2013) and goats (Jiao et al., 2017), indicating that higher rumen permeability might be necessary to facilitate greater nutrient absorption under grain feeding in early life as the papillae continue to develop. Three genes, encoding Claudins 4, 11, and 17, displayed temporal changes in expression in expression in the present study. The expression of Claudin 4 (*CLD4*) was higher at all timepoints compared to new-born animals ($P<0.05$, Fig. 3.7). Claudin 17 (*CLD17*) expression was upregulated on day 7 ($P<0.05$, Fig. 3.7), but remained stable thereafter ($P<0.05$, Fig. 3.7). Finally, Claudin 11 (*CLD11*) expression was stable throughout early life until day 96 when it was downregulated compared to the new-born calves ($P<0.05$, Fig. 3.7), but its expression was substantially lower at all timepoints than the other claudin genes, suggesting a minor role in the rumen. The bovine occludin (OCLD) gene did not change significantly in expression at any timepoint in the present study ($P>0.05$, Fig. 3.7). There were no significant changes in expression levels of any TJP genes following starter allocation on day 7, indicating that neither starter feed consumption nor the amount of feed offered had a significant impact on rumen integrity. These findings disagree with published data which showed decreased expression of claudins and occludin, and thus increased rumen permeability, following starter consumption during early life (Jiao et al., 2017, Malmuthuge et al., 2013). Offering goats a high-grain diet caused a massive disruption of rumen epithelial tight junctions (Liu et al., 2013). In adult cattle, impaired rumen barrier function is usually associated with a nutritional insult like acidosis (Aschenbach et al., 2010), and episodes of temporary hyperosmolarity during periods of rapid ruminal fermentation (Penner et al., 2010). That there was no effect of early life grain feeding on the expression of TJP in the rumen epithelium in the present study suggests that steady increases in the amount of feed offered in the first weeks of life might have allowed the rumen to adapt to solid feed digestion, and this might have been aided by straw consumption even before D7. However, more studies in young calves are required to verify this.

Beyond barrier function, there is increasing evidence that epithelial immune cells play a key role in recognising the early gut microbiota of calves (Malmuthuge et al., 2012). However, there is limited knowledge of such mechanisms in the rumen. The substantial shift in the rumen transcriptome profile between birth (naturally delivered calves) and day 7 (Fig. 3.2) included many genes encoding hallmarks of the host immune response, such as cytokines (inc. *IL36α, IL36β, IL1β*) and antimicrobial peptides (inc. *LAP, TAP*) (Table 3.1). Functional annotation of the DEG using GO Biological Process term analysis showed significant enrichment of immune-related processes including "Response to Type 1 Interferon", "Cytokine Mediated Signalling Pathway",

“Response to Cytokine”, “Innate Immune Response”, “Interleukin 1 Receptor Binding”, and “Cytokine Activity” (*P*<0.05, Table 3.2). We also observed enrichment of GO terms related to contact with foreign organisms; “Defence Response to Bacterium”, “Response to Virus”, and “Defence Response to Other Organism” (*P*<0.05, Table 3.2), suggesting that contact with microbial life in the rumen may prime early host immune function. Moreover, IPA classified the genes which were differentially expressed during early life compared to new-borns into functions like “Cell-mediated Immune Trafficking” and “Immune Cell Trafficking” (*P*<0.05, Table 3.4). Eighteen 18 IPA canonical pathways were found to be consistently enriched among the DEG when new-borns were compared to 7, 14, and 28-day old calves (*P*<0.05), including pathways activated by microbial stimuli (e.g. “Dendritic Cell Maturation”, “NF-kB Signalling”, “Toll-like Receptor Signalling”, Table 3.3), indicating sustained enrichment of host-microbial related functions during early life in the rumen.

As presented in Chapter 2 of this thesis, it proved impossible to generate microbial amplicons from contents of the new-born rumen, but found that by D7 the rumen contained a microbiota of comparable diversity to that of older milk-fed calves (days 14-28). Early life microbial exposure is thought to be key in establishing a tolerogenic environment between host and microbe in the lower gut regions (Sommer and Backhed, 2013), and our data may indicate similar mechanisms exist in the developing rumen. The ability to distinguish between pathogenic and commensal microbiota is a key feature in maintaining intestinal homeostasis, and this is performed in the gut by Toll-like Receptors (TLRs) (Rakoff-Nahoum et al., 2004). TLRs are pattern recognition receptors that activate a pro-inflammatory signalling pathway following exposure to microbial ligands, and are thought to be responsible for early monitoring of the rumen microbiota during colonisation prior to weaning (Malmuthuge et al., 2012). IPA predicted significant enrichment of the “Toll-like Receptor Signalling” pathway throughout preweaning compared to new-born calves (*P*<0.05). On day 7, the “Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses” pathway was also predicted to be activated (*P*<0.05, Table 3.3). Previous studies have reported that ruminal TLR expression generally declines with increasing age, and the expression of TLRs in the rumen is lower than in other gut regions (Jiao et al., 2017, Malmuthuge et al., 2012). We saw similar trends in our own data, whereby *TLR4* showed a trend (*P*<0.05, not evident following FDR-adjustment) toward increased expression between birth and day 7 (Fig. 3.8) but declined in expression in later life. The lack of statistical significance is likely due both to our stringent DE cut-offs and high variability in gene expression profiles among the individual calves at each timepoint. Nonetheless, activation of the TLR-

signalling cascade is supported by the observed upregulated expression of other genes involved in this pathway, including lipopolysaccharide binding protein (*LPB*), and several interleukins and their receptors (*IL1, IL36*) (*P*<0.05, Table 3.1). Most of the TLRs in our dataset (TLRs 2-7 were expressed) displayed a similar temporal expression profiles to *TLR4*, except for *TLR5*, which had greater expression on day 96 compared to new-borns (*P*<0.05, Fig. 3.8). This suggests that the mechanisms underpinning TLR-sensing of the rumen microbiota may evolve during calf development and, should be investigated further.

TLR5 is expressed on the cell surface and recognises bacterial flagella (Miao et al., 2007), and its elevated expression in postweaning in the current study corresponds to the increased richness and diversity of the luminal bacteriome on day 96 calves versus milk-fed calves discussed in Chapter 3. Prior studies that observed a temporal decrease in TLR expression in the rumen during early life (Jiao et al., 2017, Malmuthuge et al., 2012) did not examine *TLR5* expression. Activation of *TLR5* signalling was identified as a potentially important function of the commensal microbiota to maintain stability following a nutritional challenge in the adult goat rumen (Shen et al., 2017, Liu et al., 2015), but the enrichment of *TLR5* has not, to our knowledge, been associated with increasing age during early life in the rumen. Therefore, it is possible that upregulation of *TLR5* and its associated cytokines observed here indicates a role of *TLR5* signalling in maintaining rumen-microbial homeostasis in older animals, supported by the enrichment of "Toll-like Receptor Signalling" pathway on day 96 versus new-borns in IPA (*P*<0.05, Table 3.3). More studies are required to confirm this hypothesis.

TLR signalling is not the only PRR-associated pathway thought to be implicated in host-microbial homeostasis in the rumen; Malmuthuge and colleagues (2012) reported reduced expression of the *PGLYRP1* (encoding Peptidoglycan Recognition Protein 1) gene in the rumen prior to weaning compared to in older animals, but this gene was only expressed on day 14 in the present study. Expression of another member of the same family, *PLGYRP2* increased following birth in the present study (*P*<0.05) but was expressed at relatively low numbers (Fig. 3.8). *PGLYRP* mRNA expression was observed to be lower in the rumen compared to other GIT compartments (Malmuthuge et al., 2012), so these proteins may not be of great biological relevance in the rumen.

Defensins are a family of antimicrobial peptides that may also play a role in host-microbial sensing in the developing rumen (Malmuthuge et al., 2012, Yanez-Ruiz et al., 2015). These

proteins resist microbial invasion of mucosal surfaces by disrupting microbial cell membranes of a wide range of microbial groups (Ganz, 2003). Corresponding to a previous study (Malmuthuge et al., 2012), the *DEFB* (encoding a β-defensin) gene was poorly expressed prior to weaning in the current study but increased significantly by day 96 ($P<0.05$, Fig. 3.9). Two other defensin-encoding genes, *LAP* (lingual AMP) and *TAP* (tracheal AMP), were consistently among the most significantly enriched genes during early life compared to new-borns ($P<0.05$, Fig. 3.9). A previous study demonstrated that *LAP* is highly expressed in the stratum corneum of the rumen epithelium (Isobe et al., 2011), and so is in direct contact with the rumen microbiota. TAP expression is induced by proinflammatory stimuli, including interleukins and LPS (Mitchell et al., 2007). Expression of both AMPs is induced by *Mannheimia haemolytica* infection; however, this is a respiratory pathogen and was not detected in the microbial data presented in Chapter 2, indicating that it was not the cause of *LAP* and *TAP* upregulation. The expression level of both genes was similar between days 7-96, indicating their role may be critical throughout early life (Fig. 3.9). The elevated expression of these AMPs from birth corresponds to the microbial colonisation of the rumen in the first week following birth (Malmuthuge et al., 2013, Jami et al., 2013), and they may be important mediators of host tolerance to the rumen microbiota during early life. *DEFB* (β-defensin) only became significantly upregulated post weaning as previously noted (Malmuthuge et al., 2012), providing further evidence that the immune effector mechanisms regulating host response to the commensal microbiota evolve as the animal ages, as seen with the divergent TLR expression profiles discussed above, probably in response to changes in bacterial community composition. Finally, there is some evidence that stability of the ruminal microbiota may be mediated through activity of salivary immunoglobulins, including IgG and IgA (Williams et al., 2009, Fouhse et al., 2017). IgA receptors were not detected in the present study, but Fc receptors for both IgG and IgE were upregulated from day 7 onward ($P<0.05$, not shown), corresponding to microbial colonisation of the rumen following birth as discussed in Chapter 2. Further studies are warranted to identify the precise relationship between colonisation patterns and Ig activity in the rumen, as this may offer a mechanism to selectively inhibit undesirable microbial groups (e.g. methanogens).

The mature rumen epithelium is clearly delineated into four layers, as shown in Fig. 1.1, and gene expression patterns vary between each layer (Penner et al., 2011). However, as the rumen continues to develop during early life, there is no clear distinction between each layer (Graham et al., 2005). It is possible that the expression of key immune genes before full rumen development is important in protecting the host from luminal microbiota during early life, via similar

mechanisms that exist in the lower gut. Investigating spatial immune cell protein expression in the rumen tissue throughout early life might offer more information.

3.3.4 Upregulation of genes involved in stress response and collagen formation in the rumen of naturally delivered calves compared to calves delivered via elective caesarean section

There is evidence that CS delivery may affect gene expression in the neonatal jejunum and lung (Surlis et al., 2017), and have a longitudinal impact on rumen (Cunningham et al., 2018) and human gut (Korpela et al., 2018) microbial profiles during early life. However, despite the importance of rumen function for nutrient absorption, there is no data concerning any potential impact of CS delivery on gene expression profiles in the rumen. Our data showed that two genes, *FKBP5* and *P4HA3*, were differentially expressed in the rumen wall according to the mode of delivery, both of which were upregulated in naturally delivered calves compared to those born by elective caesarean section (ECS) ($P<0.05$, Table 3.1, Fig. 3.3). *P4HA3* encodes a component of prolyl 4-hydroxylase, an enzyme which is critical to collagen formation (Myllyharju, 2003), and so this upregulation in naturally delivered animals may suggest a potentially negative effect of ECS on rumen wall structure in neonates, but this should be investigated further.

FKBP5 encodes FK506-binding protein 51 (Matosin et al., 2018), which is vital to the glucocorticoid receptor (GR) complex and contributes to restored hypothalamic–pituitary–adrenal axis homeostasis following exposure to stress (Grad and Picard, 2007, Matosin et al., 2018). Delivery, regardless of the method, is a stressful event for the calf, but the longer duration of trans-vaginal delivery compared to more abrupt event of ECS may cause elevated stress levels in the naturally delivered offspring as previously shown (Cho and Norman, 2013). In a previous publication using the current animal model, the *NR3C1* gene, which encodes the glucocorticoid receptor Nuclear Receptor Subfamily 3 Group C Member 1, tended ($P=0.1$) to be downregulated in the jejunum of calves delivered via ECS (Surlis et al., 2017). High levels of neonatal corticosteroids are important for adequate colostrum absorption, thus ECS may have a negative impact on passive Ig transfer in the gut during early life (Sangild, 2003).

The "Glucocorticoid Receptor Signalling" canonical pathway was enriched in the NB.CS calves (Table 3). *FKBP5* is activated by glucocorticoids and the upregulation of *FKBP5* in this study suggests elevated GR signalling, which in turn is an indication of elevated stress experienced by NAT calves. Overall, only 2/13,154 expressed genes (0.015%) were differentially expressed

among ECS and NAT new-borns (Table 1) indicating a limited transcriptional response to mode of delivery in the rumen wall. However, a limitation of the current study is that samples were only collected from ECS calves immediately following birth, and there may be a longitudinal impact of ECS delivery on the transcriptome profile of the rumen wall, which is evident later in life. This has recently been demonstrated for calf haematological profiles (Probo et al., 2012), and rumen microbial communities (Cunningham et al., 2018) and so should be considered in any future studies.

3.4 Conclusions and summary

Physical maturation of the rumen is critical to facilitate a smooth weaning transition in calves and confers the capability to digest the high-forage diet of adult ruminants. To our knowledge, this study is among the first comprehensive investigations of early life transcriptome dynamics in beef calves. Cellular development processes and VFA transport and metabolism mechanisms are dynamic during early life and appear to be influenced by diet and age. We observed a substantial enrichment in immune related genes, processes and pathways in rumen tissue during early life. These changes occurred independently of any decrease in TJP expression, indicating that this elevated immune activity did not compromise rumen permeability. In particular, we have highlighted several mechanisms that may underpin the tolerogenic relationship between the host and the rumen microbiota during early life. Understanding the dynamics of host immune function during rumen colonisation is important in terms of early life manipulation. If a specific microbial group is prevented from establishing early in life, exposure during adulthood may elicit a detrimental immune response from the host (Yanez-Ruiz et al., 2015), and this must be considered in targeting the colonisation of undesirable microbial taxa.

The nature of the rumen wall sample used in this study may be a potential source of bias. A cross section of the entire rumen wall, which includes the rumen epithelium, papillae (where present) and the underlying muscular and vascular tissues was collected. This was necessary as it was impossible to effectively separate the epithelium from the underlying tissue in the younger calves (new-borns, day 7), and thus in the interests of consistency all samples were collected in the same manner. Future studies should devise an effective method to separate the epithelium from the underlying muscle, to ensure the accuracy of findings.

In summary, the data presented in this study provides valuable information concerning the potential interrelationship between host and microbe in rumen development, host mechanistic

control of rumen development, and the potential impact of CS delivery on rumen wall gene expression in beef calves.

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3.6 Tables and figures

Table 3.1: Details of selected DEG within each statistical contrast performed in EdgeR. The top 20 DEG within each contrast (ranked by abs. fold change) are presented.

	NB.NAT vs. NB.CS		
Ensemble Gene ID	Bovine Gene Symbol	logFC	FDR
ENSBTAG00000047502	*FKBP5*	2.53	3.64E-06
ENSBTAG00000006579	*P4HA3*	3.17	6.47E-06
	D0[1] vs. D7		
Ensemble Gene ID	Bovine Gene Name	logFC	FDR
ENSBTAG00000048171	*TAP*	10.97	1.98E-08
ENSBTAG00000027225	*LAP*	10.09	5.60E-10
ENSBTAG00000002087	*IL36A*	8.53	4.84E-10
ENSBTAG00000038033	*KRT6B*	8.30	2.49E-09
ENSBTAG00000039028	*PI3*	7.76	2.19E-08
ENSBTAG00000001785	*TGM3*	7.49	1.27E-12
ENSBTAG00000039425	*KRT6A*	7.23	7.47E-14
ENSBTAG00000002088	*IL36B*	6.70	4.69E-08
ENSBTAG00000010433	*M-SAA3.2*	6.58	5.72E-05
ENSBTAG00000004272	*ISG12(B)*	6.53	7.47E-07
ENSBTAG00000024255	*UOX*	6.48	1.16E-08
ENSBTAG00000017718	*CCL22*	6.22	1.33E-10
ENSBTAG00000014707	*ISG15*	6.13	1.63E-04
ENSBTAG00000011941	*LYZ1*	6.11	4.72E-07
ENSBTAG00000012538	*KLK14*	5.71	3.53E-07
ENSBTAG00000031750	*PLAC8*	5.58	3.71E-08
ENSBTAG00000002288	*NT5DC4*	5.57	1.65E-07
ENSBTAG00000007554	*IFI6*	5.36	1.04E-05
ENSBTAG00000009382	*KLK13*	5.32	4.46E-06
ENSBTAG00000046158	*CFB*	5.28	1.27E-08
	D0 vs. D14		
Ensemble Gene ID	Bovine Gene Name	logFC	FDR
ENSBTAG00000048171	*TAP*	11.62	3.01E-09
ENSBTAG00000038033	*KRT6B*	10.92	4.12E-12
ENSBTAG00000027225	*LAP*	10.52	1.13E-10
ENSBTAG00000002087	*IL36A*	9.25	3.19E-11
ENSBTAG00000039028	*PI3*	8.70	1.13E-09
ENSBTAG00000039425	*KRT6A*	8.69	2.01E-16
ENSBTAG00000003898	*HMGCS2*	8.50	2.53E-10

ENSBTAG00000004272	*ISG12(B)*	8.21	1.14E-08
ENSBTAG00000002288	*NT5DC4*	8.03	4.20E-12
ENSBTAG00000001785	*TGM3*	8.01	4.12E-14
ENSBTAG00000006806	*KRT17*	7.76	3.99E-12
ENSBTAG00000021118	*CYP26A1*	7.24	7.02E-16
ENSBTAG00000011941	*LYZ1*	7.20	2.54E-08
ENSBTAG00000015547	*SLC26A3*	7.09	1.46E-10
ENSBTAG00000031376	*BSP30C*	6.99	9.02E-10
ENSBTAG00000024255	*UOX*	6.83	9.20E-10
ENSBTAG00000017531	*FETUB*	6.70	6.64E-08
ENSBTAG00000016239	*DUOXA2*	6.66	1.65E-10
ENSBTAG00000014707	*ISG15*	6.65	2.31E-05
ENSBTAG00000017718	*CCL22*	6.39	1.62E-11
	D0 vs. D28		
Ensemble Gene ID	Bovine Gene Name	logFC	FDR
ENSBTAG00000038033	*KRT6B*	11.78	8.49E-13
ENSBTAG00000048171	*TAP*	11.61	1.64E-09
ENSBTAG00000002087	*IL36A*	9.78	1.03E-11
ENSBTAG00000027225	*LAP*	9.74	2.28E-10
ENSBTAG00000039425	*KRT6A*	9.62	2.53E-17
ENSBTAG00000003898	*HMGCS2*	9.44	2.84E-11
ENSBTAG00000039028	*PI3*	8.87	7.52E-10
ENSBTAG00000001785	*TGM3*	8.31	2.09E-14
ENSBTAG00000004272	*ISG12(B)*	8.23	8.78E-09
ENSBTAG00000021118	*CYP26A1*	8.06	5.72E-17
ENSBTAG00000006806	*KRT17*	7.88	2.06E-12
ENSBTAG00000016239	*DUOXA2*	7.71	8.10E-12
ENSBTAG00000002288	*NT5DC4*	7.55	1.59E-11
ENSBTAG00000020597	*FMO3*	-7.55	6.48E-15
ENSBTAG00000015252	*CHRNA9*	7.13	1.24E-12
ENSBTAG00000016234	*DUOX2*	7.11	4.29E-13
ENSBTAG00000045786	*KLRC1*	-6.90	4.04E-11
ENSBTAG00000031376	*BSP30C*	6.79	1.30E-09
ENSBTAG00000011941	*LYZ1*	6.78	6.32E-08
ENSBTAG00000011976	*CYP4B1*	-6.78	6.00E-11
	D0 vs. D96		
Ensemble Gene ID	Bovine Gene Name	logFC	FDR
ENSBTAG00000012034	KRT4	-12.65	3.61E-18
ENSBTAG00000038033	KRT6B	11.95	1.86E-13
ENSBTAG00000003898	HMGCS2	10.63	1.09E-12

ENSBTAG00000013928	WFDC2	-10.54	6.82E-10
ENSBTAG00000015547	SLC26A3	10.04	5.03E-14
ENSBTAG00000002087	IL36A	9.93	2.07E-12
ENSBTAG00000039425	KRT6A	9.75	3.83E-18
ENSBTAG00000027225	LAP	9.59	9.90E-11
ENSBTAG00000048171	TAP	9.50	1.97E-08
ENSBTAG00000020597	FMO3	-9.39	3.92E-17
ENSBTAG00000045786	KLRC1	-9.01	4.23E-13
ENSBTAG00000021118	CYP26A1	8.86	2.43E-18
ENSBTAG00000004272	ISG12(B)	8.80	9.62E-10
ENSBTAG00000005330	KRTDAP	-8.47	1.35E-15
ENSBTAG00000002974	FMO2	-8.45	4.44E-19
ENSBTAG00000011976	CYP4B1	-8.39	5.16E-13
ENSBTAG00000002288	NT5DC4	8.29	3.42E-13
ENSBTAG00000021408	FMO1	-8.23	5.65E-14
ENSBTAG00000016234	DUOX2	8.11	8.68E-15
ENSBTAG00000006806	KRT17	7.91	5.05E-13
D7 vs. D14			
Ensemble Gene ID	Bovine Gene Name	logFC	FDR
ENSBTAG00000017531	*FETUB*	4.15	5.10E-04
ENSBTAG00000015547	*SLC26A3*	4.14	4.41E-05
ENSBTAG00000046587	*Uncharacterised protein coding gene*	3.82	5.10E-04
ENSBTAG00000040393	*AKR1C1*	3.26	3.65E-04
ENSBTAG00000021118	*CYP26A1*	3.19	1.10E-06
ENSBTAG00000040019	*KRT6C*	2.79	2.02E-04
ENSBTAG00000039991	*UGT2B10*	2.59	2.09E-04
ENSBTAG00000017794	*CCDC153*	2.50	2.78E-04
ENSBTAG00000012507	*PDZD3*	2.03	6.09E-05
ENSBTAG00000003300	*MFGE8*	2.00	3.60E-04
ENSBTAG00000032821	*SCEL*	-2.18	6.85E-05
ENSBTAG00000032424	*FSHR*	-2.42	1.21E-04
ENSBTAG00000010163	*SCNN1G*	-2.56	1.74E-05
ENSBTAG00000019125	*SLC1A1*	-2.65	3.12E-05
ENSBTAG00000014296	*NCCRP1*	-2.68	8.79E-06
ENSBTAG00000021408	*FMO1*	-3.29	1.15E-05
ENSBTAG00000011976	*CYP4B1*	-3.51	2.71E-05
ENSBTAG00000016305	*ATP13A4*	-3.63	1.57E-06
ENSBTAG00000020597	*FMO3*	-3.92	9.46E-08
ENSBTAG00000037800	*APOBEC3Z1*	-5.47	1.83E-04

D14 vs. D28			
Ensemble Gene ID	Bovine Gene Name	logFC	FDR
ENSBTAG00000012034	*KRT4*	-3.68	2.40E-04
ENSBTAG00000005330	*KRTDAP*	-3.29	2.50E-04
D28 vs. D96			
Ensemble Gene ID	Bovine Gene Name	logFC	FDR
ENSBTAG00000012034	*KRT4*	-8.38	5.99E-13
ENSBTAG00000008238	*S100A7*	-6.34	1.77E-06
ENSBTAG00000021306	*CHRDL2*	-4.98	1.92E-06
ENSBTAG00000000828	*CAPN6*	-4.98	8.25E-05
ENSBTAG00000013155	*COL2A1*	-4.61	1.12E-05
ENSBTAG00000019977	*PCDH10*	-4.57	1.93E-04
ENSBTAG00000014340	*KERA*	-4.53	6.38E-05
ENSBTAG00000037899	*DLK1*	-4.46	1.99E-05
ENSBTAG00000020979	*NGFR*	-4.45	1.66E-07
ENSBTAG00000016801	*RXRG*	-4.38	1.13E-05
ENSBTAG00000002974	*FMO2*	-4.34	7.64E-09
ENSBTAG00000005330	*KRTDAP*	-4.31	1.17E-07
ENSBTAG00000044010	*EMB*	-4.25	6.85E-08
ENSBTAG00000000703	*ST6GAL2*	-4.13	6.38E-05
ENSBTAG00000006977	*PLP1*	-4.02	3.06E-05
ENSBTAG00000006451	*GAP43*	-3.87	3.30E-04
ENSBTAG00000017627	*STMN4*	-3.78	4.43E-04
ENSBTAG00000015581	*COL9A3*	-3.76	3.39E-05
ENSBTAG00000004503	*NPY*	-3.73	4.16E-04
ENSBTAG00000012909	*CRABP1*	-3.69	7.12E-05
D7 vs. D28			
Ensemble Gene ID	Bovine Gene Name	logFC	FDR
ENSBTAG00000016305	*ATP13A4*	-6.95	5.70E-11
ENSBTAG00000011976	*CYP4B1*	-6.65	1.47E-09
ENSBTAG00000020597	*FMO3*	-5.90	5.70E-11
ENSBTAG00000045786	*KLRC1*	-5.47	1.31E-07
ENSBTAG00000037800	*APOBEC3Z1*	-5.30	2.01E-04
ENSBTAG00000012034	*KRT4*	-5.04	3.35E-08
ENSBTAG00000019540	*CRNN*	-4.85	5.66E-05
ENSBTAG00000014296	*NCCRP1*	-4.63	2.96E-10
ENSBTAG00000003898	*HMGCS2*	4.50	2.41E-05
ENSBTAG00000021408	*FMO1*	-4.34	1.77E-07
ENSBTAG00000010163	*SCNN1G*	-4.06	1.96E-09
ENSBTAG00000021118	*CYP26A1*	4.01	1.47E-09

ENSBTAG00000005330	*KRTDAP*	-3.99	3.34E-07
ENSBTAG00000001021	*CYP1A1*	3.89	9.85E-05
ENSBTAG00000001595	*MT1E*	3.88	1.07E-04
ENSBTAG00000002029	*IGSF5*	-3.87	1.39E-07
ENSBTAG00000032424	*FSHR*	-3.70	1.77E-07
ENSBTAG00000015547	*SLC26A3*	3.68	5.03E-05
ENSBTAG00000002974	*FMO2*	-3.51	1.47E-09
ENSBTAG00000019125	*SLC1A1*	-3.49	2.94E-07

Table 3.2: Top Gene Ontology (GO) terms enriched among the DEG in each contrast. Only terms with an absolute NES score > |1.5| and containing 5 DEG are presented.

[1]D0 vs. D7			
Gene Ontology Term	P-value	NES	Gene Count
GO: MICROTUBULE CYTOSKELETON	5.03E-03	-2.47	43
GO: RESPONSE TO TYPE I INTERFERON	1.09E-04	2.41	20
GO: DEFENSE RESPONSE TO BACTERIUM	1.06E-04	2.38	25
GO: DEFENSE RESPONSE TO OTHER ORGANISM	1.01E-04	2.32	56
GO: RESPONSE TO BIOTIC STIMULUS	1.00E-04	2.31	78
GO: CYTOKINE MEDIATED SIGNALLING PATHWAY	1.01E-04	2.24	52
GO: CELLULAR RESPONSE TO CYTOKINE STIMULUS	1.01E-04	2.20	59
GO: INNATE IMMUNE RESPONSE	1.00E-04	2.17	81
GO: RESPONSE TO VIRUS	1.04E-04	2.17	32
GO: NUCLEOSIDE TRIPHOSPHATE METABOLIC PROCESS	2.18E-03	-2.17	9
GO: RESPONSE TO CYTOKINE	1.01E-04	2.15	69
GO: CYTOKINE RECEPTOR BINDING	3.51E-04	2.12	12
GO: CYTOKINE ACTIVITY	4.48E-04	2.08	16
GO: PROTEIN LOCALIZATION TO NUCLEUS	2.64E-03	-2.07	5
GO: DEFENSE RESPONSE	1.00E-04	2.06	131
GO: CHROMOSOME ORGANIZATION	9.17E-03	-2.04	54
GO: REG. OF SYMBIOSIS ENCOMPASSING MUTUALISM THROUGH PARASITISM	7.89E-04	2.01	15
GO: INTERLEUKIN 1 RECEPTOR BINDING	2.56E-04	2.00	7
D0 vs. D14			
Gene Ontology Term	P-value	NES	Gene Count
GO: RESPONSE TO BIOTIC STIMULUS	1.09E-04	2.57	60
GO: DEFENSE RESPONSE TO OTHER ORGANISM	1.16E-04	2.50	37
GO: DEFENSE RESPONSE TO BACTERIUM	1.31E-04	2.47	17
GO: DEFENSE RESPONSE	2.10E-04	2.11	95
GO: RESPONSE TO EXTERNAL STIMULUS	2.10E-04	2.07	95
GO: SINGLE ORGANISM CATABOLIC PROCESS	2.22E-04	2.29	51
GO: MONOCARBOXYLIC ACID BINDING	2.92E-04	2.20	8
GO: CELLULAR COMPONENT MORPHOGENESIS	4.18E-04	-2.48	16
GO: FERTILIZATION	4.72E-04	2.01	5
GO: RESPONSE TO VIRUS	5.07E-04	2.22	21
GO: PROTEINACEOUS EXTRACELLULAR MATRIX	5.54E-04	-2.61	26
GO: NEURON MIGRATION	5.84E-04	-2.40	6
GO: RESPONSE TO BACTERIUM	5.87E-04	2.10	35
GO: EXTRACELLULAR MATRIX	6.09E-04	-2.41	30
GO: STEM CELL DIFFERENTIATION	6.35E-04	-2.46	8
GO: APPENDAGE DEVELOPMENT	8.21E-04	-2.18	5
GO: EXTRACELLULAR SPACE	8.49E-04	2.02	83

GO: RESPONSE TO ORGANOPHOSPHORUS	8.50E-04	2.09	10
GO: RESPONSE TO CAMP	8.91E-04	2.05	7
GO: MICROTUBULE CYTOSKELETON	1.26E-03	-2.15	32
D0 vs. D28			
Gene Ontology Term	P-value	NES	Gene Count
GO: RESPONSE TO BIOTIC STIMULUS	1.27E-04	2.73	43
GO: DEFENSE RESPONSE TO OTHER ORGANISM	1.33E-04	2.41	28
GO: RESPONSE TO BACTERIUM	1.33E-04	2.36	29
GO: GROWTH FACTOR RECEPTOR BINDING	1.56E-04	2.30	9
GO: DEFENSE RESPONSE	1.20E-04	2.28	65
GO: INTERLEUKIN 1 RECEPTOR BINDING	1.60E-04	2.21	7
GO: CELLULAR HORMONE METABOLIC PROCESS	1.59E-04	2.17	8
GO: CYTOKINE RECEPTOR BINDING	3.04E-04	2.40	11
GO: CYTOKINE ACTIVITY	2.92E-04	2.34	15
GO: RESPONSE TO ORGANOPHOSPHORUS	4.50E-04	2.21	13
GO: HORMONE METABOLIC PROCESS	4.50E-04	2.21	13
GO: CELLULAR RESPONSE TO INORGANIC SUBSTANCE	4.76E-04	2.04	8
GO: DEFENSE RESPONSE TO BACTERIUM	5.85E-04	2.24	15
GO: ISOPRENOID METABOLIC PROCESS	6.23E-04	2.16	9
GO: CYTOKINE MEDIATED SIGNALLING PATHWAY	6.81E-04	2.13	25
GO: REGULATION OF INTERLEUKIN 6 PRODUCTION	9.35E-04	2.08	9
GO: STEROID METABOLIC PROCESS	1.29E-03	2.13	17
GO: RESPONSE TO INORGANIC SUBSTANCE	1.17E-03	2.09	34
GO: TERPENOID METABOLIC PROCESS	1.28E-03	2.03	7
GO: MONOCARBOXYLIC ACID BINDING	1.25E-03	2.02	9
D0 vs. D96			
Gene Ontology Term	P-value	NES	Gene Count
GO: ORGANELLE FISSION	2.60E-04	3.45	59
GO: CHROMOSOME SEGREGATION	2.57E-04	3.40	43
GO: MITOTIC NUCLEAR DIVISION	2.55E-04	3.36	48
GO: CHROMOSOMAL REGION	2.49E-04	3.31	35
GO: SISTER CHROMATID SEGREGATION	2.47E-04	3.28	31
GO: NUCLEAR CHROMOSOME SEGREGATION	2.50E-04	3.25	37
GO: RESPONSE TO TOXIC SUBSTANCE	2.52E-04	3.17	39
GO: CELL CYCLE	2.70E-04	3.14	94
GO: SISTER CHROMATID COHESION	2.38E-04	3.11	20
GO: MITOTIC CELL CYCLE	2.66E-04	3.11	65
GO: CELL CYCLE PROCESS	2.68E-04	3.04	82
GO: CHROMOSOME CENTROMERIC REGION	2.48E-04	3.02	30
GO: CONDENSED CHROMOSOME CENTROMERIC REGION	2.41E-04	2.93	22
GO: KINETOCHORE	2.41E-04	2.93	22
GO: CONDENSED CHROMOSOME	2.49E-04	2.89	34

GO: DETOXIFICATION	2.36E-04	2.86	16
GO: CELL DIVISION	2.56E-04	2.86	55
GO: LIPID CATABOLIC PROCESS	2.59E-04	2.86	44
GO: CELLULAR CATABOLIC PROCESS	2.78E-04	2.84	112
GO: ELECTRON CARRIER ACTIVITY	2.37E-04	2.80	18

D7 vs. D14

Gene Ontology Term	P-value	NES	Gene Count
GO: LIPID METABOLIC PROCESS	9.37E-03	1.90	12
GO: MONOCARBOXYLIC ACID METABOLIC PROCESS	1.33E-02	1.80	9
GO: DRUG METABOLIC PROCESS	2.87E-02	-1.64	5
GO: MONOCARBOXYLIC ACID BINDING	3.29E-02	1.64	5
GO: MOLECULAR FUNCTION REGULATOR	3.61E-02	1.63	6
GO: HORMONE METABOLIC PROCESS	4.12E-02	1.61	5
GO: LIPID BINDING	4.18E-02	1.62	7
GO: SMALL MOLECULE CATABOLIC PROCESS	4.98E-02	1.58	7
GO: MITOCHONDRION	4.98E-02	1.58	7

D14 vs. D28

Gene Ontology Term	P-value	NES	Gene Count
No significantly enriched terms.	N/A	N/A	N/A

D28 vs. D96

Gene Ontology Term	P-value	NES	Gene Count
GO: CHROMOSOME ORGANIZATION	4.87E-03	2.07	7
GO: PROTEIN HOMODIMERIZATION ACTIVITY	9.18E-03	1.92	6
GO: REGULATION OF RESPONSE TO WOUNDING	2.08E-02	1.80	6
GO: DEVELOPMENTAL PROCESS INVOLVED IN REPRODUCTION	2.37E-02	1.77	10
GO: CHROMATIN ORGANIZATION	2.62E-02	1.74	5
GO: HYDROLASE ACTIVITY ACTING ON ESTER BONDS	2.68E-02	1.73	10
GO: CELLULAR MODIFIED AMINO ACID METABOLIC PROCESS	2.90E-02	1.72	5
GO: RESPONSE TO ABIOTIC STIMULUS	2.69E-02	1.70	17
GO: TUBE DEVELOPMENT	3.44E-02	1.68	13
GO: IMMUNE SYSTEM DEVELOPMENT	3.62E-02	1.67	8
GO: PROTEIN DIMERIZATION ACTIVITY	4.03E-02	1.62	10
GO: SIGNALLING RECEPTOR ACTIVITY	4.20E-02	-1.61	10
GO: NEURON DEVELOPMENT	4.21E-02	-1.63	17
GO: GLYCOPROTEIN METABOLIC PROCESS	3.02E-02	-1.63	5
GO: STRUCTURAL MOLECULE ACTIVITY	2.19E-02	-1.72	11
GO: CELL PROJECTION ORGANIZATION	1.50E-02	-1.80	14
GO: NEURON PROJECTION DEVELOPMENT	1.50E-02	-1.80	14
GO: RECEPTOR ACTIVITY	1.18E-02	-1.84	13

D7 vs. D28

Gene Ontology Term	P-value	NES	Gene Count
GO: ISOPRENOID METABOLIC PROCESS	1.23E-03	2.15	5
GO: CELLULAR RESPONSE TO ORGANIC CYCLIC COMPOUND	8.20E-03	1.91	5
GO: GLAND DEVELOPMENT	1.14E-02	1.87	5
GO: CELLULAR RESPONSE TO OXYGEN CONTAINING COMPOUND	1.23E-02	1.84	5
GO: CELLULAR HORMONE METABOLIC PROCESS	1.25E-02	1.84	5
GO: CELL DEVELOPMENT	1.30E-02	1.86	7
GO: CELLULAR LIPID METABOLIC PROCESS	2.06E-02	1.77	10
GO: REGULATION OF HORMONE LEVELS	2.10E-02	1.77	9
GO: RESPONSE TO METAL ION	2.11E-02	1.76	5
GO: TISSUE DEVELOPMENT	2.11E-02	1.74	18
GO: CELLULAR RESPONSE TO ORGANIC SUBSTANCE	2.14E-02	1.76	10
GO: INORGANIC ION TRANSMEMBRANE TRANSPORT	2.44E-02	1.73	6
GO: RESPONSE TO OXYGEN CONTAINING COMPOUND	2.46E-02	1.73	11
GO: DRUG METABOLIC PROCESS	2.76E-02	-1.62	5
GO: RESPONSE TO ORGANIC CYCLIC COMPOUND	2.91E-02	1.69	12
GO: HORMONE METABOLIC PROCESS	3.46E-02	1.67	6
GO: STEROID METABOLIC PROCESS	3.83E-02	1.65	7
GO: LIPID METABOLIC PROCESS	4.22E-02	1.60	15
GO: RESPONSE TO INORGANIC SUBSTANCE	4.77E-02	1.60	6
GO: PASSIVE TRANSMEMBRANE TRANSPORTER ACTIVITY	2.89E-02	1.71	8

[1]For comparisons of new-borns with older animals, NAT calves were used to represent new-borns (D0) as there was limited difference between NAT and CS calves.

Table 3.3: Top Canonical Pathways enriched among the DEG using Ingenuity Pathway Analysis.

NB.NAT vs. NB.CS			
Pathway	logFDR	Ratio	Z-Score
NRF2-mediated Oxidative Stress Response	1.50	0.01	N/A
Glucocorticoid Receptor Signalling	1.50	0.00	N/A
[1]D0 vs. D7			
Pathway	logFDR	Ratio	Z-Score
Dendritic Cell Maturation	8.55	0.09	3.87
Role of NFAT in Regulation of the Immune Response	6.33	0.08	3.87
Acute Phase Response Signalling	5.28	0.07	3.32
p38 MAPK Signalling	4.56	0.08	3.16
Th1 Pathway	4.16	0.07	3.00
NF-κB Signalling	2.50	0.05	3.00
iCOS-iCOSL Signalling in T Helper Cells	9.10	0.13	2.89
PKCθ Signalling in T Lymphocytes	4.16	0.07	2.71
PPAR Signalling	2.77	0.07	-2.65
Interferon Signalling	6.50	0.22	2.65
Toll-like Receptor Signalling	5.21	0.12	2.65
Intrinsic Prothrombin Activation Pathway	5.02	0.17	2.65
IL-6 Signalling	2.77	0.06	2.65
Cholecystokinin/Gastrin-mediated Signalling	2.65	0.07	2.65
Phospholipase C Signalling	2.28	0.04	2.65
Adrenomedullin Signalling pathway	2.85	0.05	2.53
CD28 Signalling in T Helper Cells	5.65	0.09	2.24
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	3.38	0.07	2.24
TREM1 Signalling	1.92	0.07	2.24
Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes	1.54	0.05	2.24
D0 vs. D14			
Pathway	logFDR	Ratio	Z-Score
Oxidative Phosphorylation	7.09	0.17	4.24
Role of NFAT in Regulation of the Immune Response	3.09	0.08	3.50
Dendritic Cell Maturation	5.53	0.11	3.30
p38 MAPK Signalling	2.48	0.09	3.16
Acute Phase Response Signalling	3.01	0.09	2.89
PPAR Signalling	1.54	0.08	-2.83
Calcium-induced T Lymphocyte Apoptosis	4.41	0.17	2.71
Th1 Pathway	3.04	0.10	2.71
IL-6 Signalling	2.58	0.09	2.71
Adrenomedullin Signalling pathway	1.09	0.06	2.71
Toll-like Receptor Signalling	2.66	0.12	2.65

Cholecystokinin/Gastrin-mediated Signalling	0.97	0.07	2.65
Type I Diabetes Mellitus Signalling	2.66	0.10	2.45
Intrinsic Prothrombin Activation Pathway	2.21	0.14	2.45
TREM1 Signalling	1.15	0.08	2.45
Neuroinflammation Signalling Pathway	1.64	0.05	2.32
iCOS-iCOSL Signalling in T Helper Cells	4.44	0.12	2.31
NF-κB Signalling	1.60	0.06	2.31
Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells	2.66	0.19	2.24
Glutathione Redox Reactions I	2.45	0.21	2.24

D0 vs. D28

Pathway	logFDR	Ratio	Z-Score
p38 MAPK Signalling	1.74	0.08	3.00
Toll-like Receptor Signalling	3.35	0.13	2.83
PPAR Signalling	1.65	0.08	-2.83
Acute Phase Response Signalling	2.90	0.08	2.71
Dendritic Cell Maturation	3.70	0.09	2.67
Cholecystokinin/Gastrin-mediated Signalling	1.11	0.07	2.65
LXR/RXR Activation	3.56	0.11	-2.50
Role of NFAT in Regulation of the Immune Response	2.17	0.07	2.50
Glutathione Redox Reactions I	3.25	0.25	2.45
Superpathway of Cholesterol Biosynthesis	2.29	0.18	2.24
Glutathione-mediated Detoxification	2.13	0.16	2.24
Type I Diabetes Mellitus Signalling	1.05	0.06	2.24
TREM1 Signalling	0.81	0.07	2.24
Cyclins and Cell Cycle Regulation	0.73	0.06	2.24
Oxidative Phosphorylation	0.49	0.05	2.24
Calcium-induced T Lymphocyte Apoptosis	2.65	0.12	2.12
Ketogenesis	2.96	0.40	2.00
Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells	2.08	0.16	2.00
Salvage Pathways of Pyrimidine Ribonucleotides	0.37	0.04	2.00
Adrenomedullin Signalling pathway	2.06	0.07	1.94

D0 vs. D96

Pathway	logFDR	Ratio	Z-Score
RhoGDI Signalling	1.50	0.10	3.74
Signalling by Rho Family GTPases	1.52	0.10	-3.58
GP6 Signalling Pathway	4.46	0.16	-3.41
Glioblastoma Multiforme Signalling	2.66	0.13	-3.13
Colorectal Cancer Metastasis Signalling	1.27	0.09	-3.13
Actin Cytoskeleton Signalling	0.81	0.08	-3.05
Gαs Signalling	1.66	0.12	-2.89
Relaxin Signalling	1.37	0.10	-2.89
p38 MAPK Signalling	2.43	0.13	2.84

ILK Signalling	2.16	0.11	-2.84
Toll-like Receptor Signalling	3.04	0.17	2.71
LXR/RXR Activation	6.30	0.20	-2.68
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	1.90	0.16	-2.65
Cyclins and Cell Cycle Regulation	0.85	0.10	2.65
PCP pathway	2.33	0.16	-2.53
Superpathway of Melatonin Degradation	1.91	0.14	2.53
P2Y Purigenic Receptor Signalling Pathway	1.80	0.11	-2.50
Regulation of Actin-based Motility by Rho	0.29	0.07	-2.45
Melatonin Degradation I	1.68	0.14	2.33
Neuropathic Pain Signalling In Dorsal Horn Neurons	2.22	0.13	-2.32
D7 vs. D14			
Pathway	logFDR	Ratio	Z-Score
Nicotine Degradation II	4.88	0.06	-1.00
Oestrogen Biosynthesis	1.91	0.05	N/A
Nicotine Degradation III	1.84	0.04	N/A
Melatonin Degradation I	1.84	0.03	N/A
Superpathway of Melatonin Degradation	1.84	0.03	N/A
Xenobiotic Metabolism Signalling	1.81	0.01	N/A
UDP-N-acetyl-D-glucosamine Biosynthesis II	1.52	0.17	N/A
Salvage Pathways of Pyrimidine Deoxyribonucleotides	1.46	0.13	N/A
Pregnenolone Biosynthesis	1.40	0.08	N/A
Bile Acid Biosynthesis, Neutral Pathway	1.40	0.08	N/A
Androgen Biosynthesis	1.40	0.07	N/A
RAR Activation	1.40	0.01	N/A
Histidine Degradation VI	1.40	0.07	N/A
Ubiquinol-10 Biosynthesis (Eukaryotic)	1.39	0.05	N/A
Methylglyoxal Degradation III	1.39	0.05	N/A
LPS/IL-1 Mediated Inhibition of RXR Function	1.39	0.01	N/A
The Visual Cycle	1.39	0.05	N/A
Bupropion Degradation	1.32	0.04	N/A
Acetone Degradation I (to Methylglyoxal)	1.26	0.03	N/A
Retinoate Biosynthesis I	1.23	0.03	N/A
Retinol Biosynthesis	1.17	0.02	N/A
Thyroid Hormone Metabolism II (via Conjugation and/or Degradation)	1.17	0.02	N/A
Glutamate Receptor Signalling	1.07	0.02	N/A
Eicosanoid Signalling	1.02	0.01	N/A
Serotonin Degradation	0.98	0.01	N/A
Salvage Pathways of Pyrimidine Ribonucleotides	0.91	0.01	N/A
TR/RXR Activation	0.91	0.01	N/A
Gαs Signalling	0.88	0.01	N/A
FXR/RXR Activation	0.84	0.01	N/A

Pathway	logFDR	Ratio	Z-Score
Insulin Receptor Signalling	0.80	0.01	N/A
Gustation Pathway	0.80	0.01	N/A
Aldosterone Signalling in Epithelial Cells	0.78	0.01	N/A
NRF2-mediated Oxidative Stress Response	0.73	0.01	N/A
cAMP-mediated Signalling	0.68	0.00	N/A
G-Protein Coupled Receptor Signalling	0.61	0.00	N/A
Neuroinflammation Signalling Pathway	0.59	0.00	N/A
Glucocorticoid Receptor Signalling	0.56	0.00	N/A
D14 vs. D28			
Pathway	logFDR	Ratio	Z-Score
Glucocorticoid Receptor Signalling	1.50	0.00	N/A
D28 vs. D96			
Pathway	logFDR	Ratio	Z-Score
Integrin Signalling	0.90	0.02	-2.00
Signalling by Rho Family GTPases	0.75	0.02	-2.00
ILK Signalling	1.53	0.03	-1.34
Protein Kinase A Signalling	0.56	0.01	-1.34
Hepatic Fibrosis / Hepatic Stellate Cell Activation	4.39	0.05	N/A
Axonal Guidance Signalling	2.63	0.02	N/A
Prostanoid Biosynthesis	2.58	0.22	N/A
Intrinsic Prothrombin Activation Pathway	2.23	0.07	N/A
Putrescine Biosynthesis III	1.76	0.50	N/A
Glutathione Redox Reactions I	1.73	0.08	N/A
LXR/RXR Activation	1.66	0.03	N/A
MSP-RON Signalling Pathway	1.60	0.04	N/A
Protein Citrullination	1.37	0.20	N/A
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	1.27	0.02	N/A
Tight Junction Signalling	1.23	0.02	N/A
CDK5 Signalling	1.19	0.03	N/A
Superoxide Radicals Degradation	1.17	0.13	N/A
Tryptophan Degradation to 2-amino-3-carboxymuconate Semialdehyde	1.17	0.13	N/A
Amyotrophic Lateral Sclerosis Signalling	1.13	0.03	N/A
D7 vs. D28			
Pathway	logFDR	Ratio	Z-Score
Nicotine Degradation II	5.95	0.11	-0.38
Estrogen Biosynthesis	2.69	0.10	1.00
Nicotine Degradation III	2.33	0.07	1.00
Melatonin Degradation I	2.31	0.06	1.00
Bupropion Degradation	2.31	0.12	N/A
Xenobiotic Metabolism Signalling	2.31	0.02	N/A
Superpathway of Melatonin Degradation	2.31	0.06	1.00
LPS/IL-1 Mediated Inhibition of RXR Function	2.28	0.03	N/A

Acetone Degradation I (to Methylglyoxal)	2.28	0.10	N/A
Ketogenesis	1.82	0.20	N/A
The Visual Cycle	1.25	0.10	N/A
ErbB2-ErbB3 Signalling	1.24	0.04	N/A
eNOS Signalling	1.12	0.02	N/A
Retinoate Biosynthesis I	0.90	0.06	N/A
Glucocorticoid Receptor Signalling	0.81	0.01	N/A
Retinol Biosynthesis	0.79	0.05	N/A
L-carnitine Biosynthesis	0.79	0.33	N/A
Th1 Pathway	0.71	0.02	N/A
Protein Citrullination	0.64	0.20	N/A
UDP-N-acetyl-D-glucosamine Biosynthesis II	0.58	0.17	N/A

[1]For comparisons of new-borns with older animals, NAT calves were used to represent new-borns (D0) as there was limited difference between NAT and CS calves.

Table 3.4: Top Molecular and Cellular Functions of the DEG using Ingenuity Pathway Analysis.

NB.NAT vs. NB. CS		
Molecular and Cellular Function		
Name	Median P-value	# Genes
Cell-To-Cell Signalling and Interactions	2.8E-04	1
Cellular Growth and Proliferation	2.5E-02	1
Drug Metabolism	3.7E-04	1
Lipid Metabolism	3.7E-04	1
Small Molecule Biochemistry	3.7E-04	1
Physiological System Development and Function		
Name	Median P-value	# Genes
Haematological System Development and Function	2.8E-04	1
Endocrine System Development and Function	3.7E-04	1
Behaviour	2.4E-02	1
Nervous System Development and Function	4.9E-02	1
Tissue Development	4.9E-02	1
[1]D0 vs. D7		
Molecular and Cellular Function		
Name	Median P-value	# Genes
Cell-To-Cell Signalling and Interaction	2.4E-05	80
Cellular Movement	1.7E-05	76
Cellular Function and Maintenance	2.4E-05	87
Cellular Development	2.3E-05	81
Cellular Growth and Proliferation	2.3E-05	80
Physiological System Development and Function		
Name	Median P-value	# Genes
Haematological System Development and Function	2.4E-05	112
Tissue Morphology	2.4E-05	81
Immune Cell Trafficking	2.4E-05	75
Cell-mediated Immune Response	2.1E-05	50
Lymphoid Tissue Structure and Development	2.4E-05	86
D0 vs. D14		
Molecular and Cellular Functions		
Name	Median P-value	# Genes
Cell Death and Survival	7.5E-05	207
Cellular Movement	9.5E-05	124
Cellular Function and Maintenance	1.5E-04	116
Cell-To-Cell Signalling and Interaction	1.5E-04	96
Cell Cycle	3.4E-05	76
Physiological System Development and Function		

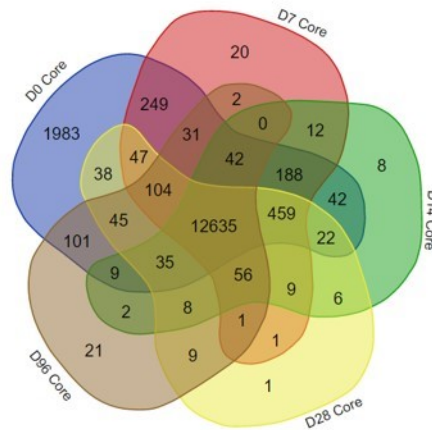
Name	Median P-value	# Genes
Haematological System Development and Function	1.5E-04	149
Tissue Morphology	1.3E-04	134
Lymphoid Tissue Structure and Development	1.5E-04	114
Organ Morphology	1.0E-04	69
Organismal Survival	3.3E-07	161
D0 vs. D28		
Molecular and Cellular Functions		
Name	Median P-value	# Genes
Cell Cycle	2.7E-04	94
Cell Death and Survival	3.3E-04	184
Drug Metabolism	3.3E-04	26
Cellular Assembly and Organization	3.3E-04	39
DNA Replication, Recombination, and Repair	2.8E-04	33
Physiological System Development and Function		
Name	Median P-value	# Genes
Organismal Survival	5.2E-07	151
Cardiovascular System Development and Function	2.3E-04	64
Hematological System Development and Function	3.3E-04	121
Tissue Morphology	2.8E-04	131
Immune Cell Trafficking	3.2E-04	76
D0 vs. D96		
Molecular and Cellular Functions		
Name	Median P-value	# Genes
Cell Cycle	2.7E-04	94
Cell Death and Survival	3.3E-04	184
Drug Metabolism	3.3E-04	26
Cellular Assembly and Organization	3.3E-04	39
DNA Replication, Recombination, and Repair	2.8E-04	33
Physiological System Development and Function		
Name	Median P-value	# Genes
Organismal Survival	5.2E-07	151
Cardiovascular System Development and Function	2.3E-04	64
Hematological System Development and Function	3.3E-04	121
Tissue Morphology	2.8E-04	131
Immune Cell Trafficking	3.2E-04	76
D7 vs. D14		
Molecular and Cellular Functions		
Name	Median P-value	# Genes
Lipid Metabolism	2.4E-02	8
Molecular Transport	2.4E-02	8
Small Molecule Biochemistry	2.4E-02	11

Name	Median P-value	# Genes
Vitamin and Mineral Metabolism	2.3E-02	5
Carbohydrate Metabolism	2.4E-02	3
Physiological System Development and Function		
Name	Median P-value	# Genes
Organ Morphology	2.2E-02	6
Organismal Development	2.2E-02	6
Reproductive System Development and Function	2.1E-02	7
Embryonic Development	2.4E-02	3
Organ Development	2.2E-02	3
D14 vs. D28		
Molecular and Cellular Functions		
Name	Median P-value	# Genes
Cellular Development	3.8E-02	1
Physiological System Development and Function		
Name	Median P-value	# Genes
Hair and Skin Development and Function	4.6E-04	1
Organ Morphology	3.0E-03	1
Digestive System Development and Function	9.7E-04	1
Organismal Development	1.6E-03	1
Tissue Morphology	1.6E-03	1
D28 vs. D96		
Molecular and Cellular Functions		
Name	Median P-value	# Genes
Cell Morphology	3.3E-03	55
Cellular Movement	1.8E-03	61
Cellular Development	3.2E-03	75
Cellular Growth and Proliferation	3.6E-03	68
Carbohydrate Metabolism	1.0E-03	6
Physiological System Development and Function		
Name	Median P-value	# Genes
Nervous System Development and Function	3.2E-03	69
Organismal Development	3.4E-03	93
Tissue Morphology	3.6E-03	71
Organ Morphology	3.4E-03	58
Organismal Survival	9.9E-04	62
D7 vs. D28		
Molecular and Cellular Functions		
Name	Median P-value	# Genes
Carbohydrate Metabolism	1.9E-03	8
Lipid Metabolism	3.7E-03	23
Molecular Transport	3.4E-03	28
Small Molecule Biochemistry	3.7E-03	32

Name	Median P-value	# Genes
Protein Synthesis	3.7E-03	5
Physiological System Development and Function		
Name	Median P-value	# Genes
Connective Tissue Development and Function	3.7E-03	9
Skeletal and Muscular System Development and Function	3.7E-03	13
Endocrine System Development and Function	3.8E-03	8
Organismal Development	3.8E-03	26
Reproductive System Development and Function	3.8E-03	14

[1]For comparisons of new-borns with older animals, NAT calves were used to represent new-borns (D0) as there was limited difference between NAT and CS calves.

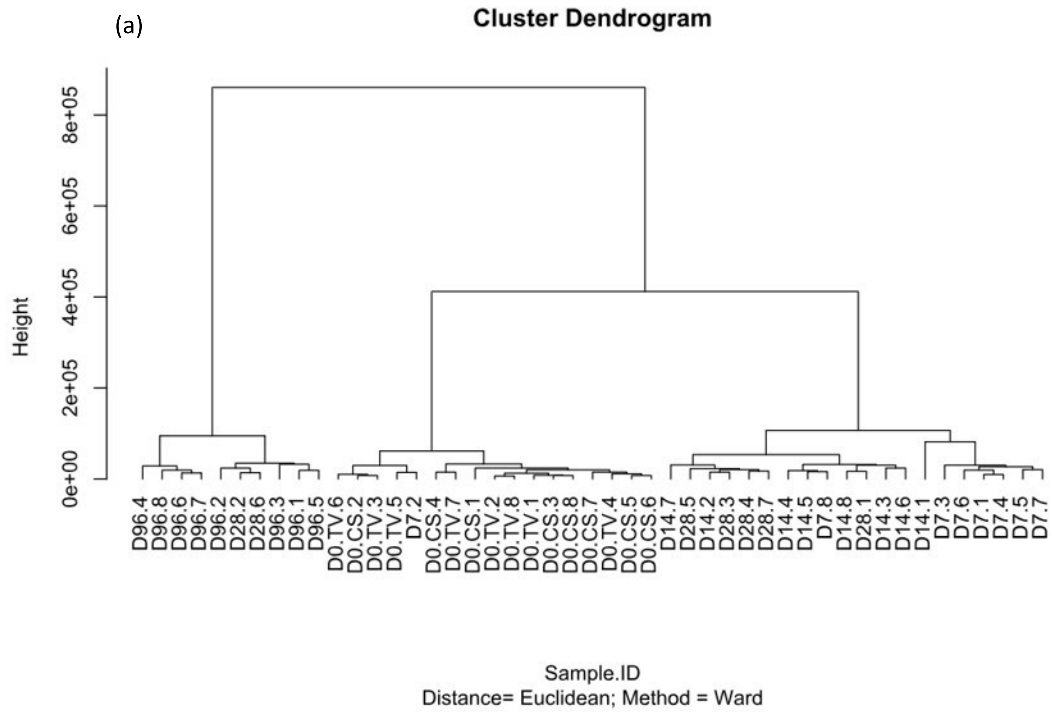
(a)



(b)

Contrast	Total DEG	Upregulated	Downregulated
1: CSD0 v TVD0	2	2	0
2: D0* v D7	347	336	11
3: D0 v D14	727	574	153
4: D0 v D28	629	438	191
5: D0 v D96	1612	587	1025
6: D7 v D14	25	13	12
7: D14 v D28	2	0	2
8: D28 v D96	213	21	192
9: D7 v D28	103	42	61

Figure 3.1: (a) Venn diagram showing numbers of expressed genes in the rumen wall in each age group. A gene was considered expressed within an age group if it had a CPM value > 1 in at least half of the samples. (b) Numbers of DEG in each statistical contrast as detected using edgeR.



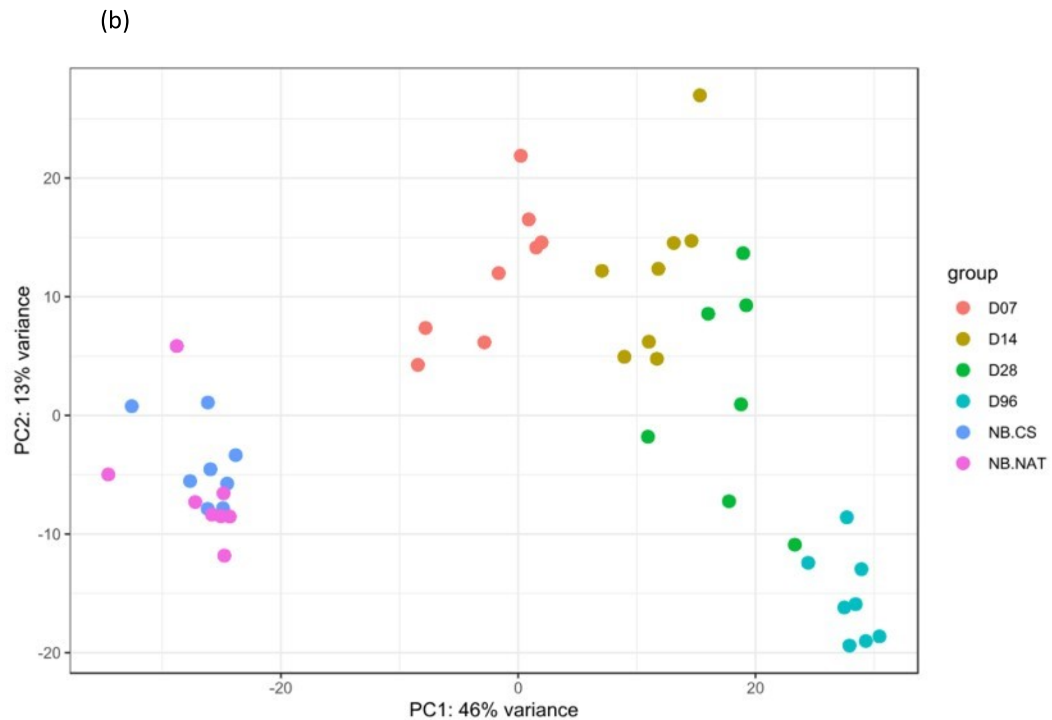


Figure 3.2: Cluster analysis generated using the Bray-Curtis Dissimilarity matrix: (a) Hierarchical clustering dendrogram using Ward disequilibrium linkage, (b) principle component analysis (PCA) plot.

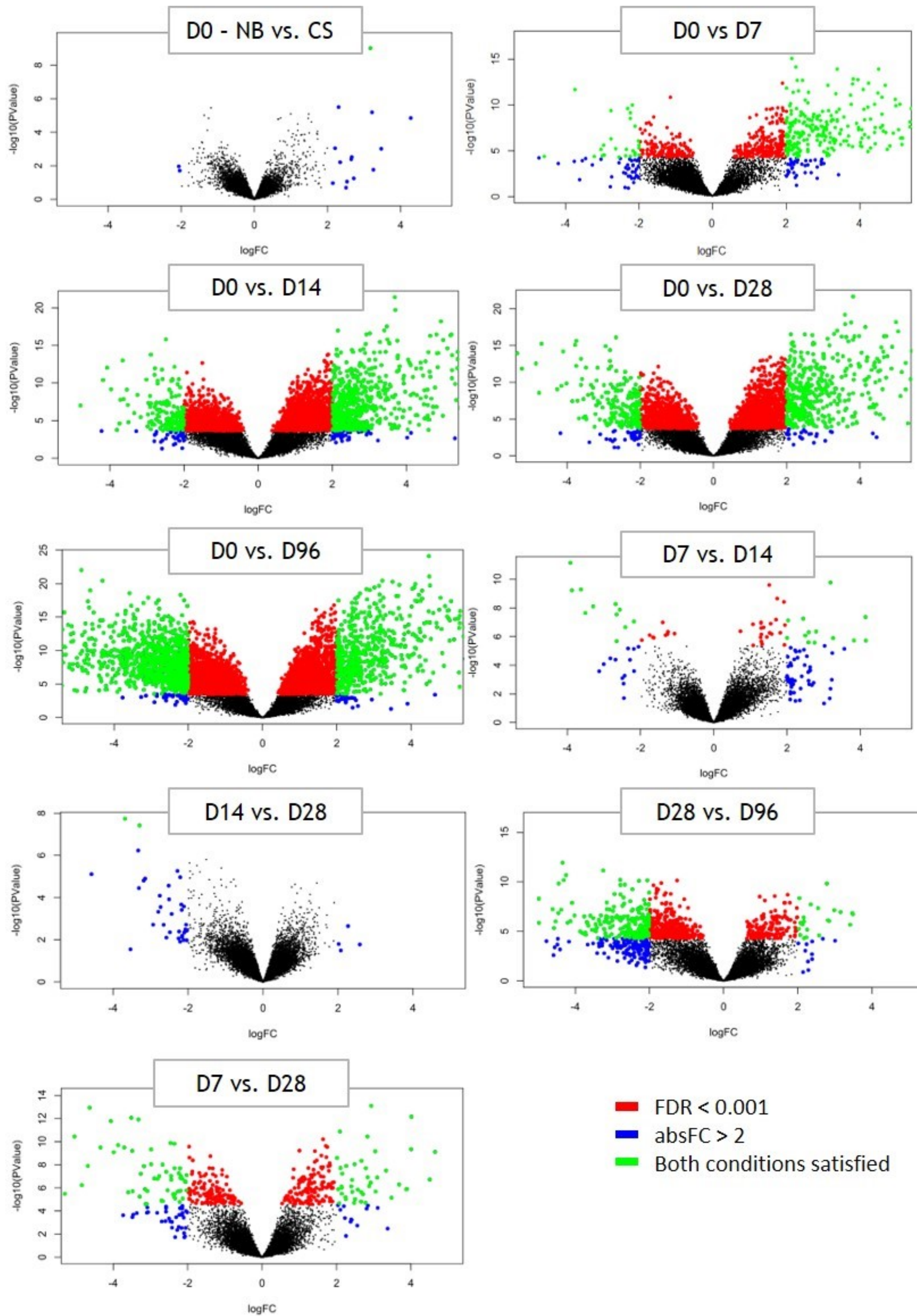


Figure 3.3: Volcano plots of differential gene expression profiles within each statistical contrast. Each data point represents an expressed gene. Red = FDR < 0.001. Blue = absolute fold change > 2. Green = both conditions met, gene is differentially expressed in this contrast.

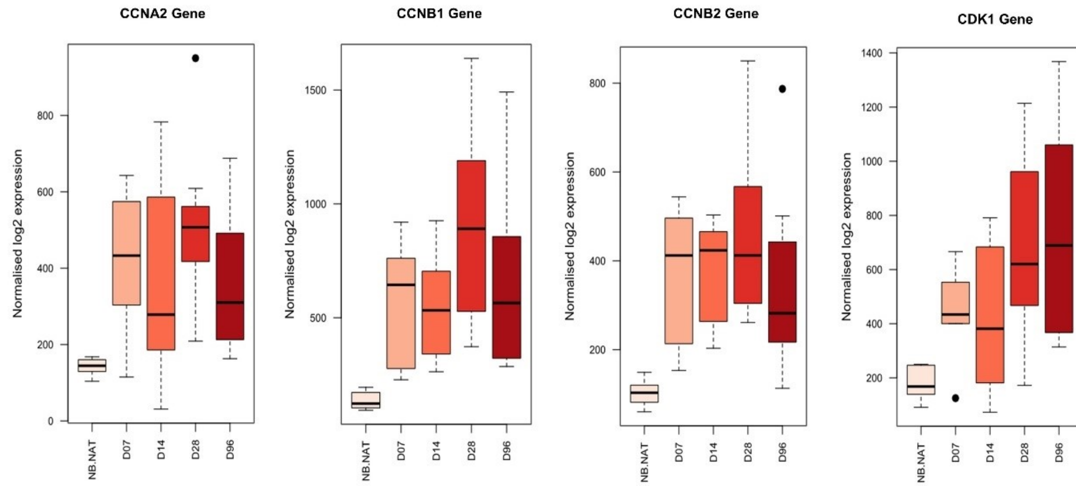


Figure 3.4: Boxplots depicting the expression profiles of selected genes involved in the cell cycle process in the rumen wall during early life. The Y-axis represents normalised CPM values.

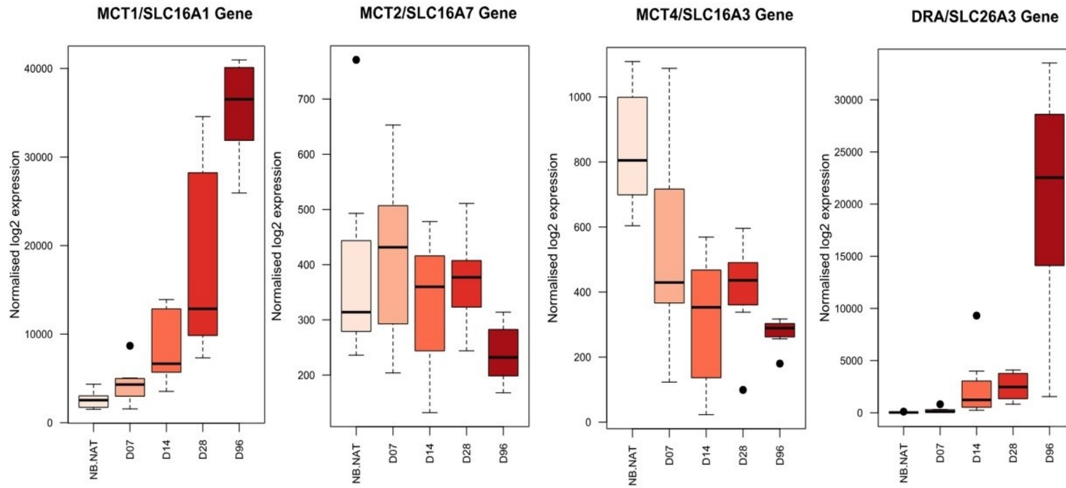


Figure 3.5: Boxplots depicting the expression profiles of VFA transporter genes in the rumen wall during early life. The Y-axis represents normalised CPM values.

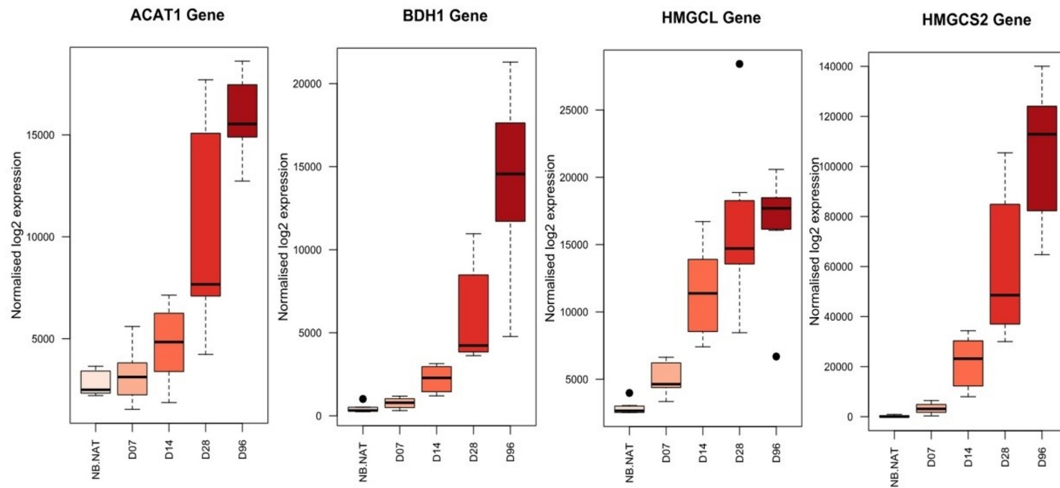


Figure 3.6: Boxplots depicting the expression profiles of genes involved in ketogenesis detected in the rumen wall during early life. The Y-axis represents normalised CPM values.

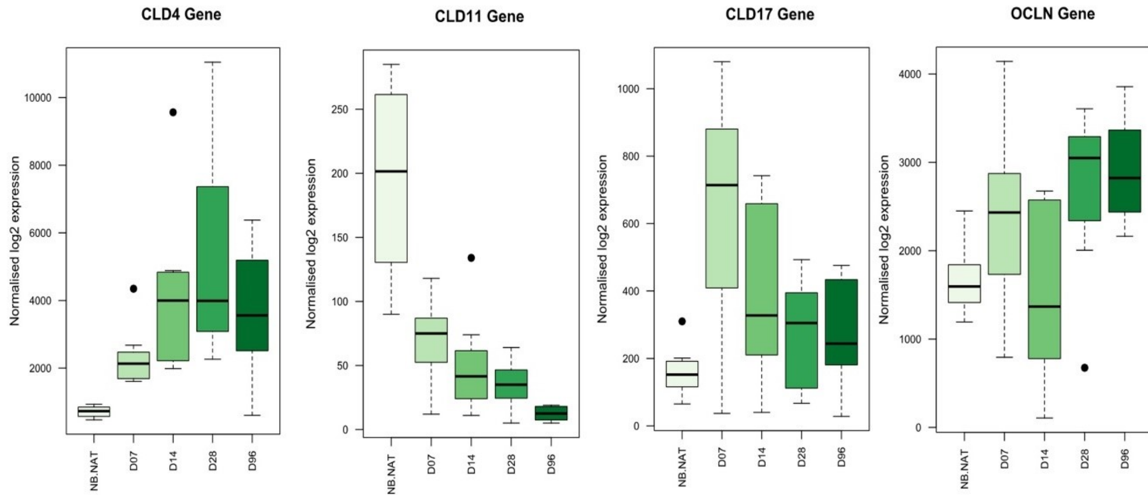


Figure 3.7: Boxplots depicting the expression profiles of tight junction protein genes detected in the rumen wall during early life. The Y-axis represents normalised CPM values.

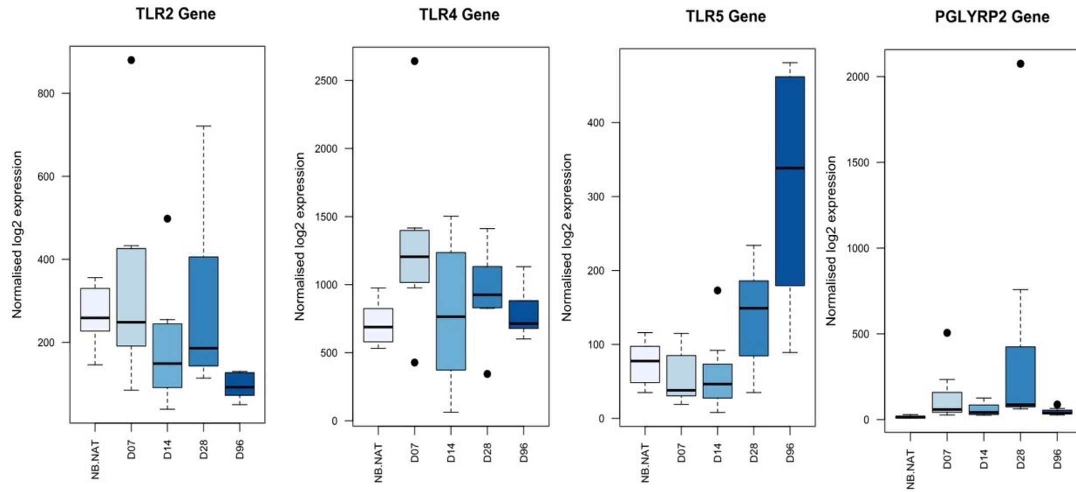


Figure 3.8: Boxplots depicting the expression profiles of pattern recognition receptors detected in the rumen wall during early life. The Y-axis represents normalised CPM values.

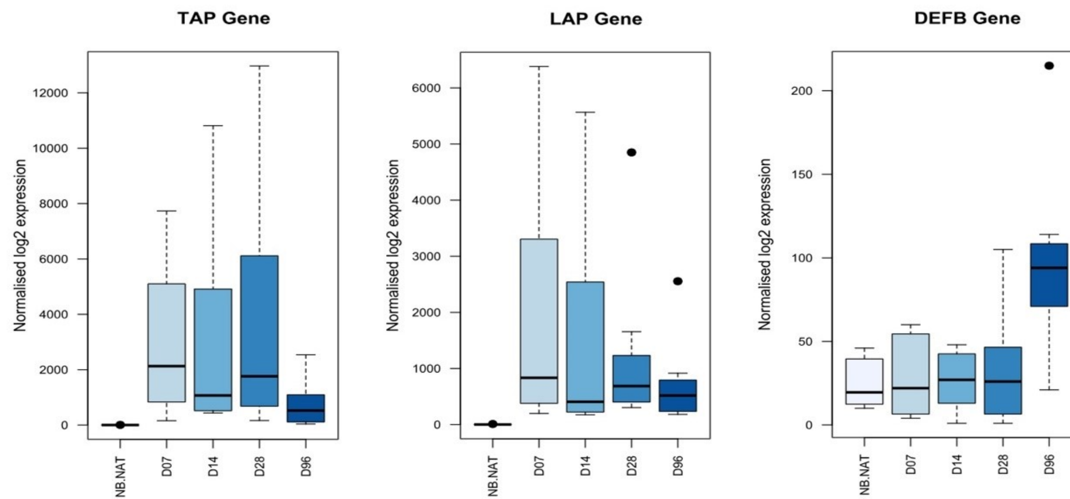


Figure 3.9: Boxplots depicting the expression of defensin genes detected in the rumen wall during early life. The Y-axis represents normalised CPM values.

Chapter 4

Effect of a butyrate-fortified milk replacer on gastrointestinal microbiota and products of fermentation in artificially reared dairy calves at weaning.

4.1 Introduction

The digestive physiology of the calf changes dramatically in the first weeks and months of life, and the transition from a nominal monogastric to functional ruminant is fraught with challenges (Steele et al., 2016, Ryle, 1992). The occurrence of gastrointestinal disorders in this period is a source of substantial economic loss in dairy production systems, responsible for around 10% of calf mortality (USDA, 2010). With rising concerns surrounding the prophylactic and growth-promoting use of antibiotics in livestock production promotion (Van Boeckel et al., 2015), there is much interest in the development of synthetic and natural alternatives to promote bovine intestinal health and development in early life.

The gastrointestinal tract (GIT) microbiota of ruminants and other production animals is well established as a key feature underscoring animal health, development and productivity (Yeoman and White, 2014). In adult cattle, the rumen microbiota is the predominant feed-degrading microbial community. However, up to 20% of milk solids may pass to the hindgut for digestion during the milk feeding phase, placing elevated importance on the hindgut microbiota in this period (Castro et al., 2016). Volatile chain fatty acids (VFAs) are organic acids produced throughout the intestinal tract by microbial fermentation, and are vital in the stimulation of intestinal growth and development (Zhou et al., 2014, Firkins and Yu, 2015). The antimicrobial properties of VFAs and their natural presence in the mammalian digestive tract suggested that VFA-derived feed additives may be an alternative to conventional antimicrobials in livestock production (Guilloteau et al., 2009b). Among the most prominent of the luminal VFAs, butyrate has been investigated for its effectiveness in enhancing animal growth and intestinal integrity and development in young livestock, with promising results (Gorka et al., 2009, Niwińska et al., 2017). Butyrate is the primary energy source for rumen epithelial cells and colonocytes, which are important mediators of water, mineral, and nutrient absorption (Bedford and Gong, 2017). Butyrate inclusion in both milk replacer and solid feed has been shown to have beneficial effects on both intestinal development and animal growth in young livestock (Xu et al., 2016, Gorka et al., 2011a, Gorka et al., 2009, Guilloteau et al., 2009b).

Enteric disorders in calves are associated with microbial dysbiosis in the gut (Oikonomou et al., 2013), and thus the health-promoting effects of exogenous butyrate may be underpinned by modulation of the GIT microbiota. There is evidence that encapsulated butyrate can reduce enteric pathogen colonisation in swine and poultry (Czerwinski et al., 2012, Hu and Guo, 2007, Xu et al., 2016), and direct infusion of butyrate into the mature cow rumen caused significant changes to the resident microbiota (Li et al., 2012c). However, there are little data concerning the effect of long-term supplementation of butyrate on GIT microbial communities in pre-weaned calves. Given the established impact of butyrate on animal growth and intestinal development, we hypothesised that provision of a butyrate-fortified milk replacer impacts microbial communities throughout the GIT while improving host performance. Therefore, the objective of this study was to assess microbial composition and fermentation in the rumen and hindgut at weaning in dairy calves offered milk replacer enriched with butyrate during early life.

4.2 Materials and methods

4.2.1 Ethical statement

All procedures involving animals were approved by University College Dublin Animal Research Ethics Committee (UCD AREC), under licence from the Irish Department of Health and Children in accordance with the Cruelty to Animals Act (Ireland 1897) and European Community Directive 86/609/EC.

4.2.2 Animal study

Forty-four male Holstein-Friesian calves (13±5 days of age) were obtained from one dairy farm and were placed at a research facility for use in this study (UCD Lyons Farm, Clane, Co. Kildare, Ireland). Calves were blocked according to age and body weight and were randomly assigned to one of two treatment groups; CON (fed unaltered milk replacer, n=22) or SB (encapsulated sodium butyrate included in milk replacer at 4g/kg of DM daily, n = 22). Calves were placed on a standard 56-day calf rearing program upon arrival at the research farm, with milk replacer (12.5% solids; Crude Protein 23% and Crude Fat 20%; Blossom™, Volac, UK) offered at 6L/day via an automatic feeder (Forester Tecknik, KFA3-MA3). Concentrates (rolled barley 26.5%; soya bean meal 25%; maize 15%; beet pulp 12.5%; soya hulls 12.5%; molasses 5%; minerals & vitamins 2.5%; vegetable oil 1%; Nutriad, Belgium) and water were offered on an *ad libitum* basis throughout the experimental period. All calves were in good health throughout the experimental period. Calves were weaned over a 7-day period (D49-56) via gradual reduction in

the allocation of milk replacer. On D56, eight animals from each group were randomly selected for euthanasia using an intravenous overdose of sodium pentobarbitone (Dolethal™, 1.4ml/kg live body-weight). Death was confirmed by lack of a corneal reflex and heartbeat. The gastrointestinal tract was quickly exteriorised and digesta samples from the rumen, cecum, and colon were collected, immediately snap frozen on liquid nitrogen, and stored at -80°C pending molecular analysis. A further digesta sample was collected from both the rumen and colon (representative of the total hindgut VFA profile, as previously shown (Elsden et al., 1946)) for VFA analysis. These samples were passed through four layers of cheesecloth and stored in H_2SO_4 at -80°C prior to VFA analysis using gas chromatography.

4.2.3 DNA isolation

Frozen digesta from the cecum, colon and rumen was ground under liquid nitrogen to a fine powder. Total DNA was extracted using the RBB+C method as previously described (Yu and Morrison, 2004); approximately 250mg of ground frozen sample was subjected to repeated bead beating followed by column purification with a QIAGEN DNeasy Stool Kit (Qiagen, UK). DNA quantity and purity were assessed by two consecutive readings at A260nm and A280nm on a Nanodrop 1000 spectrophotometer, and visualisation with UV light in a 0.8% agarose gel. Samples with DNA purity values < 1.6 were re-extracted, as were samples of low concentration (< 100ng/µl).

4.2.4 Microbial profiling using amplicon sequencing

Amplicons of the V4 hyper-variable region of the 16S rRNA gene were prepared using Illumina Nextera chemistry, as previously reported (McCabe et al., 2015). DNA concentrations recorded on the Nanodrop were used to normalise each sample to a concentration of 100ng/µl with molecular water. A 25µl PCR reaction using 20ng of DNA, and KAPA Hi-Fi PCR mix (New England Biolabs Inc.) was prepared using 515F/806R primers (Caporaso et al., 2011) to simultaneously characterise bacterial and archaeal members using the following cycle programme: 95°C for 3 minutes, and 25 cycles of: 95°C for 30sec, 55°C for 30sec, 72°C for 30sec, with a final elongation step of 72°C for 30 seconds. Amplicons were purified using the QIAGEN QIAquick PCR Purification Kit. A second PCR step was performed to add Illumina dual indices and Nextera™ adapters to the purified fragments (Illumina, San Diego, CA, USA). Following another column purification, the barcoded amplicon products were combined into two pools in equimolar quantities to ensure adequate sequencing coverage. Each pool was subjected to gel

(QIAquick Gel Extraction Kit, Qiagen) and column purification (QIAquick Purification Kit, Qiagen) to remove primer dimers and any residual agarose. Purified pools were quantified by qPCR using the KAPA SYBR FAST Universal kit with Illumina Primer Premix (New England Biolabs Inc.). Pools were then diluted and denatured according to the Illumina MiSeq library preparation guide. A 6pM amplicon library was spiked with 30% denatured and diluted PhiX Illumina control library (version 3, 12.5 pM), and subjected to sequencing on the Illumina MiSeq platform with one pool per run.

4.2.5 Sequence data quality control and pre-processing

Demultiplexed paired end reads were trimmed and filtered to remove low quality reads and bases (Phred quality score threshold of 20), and simultaneously merged using the BBTools suite (Bushnell, 2015). The resulting merged reads were then size selected to retain only reads ± 2 standard deviations from the mean read length, to minimise spurious OTU creation. Finally, merged pairs were combined into a single file for downstream processing using the Quantitative Insights Into Molecular Ecology (QIIME v.1.9) tool (Caporaso et al., 2010).

4.2.6 Bioinformatic analysis

Operational taxonomic unit (OTU) identification using a similarity level of 97% was carried out using the open reference picking method implemented in QIIME (Caporaso et al., 2010). A representative sequence from each identified OTU was then aligned against the reference Greengenes database (v.13_8) (Guilloteau et al., 2010). A graphical representation of the phylogenetic trees created in QIIME was generated using the Interactive Tree of Life software (Letunic and Bork, 2016). The raw and unfiltered OTU table created in QIIME was imported into R to create a Phyloseq class object (McMurdie and Holmes, 2013). α-diversity was computed by first randomly subsampling (rarefying) the OTU table to the lowest read number, to reduce bias due to differential sequencing depth. The Shannon and Chao1 metrics were used to assess diversity and evenness of the rumen and hindgut microbiota. β-diversity was calculated in a similar manner, with a Bray Curtis Dissimilarity matrix constructed from the rarefied OTU table. A cluster dendrogram using Ward linkage equilibrium was generated from the same OTU table in R. Principle Coordinate Analysis (PCoA) was performed in Phyloseq and used to visualise these distance matrices in 2-dimensional space. Singleton OTUs were removed, and relative abundances of taxa at the phylum, family, and genus levels were computed in R.

4.2.7 Statistical analysis

Permutation based Analysis of Variance (PERMANOVA) analysis based on the Bray Curtis Dissimilarity Matrix was carried out in R using the Vegan package to compare microbial structure between groups and GIT region (Dixon, 2003, Anderson, 2001). Taxonomic abundances at the phylum and genus levels were compared across treatments (within GI compartment) using a Wald parametric test, offered within the DESeq2 Bioconductor package in R (Love et al., 2014). A false-discovery rate (FDR) threshold of 0.15 was used to determine statistical significance (Benjamini and Hochberg, 1995). Only taxa represented by ≥ 0.01% of all 16S rRNA sequences in either treatment group were considered present. Exploratory investigation of taxonomic profiles revealed two outlier animals (one from each group), and they were removed from subsequent analysis leaving a total of 7 animals in each treatment group.

4.3 Results

4.3.1 Animal performance

This experiment was conducted in association with a larger study designed to examine the effect of SB supplementation on the performance, feed efficiency and immune status of artificially reared dairy calves (Pierce et al., 2014). Briefly, from this perspective, calves supplemented with SB tended (*P*=0.08) to have a higher pre-weaning growth rate compared to CON (0.69 versus 0.59 kg/day). At weaning SB calves (80.2 kg) were 3.1 kg heavier than the CON group (76.9 kg) with bodyweight differences detected from day 42 until weaning. Total DMI was not different between dietary treatments but pre-weaning SB supplementation tended (*P*=0.08) to improve feed efficiency (measured using feed conversion ratio) of the calves (SB; 1.7:1 compared to CON; 2.5:1; *P*=0.07). Feed intakes and growth rates are presented in Appendix F.

4.3.2 Fermentation profiles in the rumen and hindgut at weaning

Volatile fatty acid (VFA) profiles of the rumen and colon contents at weaning are presented in Table 4.1. Colonic concentrations of total VFA, propionate, and acetate were higher for SB fed calves (*P*<0.05). SB supplementation reduced ruminal butyrate concentration (*P*<0.05), but total VFA concentration was unaffected.

4.3.3 Microbial structure and diversity in the rumen and hindgut in response to SB

Amplicon sequencing of rumen and hindgut digesta samples from calves at weaning yielded a total of 10,348,464 high quality reads, with an average of 215,593 ± 75,380 sequences

per sample. Taxon abundance was agglomerated at the genus and phylum levels for comparisons across treatments, and relative abundances of all detected taxa are summarised in Appendix E.

Alpha diversity measured using the *Shannon* index was not affected by treatment in any region studied, though was higher in both hindgut regions than in the rumen (P<0.05, Table 4.2). The *Chao1* index of species richness was lower in the rumen of SB animals (P<0.05), but was similar across treatments in the hindgut (Table 4.2), and was higher in the colon than both other compartments (P<0.05, Table 4.2). Principal Coordinate Anlaysis (PCoA) and cluster analysis showed some evidence of separation according to treatment, independent of GIT region in the hindgut (Fig. 4.1), but comparisons using PERMANOVA failed to detect any differences (P<0.05, Table 4.3). There was, however, clear separation according to gastrointestinal region, with the rumen community clustering away from both hindgut regions (P<0.05), while both hindgut regions appeared to harbour a similar microbial community (Fig 4.1).

4.3.4 Microbial composition in the rumen and hindgut in response to SB

4.3.4.1 Rumen

Among the bacterial phyla detected in the rumen, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* were predominant, followed by *Actinobacteria* and *Cyanobacteria* (Fig. 4.2), while the remaining minor phyla (< 1% 16S rRNA reads) are presented in Fig. 4.2. Archaea were represented by the *Euryarchaetota* phylum. 60 genus-level assignements were reported from the rumen, with *Prevotella*, *f.Succivibrionaceae* and *f.Lachnospiraceae* predominant at weaning, regardless of dietary treatment. Notably, only 48.14% of reads recovered from the rumen could not be confidently assigned at the genus level. This is reflected in the high abundances of f.*Succinivibrionaceae* (f = family level, unassigned at genus level in QIIME), f.*Lachnospiraceae* and o.*Clostridiales* in the rumen samples, as well as a further 21 unclassifed genus-level taxa (Appendix E, Fig. 4.3a). Comparisons of taxon abundance in DESeq2 between SB and CON animals showed no statistically significant effect of dietary treatment on the rumen microbiota at either phylum or genus level following adjustment into FDR.

4.3.4.2 Hindgut

Twelve bacterial phyla and a single archaeal phyla were detected in the hindgut, among which *Defferibacteres* was unique to the colon (Fig. 4.2). Like the rumen, a significant proportion of 16S rRNA reads recovered from the cecum and colon could not be resolved taxonomically to the genus level (~59%). Ninety-three and 88 genera were detected in the cecum and colon,

respectively. Genera annotated only as f.*Lachnospiraceae* and f.*Ruminococcaceae* were the most abundant in both compartments (Fig. 4.3). There was a minor impact of treatment on composition of the hindgut microbiota. For instance, in the colon, *Prevotella* was enriched in SB animals (*P*<0.05). In the cecum, several taxa were different between treatments; as in the colon *Prevotella* (4.31- 9.48%) was numerically higher in the SB cohort, but this difference was not signficant, possibly due to the large inter-animal variaiton observed (Appendix E).

An additional 9 genera were different between dietary treatments in the cecum (*P*<0.05); *Shuttleworthia* (0.01 vs. 0.06%), *Butyrivibrio* (0.13 vs. 0.81), *Sharpea* (0.32 vs. 1.09%), and *Mogibacterium* (0.12 vs. 0.26%) were all reduced by SB supplementation (*P*<0.05), as well an unidentified member of the f.[*Mogibacteriaceae*] (0.65 vs. 1.56%) (Fig. 4.3b). A genus belonging to the Cyanobacterial *YS2* order was increased by SB, as were *Lachnospira* (0.13 vs. 0.06%), *Phascolarctobacterium* (1.40% vs. 0.66%), and a genus annotated as *p-75-af* belonging to *Erysipelotrichaceae* (0.31 vs. 0.11%, *P*<0.05). A single genus from the *Tenericutes* phylum classified only as o. *ML615J-28* was also increased in the SB group (0.19 vs. 0.04%, *P*<0.05). Additionally, an undetermined genus assigned to the *Coriobacteriaceae* family was reduced by SB in the cecum (3.96 vs. 8.17%, *P*<0.05, Fig 4.3b).

4.4 Discussion

The beneficial effects of dietary butyrate supplementation (often included in salt form as calcium or sodium butyrate) on animal growth and intestinal development have been demonstrated in calves (Gorka et al., 2009, Guilloteau et al., 2009a, Gorka et al., 2011a), chickens (Hu and Guo, 2007) and pigs (Kotunia et al., 2004). While there is now an established body of evidence supporting the potential of butyrate as a beneficial feed additive, its impact on the gut microbiota is unknown. In adult animals, hindgut fermentation typically provides 5-10% of dietary energy, but this may be elevated during the pre-weaning phase of calf growth, when up to 20% of ingested milk solids may pass to the hindgut (Gressley et al., 2011, Castro et al., 2016). Thus microbial fermentation in the cecum and colon is an important host energy source during this period (Castro et al., 2016). Given that the importance of the hindgut in feed digestion is accentuated during early ruminal development, it is of interest to ascertain what changes may occur in the microbiota and fermentation patterns following SB supplementation. In a previous study, our group showed positive effects on growth and efficiency when dairy calves were supplemented with SB (Pierce, 2014). Here, we provide evidence that such improved performance

is accompanied by changes in microbial composition and fermentation in the hindgut compartments, while the rumen microbiota is mostly unaffected.

4.4.1 Sodium butyrate does not induce substantial changes in the rumen microbiota or fermentation profile

In terms of bacterial composition, the rumen microbiota was unaffected by SB. However, species richness (assessed using the Chao1 estimator) was lower in the SB animals, indicative of a greater number of sparsely abundant OTUs being present the rumen of CON animals than the SB group. Interestingly, we also observed a reduction in ruminal butyrate concentration in the SB cohort. The digestive physiology of the milk-fed calf effectively precludes entry of liquid feed into the reticulorumen via action of the reticular groove (Black and Sharkey, 1970), and so these changes are likely due to an indirect effect of SB on the rumen microbiota, as the exogenous butyrate in the milk replacer did not enter the rumen. Such indirect influences of SB on the rumen have previously been observed; SB-fortified MR significantly improved rumen growth and papillae development compared to calves fed conventional MR (Gorka et al., 2011b), but we did not observe such effects in the present study where rumen papillae length, width, and perimeter were not affected by SB supplementation (data not shown). Thus, though we observed a reduction in the concentration of ruminal butyrate, this does not appear to have had any detrimental effects on rumen development. It is possible that if the excess dietary butyrate was absorbed in the gut, it may have reduced the requirement for ruminal butyrate in the SB calves. It is also worth noting that many inconsistent results have been reported in the literature when butyrate or its derivatives are used as supplements in livestock diets, as recently reviewed (Bedford and Gong, 2017). Nonetheless, this suggests cross-talk mechanisms may exist between the lower gut and the rumen and warrant further investigation. In studies where SB was included in calf starter, significant development of the rumen epithelium was observed (Górka et al., 2011, Gorka et al., 2009), and future work should also examine changes in the rumen microbiota and fermentation profiles when calves are supplemented with SB in solid feed.

4.4.2 Sodium butyrate modifies the hindgut microbiota and fermentation profiles in early life

The microbial profiles of the cecum and colon were highly similar. No significant clustering was observed in the PCoA plot according to treatment within either compartment, but finer shifts in the microbial profile were evident in both. In the colon, the proportion of *Prevotella*

was increased by SB, with a similar numerical increase observed in the cecum. Enrichment of *Prevotella* in the colon and stomach of neonatal piglets has previously been reported following SB supplementation (Xu et al., 2016). *Prevotella* is established as a primary member of the mammalian gut ecosystem, comprising species capable of fermenting a wide range of non-cellulosic plant polysaccharides and protein (Purushe et al., 2010a). *Prevotella* spp. positively correlated with intestinal butyrate concentrations in growing pigs (Ivarsson et al., 2014), and it is possible that excess dietary butyrate reaching the colon conferred a competitive advantage on *Prevotella*, as they are not notable butyrate producers (Emerson and Weimer, 2017), aligning with the significant reduction in known butyrate producing taxa discussed below. Supplementing the diet of neonatal piglets and poultry with SB has previously been reported to reduce the abundance of known gut pathogens (e.g *E. coli*) (Xiong et al., 2016). We did not observe similar effects in our study, which may be attributable to differences in analytical approach (e.g. qPCR for specific scour causing bacteria). We did detect *Escherichia* in our dataset, but its proportion was very low (<0.005% of total 16S rRNA sequences) and so was not considered in our final analysis. This highlights a limitation of amplicon sequencing surveys, whereby potentially important taxa may be under- or over-represented due to variation in 16S rRNA gene copy number among microbial species (Klappenbach et al., 2001).

We observed most evidence of microbial manipulation through SB supplementation, in the cecum. Most notably, the abundances of several important VFA producers were changed. *Phascolarctobacterium* rapidly converts succinate to propionate in the gut (Watanabe et al., 2012, Aschenbach et al., 2010). The higher abundance of this genus in the cecum of SB animals may have contributed to improved growth via increased host energy substrate, as propionate is the primary precursor for gluconeogenesis in ruminants (Aschenbach et al., 2010). This, combined with our observation of higher levels of propionate and total VFA, provides evidence that improved rates of bacterial fermentation in the hindgut may also contribute to SB-driven performance improvements, as well as the increased activation of the IGF-1 pathway previously reported (Guilloteau et al., 2009b). Abundances of known butyrate-producing *Butyrivibrio* and *Shuttleworthia* were reduced in the cecum under SB supplementation, suggesting that exogenous butyrate suppresses microbial biosynthesis of butyrate in the gut. The reduction of the lactate producer *Sharpea* may also contribute to lower microbial butyrate as lactate is an intermediate molecule formed by bacterial action in the GIT. Lactate is usually rapidly utilised for VFA (primarily butyrate) synthesis, as accumulation can lead to harmful acidotic conditions (Flint et al., 2014, Bourriaud et al., 2005). While the mechanisms and occurrence of ruminal acidosis has been extensively investigated in cattle (Gao and Oba, 2016, Kim et al., 2016), there is little

knowledge of the prevalence of hindgut acidosis in calves. Lactate was not measured in the present study, but our results suggest that lactate metabolism may be an important intermediary in the response of the gut microbiota to exogenous butyrate, warranting further investigation.

Sodium butyrate supplementation in reduced cecal abundance of taxa associated with lowered gut health and integrity, and elevated inflammation. For instance, *Mogibacterium,* a known genus of the oral microbiota, was reduced in response to SB supplementation. Whilst the role of *Mogibacterium* in the gut is not fully understood, previous studies have observed a decreased faecal abundance of this genus in response to beneficial prebiotic supplementation in neonatal piglets (Berding et al., 2016), and mucosal abundance of *Mogibacterium* was higher in the distal gut of human colorectal cancer patients than healthy controls (Chen et al., 2012). Therefore, while the dearth of knowledge concerning the characteristics of *Mogibacterium spp.* in the gut ecosystem make it difficult to speculate as to why SB may affect it, it's reduction may be indicative of favourable changes in the gut microbiota of calves fed SB. Similarly, the abundance of *Actinobacteria* was also significantly lower in the cecum of SB calves, driven by a significant reduction in a genus classified only as part of the *Coriobacteriaceae* family (reported as "f__*Coriobacteriacea*e__" in QIIME). There were several other low-abundance genera assigned to *Coriobacteriaceae* (<0.01%), so this is likely an undescribed genus or genera which may have an important role in the maintenance of gut health. Several novel members of this family have been described recently (Kobayashi et al., 2017, Looft et al., 2015), and further advances in our knowledge of the role of *Coriobacteriaceae* in the gut may resolve the possible role of as-yet undefined *Coriobacteriaceae* species in SB-driven growth improvements. The *Coriobacteriaceae* in the gut have been associated with a suppression in host inflammatory response. Reduced abundance of this family was previously observed in tandem with lower detection of the pro-inflammatory IL-6 in blood plasma (Kemp and Lander, 1984), and so our results may indicate reduced immunogenicity among the cecal microbiota of SB fed calves.

The higher abundance of *Cyanobacteria* observed in the cecum of SB animals was driven by significant increases of a genus assigned to the *YS2* order. This highlights a wider issue concerning 16S rRNA gene investigations of intestinal microbial communities. Although *Cyanobacteria* have been widely reported as minor contributors to GIT microbial diversity in mammals (Meale et al., 2017, Kittelmann et al., 2013, Jenkins et al., 2008), the validity of their role in the anaerobic gut ecosystem is questionable, as many species of this phylum are native to marine environments and are notable performers of complex oxygenic photosynthesis (Ley et al., 2005). Recent studies have revealed that the *Cyanobacteria* found in the gut are genetically

dissimilar to their photosynthetic relatives, and likely diverged prior to the latter developing the capability for photosynthesis (Veneman et al., 2015, Bickerstaffe et al., 1972). Two such novel *Cyanobacteria*-like lineages have been described in the human GIT to date, the *Melainabacteria* (Veneman et al., 2015), and the *Sericytochromatia* (Bickerstaffe et al., 1972), but there is not yet a consensus on the correct nomenclature (Soo et al., 2014). Neither is it known if these novel taxa are also the same *Cyanobacteria*-derivatives present in the ruminant gut, and this warrants urgent investigation. Regardless, increased abundance of *Cyanobacteria* has not been previously reported in the gut of SB supplemented calves, suggesting a potential role of the newly described *Cyanobacteria* groups in the developing intestine, but further work is needed to confirm their role in the ruminant gut ecosystem.

4.4.3 The rumen and hindgut harbour significantly different microbial communities at weaning

While patterns of microbial colonisation in the pre-functioning rumen have been the subject of several investigations recently (Jami et al., 2013, Rey et al., 2013, Malmuthuge et al., 2014, Malmuthuge et al., 2013), there are noticeably fewer published reports concerning the hindgut microbiota of young ruminants. In agreement with the available literature, we found that the rumen and hindgut microbiota differed significantly at weaning (Meale et al., 2017, Meale et al., 2016). In addition to lower rumen bacterial diversity, VFA levels were higher in the rumen than in the colon, suggesting that at weaning, the rumen microbiota ferments plant biomass at a greater rate than that of the hindgut. It is likely that the greater range of secondary fermentation products entering the lower gut is the driver of the increased bacterial diversity of the cecum and colon. The bacterial profile of the rumen was resembled that previously reported in young animals and was dominated by *Firmicutes* and *Bacteroidetes*. *Bacteroidetes* have previously been reported as the predominant bacterial phyla in the rumen and hindgut of 3-week old and weaned diary calves (Malmuthuge et al., 2014, Meale et al., 2016), and in the rumen of 6-week old lambs (Wang et al., 2016b). *Prevotella* was the most abundant bacterial genus in the rumen at weaning which is in agreement with published reports (Meale et al., 2016). Our data showed the principal bacterial phylum *Firmicutes* was dominated by unclassified *Succinivibrionaceae* in the rumen, but that the hindgut regions harboured higher relative abundances of unclassified genera from the *Lachnospiraceae* and *Ruminococcaceae* families while the *Succinivibrionaceae* members were minor contributors. *Succinivibrionaceae* has been reported as a member of the core active rumen microbiota in adult cattle (Li and Guan, 2017), and is implicated in reduced methane formation in both ruminants and macropods (Danielsson et al., 2017, Pope et al., 2011, McCabe

et al., 2015). The predominance of *Prevotella* and *Succinivibrionaceae* has been previously documented in the rumen of adult dairy cows (Dill-McFarland et al., 2017), but the high abundance of uncharacterised *Succinivibrionaceae* in the rumen at weaning has not, to our knowledge, been reported to date. However, caution should be exercised when comparing results of multiple amplicon sequencing surveys, as amplification primer choice can significantly bias results (Nelson et al., 2014). Popova et al. (2013) and Zhou et al. (2014) have previously described the hindgut methanogen populations in lambs and dairy calves, and our findings are largely similar to theirs, with *Methanobrevibacter* as the predominant genus.

Unclassified genera of the *Lachnospiraceae* were previously reported as comprising just 5.58% of faecal 16S rRNA sequences 5 days after weaning, in contrast to our observation of high abundance in the cecum and colon (Meale et al., 2016). The same study revealed high abundance of an unclassified *Ruminococcaceae* genus in the faeces of dairy calves shortly after weaning which is consistent with our results (Meale et al., 2016). Both taxa have been widely reported as important members of the gut microbiota, containing prominent plant polysaccharide hydrolysing species (Flint et al., 2012). Interestingly, visualisation of the phylogenetic tree generated in QIIME shows *Prevotella* sequences recovered from the rumen appeared to cluster away from the other *Bacteroidetes* taxa (Fig. 4.3(a)), suggesting that at weaning the rumen may contain a phylogenetically distinct cohort of *Prevotella spp.* compared to that of the hindgut, where *Prevotella* sequences clustered broadly as expected (Fig. 4.3(b), 4.3(c)). This warrants further investigation, given the ubiquitous and abundant presence of *Prevotella* in the mammalian digestive tract. Also evident in our dataset is the dominance of undescribed microorganisms in the mammalian GIT. Indeed, among the ten most abundant genus level taxa reported in the hindgut regions, only four (*Prevotella, Clostridium, Bacteroides* and *Ruminococcus*) were annotated as a known bacterial genus. This underlines the large number of as-yet uncharacterised bacteria that exist within the mammalian gut, and highlights the inherent difficulties in accurate compositional and functional profiling of the GIT microbiota.

4.5 Conclusions

The data presented here and in our companion study (Pierce, 2014) provide evidence that the improved performance recorded for SB supplemented calves may be mediated through minor changes in the rumen and hindgut microbiota, with a particularly notable response to SB evident in the cecum. However, it is impossible to conclude whether changes in microbial composition are actively contributing to this improved growth and performance, or whether the host

phenotype is driving changes in the microbial community. It is possible that the major effects of exogenous butyrate supplementation on the GIT microbiota may occur during the first weeks of life and are not evident at weaning, and indeed previous work has suggested that for maximum impact, butyrate should be supplemented from the first day of life (Bedford and Gong, 2017). The present study may also be limited by the fact that the calves had already undergone a weaning process (between days 49-56) when the samples were collected, and the amount of exogenous butyrate entering the GIT was thus reduced in the week preceding slaughter. It may be advantageous to collect digesta samples throughout the milk-feeding period in future studies, to assess if SB supplementation may facilitate a smoother weaning transition. Nonetheless, considering the significant differences that were still evident one week following the onset of the weaning process, SB supplementation appears to impart persistent changes on gut microbial composition and fermentation in dairy calves, and may be a candidate additive for "microbial programming" of gut microbial communities in early life (Yanez-Ruiz et al., 2015). In summary, we conclude that positive trends in growth rate and feed efficiency associated with SB supplementation in early life occur in tandem with changes in bacterial composition and fermentation in the hindgut. More thorough investigations using metagenomic or metatranscriptomic approaches may offer further information as to the mechanisms by which sodium butyrate modulates the gut microbial community in young ruminants.

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4.7 Tables and figures

Table 4.1: The effect of SB inclusion in milk replacer on Volatile Fatty Acid (VFA) profiles in the rumen and colon. P-values were obtained using a Monte-Carlo permutational t-test in R.

	Rumen			**Colon**		
Item	CON	SB	P-value	CON	SB	P-value
Total VFA Concentrations (mmol/L)						
Acetate	[1]90.56 ± 7.51	78.46 ± 1.93	[2]*NS*	39.48 ± 3.49	60.84 ± 6.03	0.01
Propionate	62.43 ± 7.51	58.26 ± 1.73	*NS*	10.77 ± 1.20	17.06 ± 2.00	0.02
Butyrate	16.21 ± 1.17	11.49 ± 0.57	0.04	3.56 ± 0.40	5.01 ± 0.84	*NS*
Isobutyrate	0.71 ± 0.37	0.33 ± 0.06	*NS*	0.53 ± 0.05	0.50 ± 0.09	*NS*
Valerate	4.91 ± 0.61	3.43 ± 0.09	*NS*	0.75 ± 0.13	0.85 ± 0.09	*NS*
Isovalerate	1.79 ± 0.28	1.05 ± 0.08	*NS*	0.37 ± 0.06	0.31 ± 0.06	*NS*
Total VFA	176.44 ± 16.02	152.82 ± 3.67	*NS*	55.46 ± 4.87	84.57 ± 8.60	0.02
Molar Proportions of VFA						
Acetate	0.517 ± 0.01	0.513 ± 0.003	*NS*	0.712 ± 0.01	0.721 ± 0.01	*NS*
Propionate	0.346 ± 0.01	0.379 ± 0.004	*NS*	0.192 ± 0.01	0.200 ± 0.01	*NS*
Butyrate	0.094 ± 0.004	0.077 ± 0.004	*NS*	0.064 ± 0.003	0.057 ± 0.01	*NS*
Isobutyrate	0.003 ± 0.002	0.001 ± 0.001	*NS*	0.010 ± 0.001	0.007 ± 0.001	*NS*
Valerate	0.028 ± 0.003	0.023 ± 0.001	*NS*	0.014 ± 0.001	0.010 ± 0.001	*NS*
Isovalerate	0.011 ± 0.002	0.007 ± 0.001	*NS*	0.007 ± 0.001	0.004 ± 0.001	*NS*

[1]Mean±SEM, [2]Not significantly different.

Table 4.2: Comparisons of alpha diversity metrics in the rumen, cecum, and colon of calves at weaning. *P*-values show significant differences according to dietary treatment. Significant differences according to gastrointestinal region are denoted with different letters.

	Chao1				Shannon			
GI Region	Overall	CON	SB	P-value	Overall	CON	SB	P-value
Rumen	1698.0[a]	1887.2	1508.7	0.01	3.6[a]	3.7	3.6	0.15
Cecum	1728.7[a]	1630.3	1827.0	0.30	4.9[b]	4.8	5.0	0.28
Colon	2849.0[b]	2827.3	2870.7	0.87	5.1[b]	5.1	5.1	0.76

Table 4.3: Comparing microbial communities between treatments and gastrointestinal region in the rumen, cecum and colon. *P*-values obtained using PERMANOVA analysis based on Bray Curtis dissimilarity matrices.

Treatment			GI Region		
GI Region	F-value	P-value		F-value	P-value
Rumen	1.04	0.37	Rumen vs. Cecum	21.44	0.001
Cecum	1.30	0.16	Rumen vs. Colon	21.15	0.001
Colon	1.28	0.12	Cecum vs. Colon	0.82	0.700

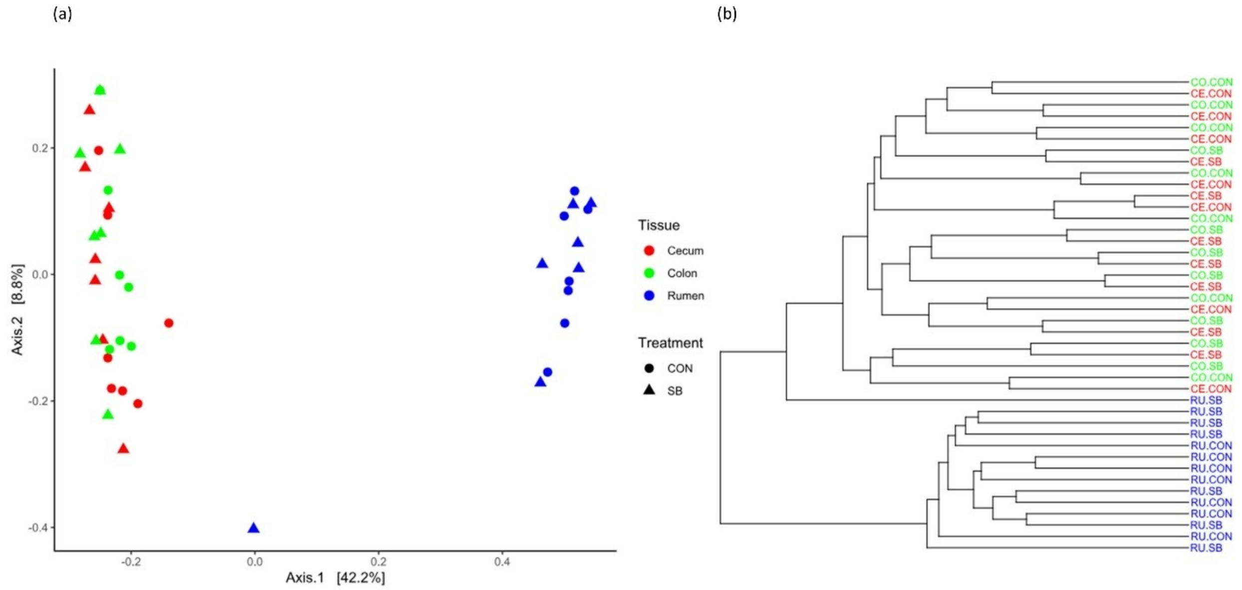

Figure 4.1: (a) Principal Coordinate Analysis plot and (b) cluster dendrogram plot generated using a Bray-Curtis dissimilarity matrix of operational taxonomic units (OTUs) present in the rumen, cecum, and colon at weaning in calves fed milk replacer ± sodium butyrate.

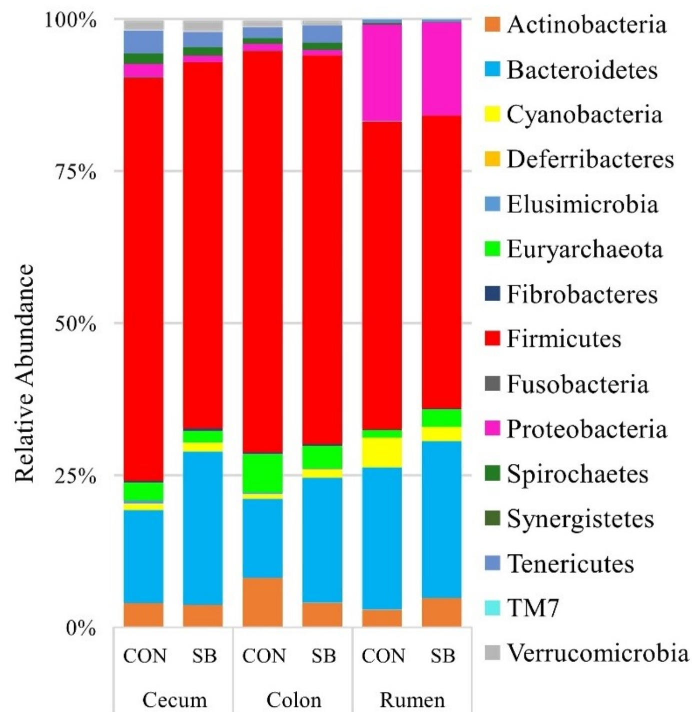

Figure 4.2: Stacked bar chart of microbial abundances at the phylum level, calculated as a percentage of total 16S rRNA reads within each group.

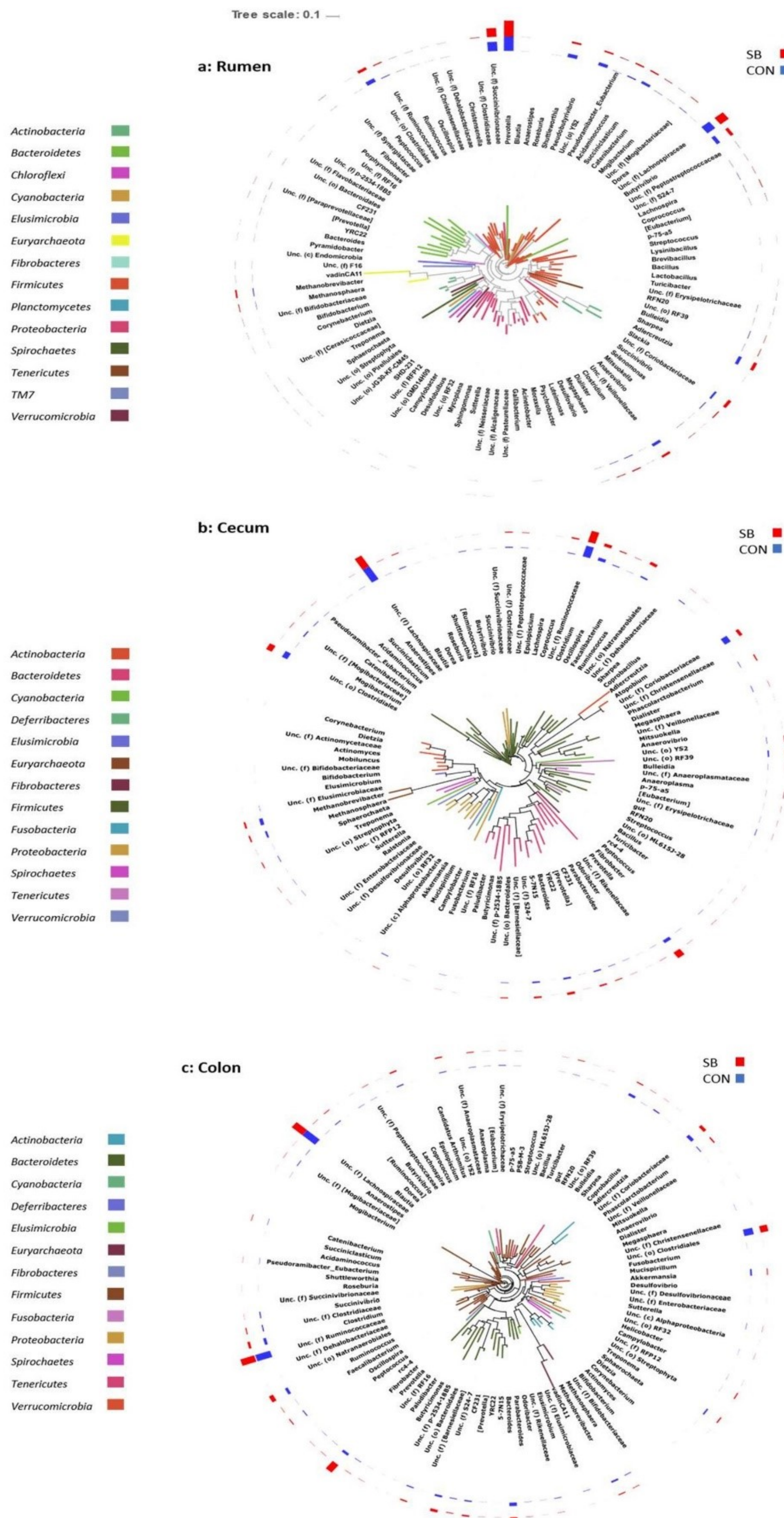

Figure 4.3: Phylogenetic Trees of (a) rumen, (b) cecum, and (c) colon microbiota, built from a multiple sequence alignment generated in QIIME. OTUs were agglomerated at the genus level in R. The trees were visualised using the Interactive Tree of Life (ITOL) software package

Chapter Five
General Discussion

5.1 Background

There is increasing evidence that dietary modification in the first days and weeks of life may offer an opportunity to permanently manipulate rumen microbial composition and function, in order to improve nutrient utilisation and/or reduce wasteful processes like methanogenesis (Abecia et al., 2017, Abecia et al., 2014, Abecia et al., 2013). However significant gaps in the knowledge remain concerning the optimum timeframe for such interventions, and existing data is largely drawn from studies in small ruminants and dairy calves. Furthermore, despite the importance of proper physical maturation of the rumen during early life, the molecular mechanisms underpinning its development during microbial colonisation are largely unknown in calves. The data presented in this thesis outlines the ontogeny of the rumen microbiota (Chapter 2) and the molecular dynamics of physical rumen development (Chapter 3) during early life. These findings fill a major gap in the knowledge, providing an integrative understanding of the co-development of both host and microbe in the young ruminant, which will be key in optimising potential modification strategies. Moreover, these are among the first comprehensive investigations of the developing rumen and its microbial populations in beef calves.

In contrast to the rumen, little is known regarding the composition and activity of the hindgut microbiota during early life, though this was partially addressed in a recent study (Song et al., 2018). There is increasing evidence that the lower GIT, and its resident microbiota, make important contributions to animal health (Malmuthuge and Guan, 2017). As such, targeting the function of the hindgut microbiota is an alternative approach to improving calf health and performance during rumen development in the pre-weaning period. Chapter 4 examined the effect of a butyrate-enriched milk replacer on animal performance and rumen and hindgut microbiota in dairy calves, showing an association between elevated growth and microbial community composition and fermentation in the hindgut in response to exogenous butyrate. Overall, the data presented in this thesis contribute to our fundamental understanding of rumen microbial composition and molecular development during early life (Chapters 2 & 3) and highlights that enhanced activity of the hindgut microbiota may contribute to early life calf growth (Chapter 4).

5.2 Understanding microbial establishment and development in the developing rumen

5.2.1 Does mode of delivery influence the rumen and its resident microbes?

At present there is extensive disagreement surrounding the validity of the "in utero colonisation" hypothesis in mammals, whereby the mammalian GIT acquires a microbiota prior to birth (Perez-Muñoz et al., 2017), and the source of the initial microbial inoculum of the rumen is unknown. While there is some evidence of a microbial presence in the bovine uterus (Machado et al., 2012, Santos and Bicalho, 2012), both the foetal environment and GIT were recently shown to be sterile during the third trimester of pregnancy in cattle (Malmuthuge and Griebel, 2018). This indicates that microbial colonisation of the rumen occurs during or after birth. However, in this thesis (Chapter 2), attempts to characterise the pioneer rumen microbiota of new-born calves delivered naturally or via elective caesarean section were unsuccessful, possibly due to low microbial density which rendered our sampling procedure (swabbing of the interior rumen surfaces) insufficient. In order to examine the effect of the birthing process on rumen colonisation in new-borns, it may be necessary to collect the entire rumen compartment as previously reported (Malmuthuge, 2016). Studies in neonates might also be limited by external contamination, as samples cannot be collected under completely sterile conditions. Moreover, commercial reagents used in laboratory analysis are known sources of contamination, which could be magnified in studies of low-density microbial communities (Hilali et al., 1997, Corless et al., 2000). Every reasonable precaution was taken to ensure that no environmental microbiota contaminated our samples, so it may be that microbial colonisation of the rumen only occurs following birth, from maternal or environmental microbial reservoirs. The use of RNA-based approaches may also be preferential, as these will assess only the active (live) microorganisms present.

A recent study in dairy calves reported that while there was a measurable longitudinal effect of delivery process and other maternal factors on the rumen microbiota, this was outweighed by other factors like diet and age (Cunningham et al., 2018), and there was not a major effect of birthing process on the transcriptomic profile of the rumen wall as discussed in Chapter 3. Therefore, it may be beneficial to focus future research efforts toward postnatal colonisation patterns rather than further work concerning in utero colonisation, or exposure to maternal birth canal microbiota.

5.2.2 Understanding colonisation and succession among the early rumen microbiota

Identification of the precise "window of opportunity" for early life rumen manipulation, outside of which intervention is ineffective, is currently of great priority (Huws et al., 2018). Most studies to date used rumen fluid sampling from dairy calves, which may not be sufficient to describe the full extent of rumen microbial diversity (Rey et al., 2014, Jami et al., 2013, Cammack et al., 2018). Examining the temporal development of both rumen solid and liquid digesta microbiota during early life in Chapter 2 revealed that establishment patterns of bacteria and archaea was highly similar across rumen fraction after day 7, despite previous reports to the contrary (Sadet et al., 2007, Deusch et al., 2017). This discrepancy may be explained by different methods of fraction separation, and also by the fact that most previous studies were performed in adult ruminants. Nonetheless, based on these findings and another recent study in dairy calves (Dill-McFarland et al., 2018), it may not be necessary to analyse solid- and liquid-phase microbiota separately during early life.

The temporal data showed that the pioneer rumen microbiota settled by day 21, suggesting that for optimum effectiveness, interventions to modify the rumen microbial community must occur prior to this point. In practise, it is likely that to be effective, dietary modification/supplementation should begin as early as possible after birth, as we observed a migration toward a mature-like composition even in the first week of life. Moreover, that a major difference across rumen fractions was only observed on day 7 also indicates that the first week of life might be a critical period for intervention, to take advantage of the more heterogenous microbiota. Future investigations should also record solid feed intakes, as this was a limitation of this study work, given the major influence of diet on rumen microbial composition (Henderson et al., 2015). These data are further limited by the fact that VFA profiles could not be analysed. This should be prioritised in future studies, as it offers insight into changes in microbial function to accompany those found in microbial composition using 16S rRNA sequencing. The microbial succession process evident in Chapter 3, whereby major rumen taxa become established in the first 3 weeks of life, should also be validated using qPCR, as amplicon sequencing is biased by gene copy number variation (Louca et al., 2018). Moreover, samples were only available at three timepoints prior to settlement of the microbiota, so future studies should include more sampling points to clearly define the precise "window of opportunity" for intervention.

It was unavoidably necessary to relocate heifers in replicates 3 and 4 to a second research facility for calving and subsequent calf rearing. We noted a substantial farm effect on the microbial communities of the rumen throughout early life. Both bacterial and archaeal communities were influenced by farm, but the effect of farm on the archaea appeared to outweigh that of age.

Animals raised on F1 harboured high abundances of *Mbb. smithii* throughout early life, and greater abundances of *Mbb. boviskoreani* on days 28 and 96, indicating an evolution of the farm effect with advancing age. These findings are particularly relevant as the methanogens are often the target of efforts to manipulate the rumen microbiota to reduce methane emissions (Abecia et al., 2013). Future experimental manipulation of the rumen microbiota should be replicated at multiple locations, to ensure consistency of effect. These studies should also include methane measurements where possible, to assess if a farm effect on the rumen archaeal composition corresponds to changes in methane production.

It is difficult to define exactly the source of this farm effect. As detailed in Chapter 2, heifers and calves received the same dietary and veterinary care pre- and post-partum and were housed in a similar manner. A farm effect on the rumen microbiota has been reported previously (Weese and Jelinski, 2017, Indugu et al., 2017), but as in the present study, the authors could not point to any significant geographical or management factors which may have underpinned this. One difference which may have contributed to this discrepancy was that calves on F2 were housed in the same barn as the cows (with no physical contact after 48h), while those on F1 were housed in a different building. Previous studies have reported that maternal contact is a significant determinant of microbial composition in the rumen (Cunningham et al., 2018, Fonty et al., 1989, Stewart et al., 1988). Whatever the underlying reason might be, these are, to the best of our knowledge, the first data to show significant differences in rumen colonisation patterns among animals raised on different farms, and these findings must be considered in future attempts at redirection.

5.2.3 Understanding ruminal transcriptomic dynamics during early life

Proper physical maturation of the rumen is critical to the lifetime performance of the animal. The close relationship between the rumen and its resident microbiota indicates the need to study both to fully understand the implications of microbial shifts during early life (Chapter 2) for the host animal. Studies in young bovines to date have mostly used dairy bull calves (Naeem et al., 2014, Jiao et al., 2016). Therefore Chapter 3 examined the molecular mechanisms underpinning ruminal ontogeny during early life in heifer and bull beef calves. There was a major effect of location on the rumen microbiota, but the transcriptome profile was unaffected, indicating that microbial shifts may not necessarily be important to the host, possibly due to the functional redundancy of the microbiota (Weimer, 2015). Furthermore, Chapter 2 showed that diet was a major driver of microbial changes during early life, but this was less evident for the

transcriptome, with limited temporally adjacent changes evident after day 7. Genes and pathways involved in VFA absorption and metabolism increased linearly during early life, probably driven by elevated starter consumption as previously reported (Jiao et al., 2016). However, functions which underpin the morphological development of the rumen (cellular proliferation, apoptosis) did not appear to change until post-weaning, indicating that high levels of solid feed consumption, and/or a greater density of bacterial life (as reported in Chapter 2) might be necessary for significant papillary growth. Calf starter consumption and VFA profiles were not recorded in the present study, which limits analysis of these potentially important factors, and this could be addressed in future studies.

There was a striking upregulation of immune-related genes and processes in the rumen wall following birth, corresponding to microbial colonisation within the first week as discussed in Chapter 2. The role of the rumen in host immunity is poorly understood, but the tolerogenic relationship between the host and microbes is one which could potentially be manipulated for the purposes of microbial programming in rumen (Yanez-Ruiz et al., 2015). Existing knowledge is largely drawn from qPCR studies in small ruminants and dairy calves (Liu et al., 2015, Liu et al., 2013, Malmuthuge et al., 2013, Malmuthuge et al., 2012). This enrichment of host immune processes did not appear to be influenced by diet after the first week, as there was little change in activity of immune-related genes or pathways from day 7 onward. This indicates that host-microbial homeostasis in the rumen may be mediated less by salivary Ig and more extensively by host-expressed genes than previously speculated (Fouhse et al., 2017). Taken together, these data point to extensive activity of cellular immune genes and pathways in the rumen tissue during early life and post-weaning. Moreover, previous work has shown that starter consumption might increase rumen permeability (Aschenbach and Gäbel, 2000), but we found TJP gene expression generally remained constant throughout early life, indicating that beef animals may be more resistant to grain-induced ruminal dysfunction. Correlation analysis (not shown) of differentially expressed genes in rumen tissue with the differentially abundant taxa in the digesta did not show any significant associations, indicating that rumen development might be more closely related to microbial function rather than simply microbial composition. It might also be more influenced by the epimural microbiota, which should be assessed in future studies. Finally, the mature rumen comprises four clearly delineated layers (Fig. 1.2), and gene expression varies spatially across these layers. The data in this thesis was derived from transcriptomics of a cross section of the entire rumen wall, and so represent gene expression profiles in all four layers of the rumen epithelium. Devising a method to effectively separate the four rumen epithelial layers, even during early life development, will allow these data to be validated.

5.2.4 Butyrate supplementation modifies hindgut microbiota and fermentation

Gastrointestinal disorders are the leading cause of preweaned calf mortality (USDA, 2010), and are therefore of major economic concern. Conventional antibiotics have traditionally been widely applied to prevent or reduce occurrence of gut infections in young calves (Trevisi et al., 2014). However, concerns surrounding the emergence of antibiotic resistant pathogens and their subsequent transfer to the food chain have led to much interest in the development of natural and synthetic alternatives to promote enhanced gut health during early life. Butyrate is a VFA produced by the gut microbiota, and has shown promise in improving gut health and animal performance in a range of livestock species (Bedford and Gong, 2017). Despite knowledge of the close relationship between gut function and the resident microbiota, there was little data concerning the impact of butyrate supplementation on gut microbial communities. Offering dairy calves a milk replacer enriched with protected butyrate during early life (Chapter 4) tended to improve both growth rates and feed efficiency at weaning. This was accompanied by an increase in VFA concentrations in the colon of supplemented animals, compared to untreated calves, indicating that enhanced hindgut fermentation could contribute to the performance improvements under exogenous butyrate supplementation. Moreover, while butyrate concentration in the hindgut was unchanged by treatment, there was a decrease in the abundance of several native butyrate producing bacteria (e.g. *Butyrivibrio*) in the cecum. This suggests that the microbial community responded to butyrate supplementation by suppressing microbial biosynthesis of this acid. While this is an intriguing finding from a microbiological perspective, it may be a limiting factor for the effectiveness of butyrate as a feed supplement to promote gastrointestinal health. This study was limited in that the calves had undergone a weaning process in the week prior to slaughter, reducing the amount of exogenous butyrate present at sample collection. However, this in itself indicates that feeding organic acids during early life might be a mechanism for persistent change of the gut microbiota. Further studies using metagenomic or metatranscriptomic approaches might offer deeper insight into the mechanisms by which the early life hindgut microbiota respond to exogenous butyrate and contribute to host growth. Moreover, examination of the mucosal microbiota may offer further clues as to the extent to which host-microbial interactions modulate the effect of exogenous dietary butyrate.

5.3 Caveats, implications, and future directions

The data presented in this thesis offers more fundamental understanding of early life dynamics of rumen and hindgut microbiota, and molecular control of rumen development. There

are several caveats to these studies, which must be noted when considering their application outside of a research setting. High-throughput sequencing efforts are known to be subject to a range of biases, including method of sample collection (Paz et al., 2016), method and duration of sample preservation prior to analysis (Granja-Salcedo et al., 2017), and choice of nucleic acid extraction protocol (Henderson et al., 2013, Villegas-Rivera et al., 2013). Furthermore, a large variety of bioinformatic tools has been developed for the analysis of high-throughput sequencing data in recent years but have not been widely compared for their consistency.

The studies outlined in Chapters 2 and 4 relied on 16S rRNA gene surveys to generate a snapshot of bacterial and archaeal populations in the rumen and hindgut. While rapid and cost-effective, amplicon sequencing is not quantitative, and offers limited resolution beyond the genus level, particularly for bacteria. Primer biases inherent to amplicon sequencing were discussed in Chapter 1. The studies outlined in both Chapters 2 and 4 used a primer set which targeted the V4 hypervariable region of the 16S rRNA gene (515F/806R). This primer pair was originally chosen as it allows for simultaneous investigation of both bacterial and archaeal communities using a single amplicon (Caporasso et al., 2011), and a modified version is recommended by the Earth Microbiome Project (www.earthmicrobiome.org/). However, previous studies have shown that combined amplification of archaeal and bacterial 16S rRNA domains may not be accurate, and there are concerns that V4 amplification is biased against the *Methanobrevibacter gottshalkii* clade (Klindworth et al., 2013, Fischer et al., 2016, Zhou, M., personal communication). Therefore, for the study detailed in Chapter 2, we selected a second primer pair (915aF/1386R) to amplify the V6-V8 region of the archaeal 16S rRNA gene, previously reported as the region of choice to study the rumen archaea (Snelling et al., 2014). For the analysis described in Chapter 4, the combined (515F/806R) primer was retained for co-analysis of the bacteria and archaea, and so this may have biased our findings. It is advisable that to avoid this, future studies should amplify bacterial and archaeal 16S rRNA gene fragments separately.

We must also consider that amplicon sequencing does not offer the opportunity to robustly assess microbial function within an ecosystem. While tools like CowPi (Wilkinson et al., 2018), PICRUSt (Langille et al., 2013), and Tax4Fun (Abhauer et al., 2015) allow predicted function to be inferred from amplicon sequencing data, these are extrapolated from the compositional profile and so are subject to the same biases described above and in Chapter 1. Metatranscriptomics has recently been applied in the rumen to verify the existence of a relationship between the active rumen microbiota and feed efficiency (Li and Guan, 2017). A similar survey of the early rumen microbiota would offer a more comprehensive picture of microbial composition and function

during early life, its contribution to rumen development, and serve to verify our findings. This would also allow for concurrent investigation of the rumen eukaryotes (i.e. fungi and protozoa) that play important roles in fibre degradation, but which were not assessed in this thesis. Moreover, such an approach could elucidate if compositional variation due to farm environment or diet is replicated in the functional profile, which may be a more favourable way to assess changes in the rumen microbial environment moving forward.

This range of potential sources of variation in studies of the ruminant gut microbiome suggests that there is an urgent need for comprehensive discussion between research groups internationally to standardise all protocols, from sample collection and storage through to laboratory processing, sequencing, and data analysis. Steps have been taken in this regard in recent years with the formation of international research consortiums like Ruminomics (http://www.ruminomics.eu/) and the Rumen Microbial Genomics Network (http://www.rmgnetwork.org/). Further expansion of these forums will allow for reliable comparisons of published literature, but in the meantime, scientists should remain reticent of these potential biases when comparing results obtained across different studies.

In a larger context, there is perhaps an overreliance currently on reporting associative interactions between host phenotypes and taxonomy of the rumen microbial community (e.g. feed efficiency (Ellison et al., 2017, Carberry et al., 2012, McGovern et al., 2018), though this is not limited only to studies of the rumen microbes. Such studies (including those presented in this thesis) typically produce a list of "biomarker" taxa, associated with a particular diet/age/disease state etc., but often without any clear biological relevance to the study at hand (Surana & Kasper, 2017). With such an approach, it cannot be concluded if microbial changes are a driver or a product of phenotypic variation. There is scant evidence of any robust cause-effect relationships between the microbiome and host phenotype in ruminants, and for all the recent advances in our knowledge of the rumen microbiome, including during early life, much remains unknown. For instance, the "million-dollar question" is unanswered: what is the "ideal" rumen microbiome? Can it be determined if one exists? And if so, can the microbial colonisation patterns of a young animal be modulated effectively enough to ensure the desired community becomes established? The vast functional redundancy among the microorganisms makes it unlikely that the removal of a small number of bacterial groups from the rumen would have any lasting impact on community function or host metabolism (Weimer, 2015). Conversely, in order to seed a more favourable microbiota, functional niches for these microbial groups to occupy would need to be available, so measuring the effectiveness of manipulation via functional changes rather than taxonomic

changes is preferable. Early life manipulation is a promising strategy to improve host production, but much remains to be discovered in this regard. A shift in thinking from associative to causal relationships between the microbe and host traits will likely be required for the field of "Rumen-Omics" to contribute significantly to enhanced production strategies. A limited number of recent studies have proposed a degree of host genetic control over the rumen microbiota (Sasson et al., 2017, Roehe et al., 2016), but it is unknown if host genetics might influence colonisation patterns during early life. If strongly defined heritable relationships between the host and the early microbiome can be fully elucidated, it might be possible to target the host (e.g. via genetic selection) to improve the microbiome, rather than vice versa, as is the current practise. It is likely that multi-omic frameworks incorporating a several datasets may be necessary to fully elucidate causal relationships between host and microbe in the ruminant gut, and future studies of the developing rumen microbiota should also include other omics data (e.g. host genotype, metabolomics etc.) in their analysis where possible, to define such relationships.

In summary, and despite the limitations described above, this thesis contributes fundamental knowledge concerning early life dynamics of the rumen and hindgut microbiota, as well as the molecular mechanisms underpinning rumen development in young calves. The data discussed in Chapter 2 indicates that the optimum timeframe for early life manipulation occurs within the first three weeks of life, and that the first week of life could be key. This is the first study to show that the early life rumen microbiota might be significantly influenced by local environment, a finding which warrants urgent validation. Lack of reproducibility due to a farm effect could be a significant limiting factor in efforts to module the rumen microbiota in large-scale beef or dairy operations. Chapter 3 shows that the rumen transcriptome evolves with age, but to a lesser degree than the microbiota. There was no measurable impact of farm at the transcriptomic level, indicating that it might be somewhat more resilient to external pressures than the microbiota. The substantial enrichment of immune-related functions following birth has not been reported previously, to our knowledge, and building on this knowledge might offer a mechanism to selectively inhibit certain microbial groups from becoming established. The microbial signature associated with exogenous butyrate supplementation in Chapter 4 indicates that the hindgut microbiota may also be a promising target to improve animal performance during the milk-feeding period. These microbial data require validation at the RNA level, and/or quantitative analysis using qPCR. The transcriptomic data should be verified using proteomic analysis, as mRNA expression does not necessarily reflect protein production. Nonetheless, the studies detailed in this thesis provide further understanding of the co-evolution of the rumen and its resident microbiota during the first weeks of life, and indicate that manipulation of the hindgut

microbiota might offer further possibilities to improve animal performance via early life management.

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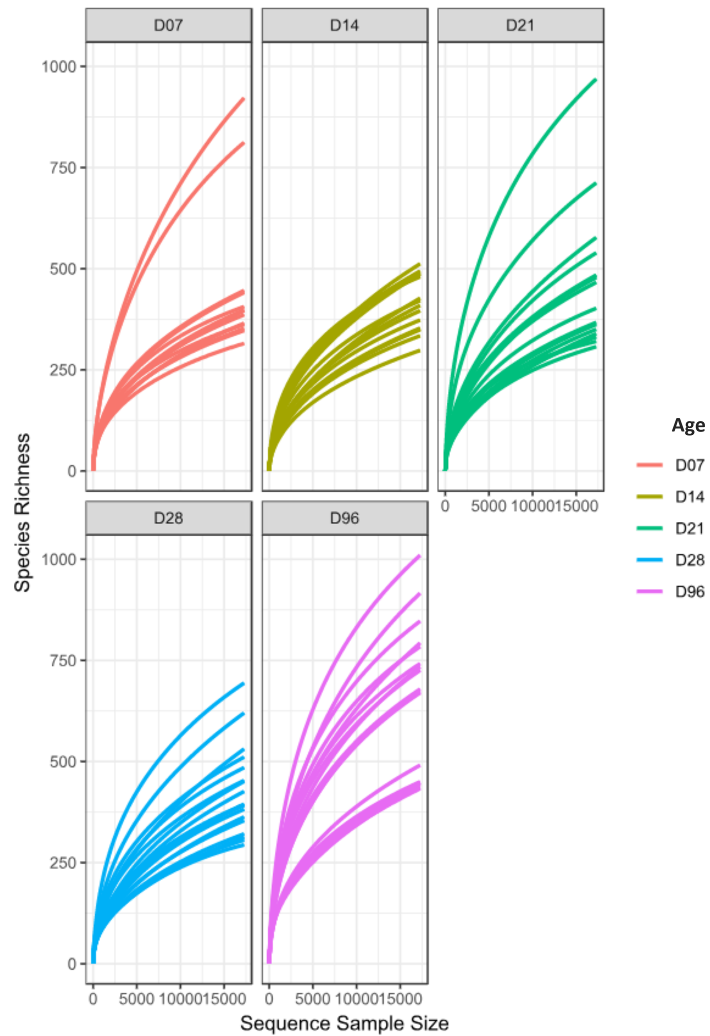
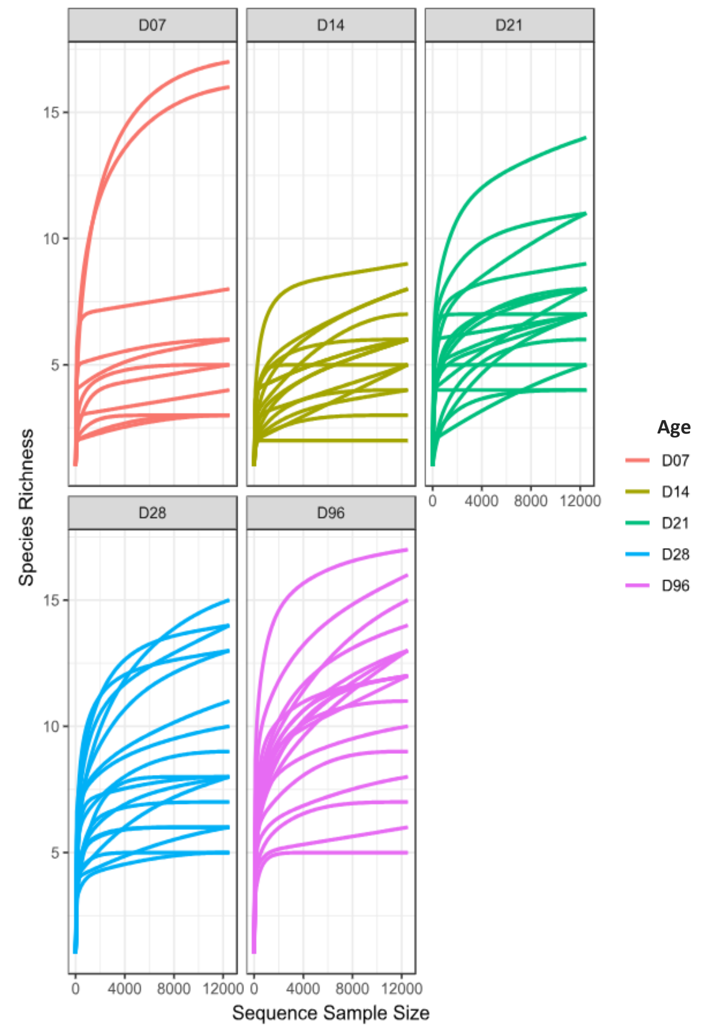
Appendices

Appendix A: Rarefaction curves of rumen digesta (a) bacterial and (b) archaeal 16S rRNA amplicon sequencing libraries during early life.

(a)



(b)



Appendix B: Details of bacterial phylum abundances and temporal changes in the rumen digesta during early life revealed by amplicon sequencing. Values are the percentage abundances calculated as a proportion of the total bacterial population. *P*-values were calculated from temporally adjacent contrasts in DeSeq2.

Rumen solid digesta										
	Mean Abundances						**P-values**			
Phylum	**D07**	**D14**	**D21**	**D28**	**D96**	**SEM[1]**	**D7-D14**	**D14-D21**	**D21-D28**	**D28-D96**
Firmicutes	27.89	50.98	49.25	42.91	51.29	3.95	<0.01	0.88	0.99	0.14
Bacteroidetes	47.36	32.71	29.49	36.37	28.46	3.05	0.15	0.81	0.61	0.74
Actinobacteria	5.43	9.54	8.05	8.50	6.89	0.63	0.03	0.81	0.99	0.94
Proteobacteria	11.86	2.98	7.69	6.77	5.73	1.30	0.02	0.38	0.99	0.20
Fibrobacteres	1.40	1.00	1.12	1.77	2.72	0.28	0.62	1.00	0.99	0.72
Spirochaetes	0.57	0.76	1.44	1.59	2.11	0.25	0.90	0.81	0.99	0.74
Cyanobacteria	0.11	0.16	1.17	0.65	0.98	0.19	0.62	0.04	0.99	0.03
Tenericutes	0.15	0.08	0.14	0.42	0.78	0.12	0.60	0.81	0.99	0.05
Verrucomicrobia	0.56	0.58	0.46	0.45	0.41	0.03	0.42	0.97	0.99	0.74
TM7	0.03	0.02	0.03	0.02	0.20	0.03	0.16	0.34	0.37	<0.01
Synergistetes	0.06	0.19	0.54	0.12	0.16	0.07	0.89	0.31	0.97	0.44
Planctomycetes	0.02	0.01	0.42	0.32	0.11	0.07	NA	NA	NA	NA
Elusimicrobia	ND	0.16	0.02	0.04	0.07	0.02	NA	NA	NA	NA
Lentisphaerae	ND	0.01	0.05	0.02	0.04	0.01	<0.01	0.34	0.99	0.03
Fusobacteria	4.47	0.82	0.04	0.04	0.02	0.77	0.06	<0.01	0.99	0.08
Chloroflexi	ND	ND	ND	ND	0.01	<0.01	0.90	0.88	0.99	0.30

WPS-2	ND	ND	0.07	ND	0.01	0.01	NA	NA	NA	NA
SR1	0.06	ND	ND	ND	ND	0.01	0.38	1.00	0.99	0.94
[Thermi]	0.01	ND	ND	ND	ND	<0.01	0.60	1.00	0.99	0.94
GN02	0.01	ND	ND	ND	ND	<0.01	0.42	1.00	0.99	0.94
Rumen Liquid Digesta										
	Mean abundances						**P-values**			
Phylum	**D07**	**D14**	**D21**	**D28**	**D96**	**SEM**	**D7-D14**	**D14-D21**	**D21-D28**	**D28-D96**
Firmicutes	19.05	51.74	42.06	39.49	45.15	4.93	<0.01	0.06	0.99	0.05
Bacteroidetes	51.33	31.41	38.16	38.69	30.91	3.30	0.03	0.94	0.92	0.75
Proteobacteria	19.75	4.01	10.61	10.92	7.59	2.34	<0.01	0.06	0.92	0.49
Cyanobacteria	0.05	0.06	1.59	1.03	4.50	0.73	0.72	<0.01	0.92	<0.01
Actinobacteria	2.80	7.71	4.66	5.49	4.27	0.72	0.01	0.20	0.92	0.48
Spirochaetes	0.27	0.23	0.99	1.23	2.05	0.30	0.70	0.65	0.92	0.48
Tenericutes	0.21	0.10	0.13	0.17	2.00	0.33	NA	NA	NA	NA
Fibrobacteres	0.85	0.52	0.61	1.10	1.37	0.14	0.41	0.76	0.92	0.56
Verrucomicrobia	3.05	2.96	0.43	1.51	0.97	0.47	0.72	<0.01	0.13	0.56
TM7	0.01	ND	ND	ND	0.41	0.07	0.02	0.26	0.92	<0.01
Elusimicrobia	ND	0.50	0.03	0.11	0.28	0.08	<0.01	<0.01	0.92	0.02
WPS-2	ND	ND	0.02	ND	0.18	0.03	0.19	0.02	<0.01	<0.01
Synergistetes	0.06	0.54	0.53	0.13	0.13	0.09	NA	NA	NA	NA
Planctomycetes	0.01	ND	0.12	0.10	0.12	0.02	0.11	0.02	0.92	0.03
Lentisphaerae	ND	0.01	0.04	0.01	0.06	0.01	0.99	0.79	0.92	<0.01
Chloroflexi	ND	ND	ND	ND	0.01	<0.01	0.99	0.85	0.92	0.04
Fusobacteria	2.47	0.20	0.02	0.01	ND	0.43	<0.01	<0.01	0.92	0.08
[Thermi]	0.02	ND	ND	ND	ND	<0.01	<0.01	0.24	0.99	<0.01
SR1	0.05	ND	ND	ND	ND	0.01	0.20	0.65	0.92	0.90

GN02	0.01	ND	ND	ND	ND	<0.01	0.33	0.81	0.92	0.89

[1]SEM = Standard Error of the Mean.

Appendix C: Genus-level bacterial composition of the rumen digesta during early life as revealed using amplicon sequencing. Values are mean abundances calculated from the total number of bacterial reads within each sample. Only genera contributing ≥ 1% of total abundance at one timepoint are presented. P-values were calculated from temporally adjacent comparisons in DeSeq2.

Rumen Solid Digesta										
	Mean Abundances						**P-values**			
Genus	**D07**	**D14**	**D21**	**D28**	**D96**	**SEM**	**D7-D14**	**D14-D21**	**D21-D28**	**D28-D96**
Prevotella	9.90	23.67	24.97	30.13	19.90	3.02	0.06	0.81	1.00	0.12
o. Clostridiales	5.26	1.35	4.92	4.67	10.80	1.35	0.01	0.01	1.00	0.21
Butyrivibrio	0.33	0.41	6.74	3.28	7.00	1.30	0.96	<0.01	1.00	0.83
f. Coriobacteriaceae	0.06	2.27	6.45	6.79	6.30	1.21	<0.01	0.31	1.00	0.93
f. Lachnospiraceae	2.27	5.62	3.67	4.82	5.60	0.57	0.96	0.21	1.00	0.81
f. Ruminococcaceae	1.34	2.02	2.14	2.04	5.30	0.63	0.68	0.72	1.00	0.01
Succiniclasticum	1.56	0.48	3.25	2.56	5.10	0.69	0.04	0.01	1.00	0.53
o. Bacteroidales	2.07	0.26	2.05	3.05	4.80	0.66	0.13	0.13	1.00	0.18
f. Succinivibrionaceae	<0.01	0.20	0.15	0.08	4.20	0.73	0.10	0.44	1.00	0.01
Megasphaera	<0.01	8.31	4.54	5.13	3.00	1.22	<0.01	0.61	1.00	0.25
Fibrobacter	1.44	1.02	1.14	1.81	2.80	0.29	0.68	0.88	1.00	0.58
Ruminococcus	0.73	2.15	4.10	3.06	2.60	0.50	NA	NA	NA	NA
Treponema	0.50	0.69	1.13	1.07	2.00	0.23	0.85	0.63	1.00	0.29
Sharpea	<0.01	<0.01	0.23	0.16	1.60	0.28	0.97	<0.01	1.00	0.02
YRC22	0.01	0.05	0.08	0.08	1.50	0.25	0.67	0.58	1.00	0.06
f. S24-7	<0.01	0.01	1.11	1.65	1.40	0.31	0.02	<0.01	1.00	0.87

Acidaminococcus	<0.01	2.52	2.14	1.94	1.10	0.40	<0.01	0.76	1.00	0.25
Mitsuokella	<0.01	0.17	0.71	0.40	1.10	0.18	<0.01	0.48	1.00	0.65
Shuttleworthia	<0.01	0.04	<0.01	<0.01	1.10	0.20	0.19	0.44	1.00	<0.01
Coprococcus	0.31	0.08	0.12	0.06	1.00	0.16	0.04	0.96	1.00	<0.01
o. YS2	<0.01	<0.01	1.08	0.55	0.90	0.20	0.97	<0.01	1.00	0.12
Succinivibrio	0.03	0.37	5.62	5.49	0.90	1.13	<0.01	0.57	1.00	<0.01
o. RF39	0.11	0.03	0.06	0.35	0.70	0.12	NA	NA	NA	NA
BF311	0.04	0.05	0.07	0.10	0.60	0.10	0.96	0.81	1.00	0.25
Catenibacterium	<0.01	4.14	4.73	4.75	0.60	0.94	<0.01	0.91	1.00	0.01
Bifidobacterium	0.01	5.90	1.31	1.58	0.60	0.93	<0.01	0.06	1.00	0.15
Bulleidia	<0.01	0.04	0.59	0.35	0.60	0.11	<0.01	0.09	1.00	0.86
Lachnospira	0.02	2.39	1.00	1.43	0.50	0.36	<0.01	0.06	1.00	0.19
f. Veillonellaceae	0.49	0.66	1.57	0.28	0.50	0.20	0.42	0.39	1.00	0.52
[Eubacterium]	<0.01	0.24	0.54	0.23	0.50	0.09	<0.01	0.57	1.00	0.52
f. [Mogibacteriaceae]	0.78	0.18	0.44	0.89	0.50	0.11	0.02	0.33	1.00	0.46
f. [Paraprevotellaceae]	0.24	1.13	0.34	0.48	0.50	0.14	0.64	0.98	1.00	0.45
Dialister	0.71	0.30	1.46	1.32	0.30	0.22	0.68	1.00	1.00	0.05
Roseburia	<0.01	4.00	1.83	1.40	0.30	0.63	<0.01	0.57	1.00	0.04
f. RFP12	<0.01	<0.01	0.33	0.40	0.30	0.08	0.02	<0.01	1.00	0.65
f. Christensenellaceae	0.02	<0.01	0.05	0.01	0.30	0.05	0.25	0.12	1.00	0.01
Desulfovibrio	0.58	0.32	0.29	0.53	0.30	0.06	0.54	0.63	1.00	0.81
Mogibacterium	0.17	0.11	0.14	0.17	0.20	0.02	0.24	0.51	1.00	0.89
Pseudobutyrivibrio	0.19	0.45	0.23	0.04	0.20	0.06	NA	NA	NA	NA
o. RF32	<0.01	0.14	0.02	0.01	0.20	0.04	<0.01	0.25	1.00	0.01
f. F16	<0.01	<0.01	0.01	<0.01	0.20	0.03	0.41	0.37	0.59	<0.01
Oscillospira	0.93	0.53	0.20	0.36	0.20	0.12	0.04	0.03	1.00	0.51

Pseudoramibacter_Eubacterium	0.01	0.29	0.65	0.39	0.20	0.10	<0.01	0.35	1.00	0.11
Moryella	<0.01	<0.01	0.01	0.05	0.20	0.03	0.87	0.14	1.00	0.07
Sphaerochaeta	0.04	0.04	0.28	0.51	0.20	0.08	0.69	0.10	1.00	0.10
Pyramidobacter	0.05	0.18	0.52	0.09	0.10	0.08	0.97	0.10	1.00	0.46
f. Clostridiaceae	<0.01	<0.01	0.01	0.08	0.10	0.02	NA	NA	NA	NA
Atopobium	0.08	0.04	0.01	<0.01	0.10	0.02	0.54	0.10	0.56	<0.01
f. Pirellulaceae	0.01	<0.01	0.42	0.33	0.10	0.08	<0.01	<0.01	1.00	0.38
Blautia	0.21	1.50	0.91	0.39	0.10	0.23	0.01	0.13	1.00	0.03
[Prevotella]	10.02	0.27	0.22	0.32	0.10	1.75	NA	NA	NA	NA
CF231	5.98	1.81	0.24	0.44	0.10	0.99	NA	NA	NA	NA
Clostridium	0.24	0.19	0.34	0.70	0.10	0.10	0.01	0.57	1.00	0.03
Anaerovibrio	0.14	0.75	0.12	0.49	<0.01	0.12	NA	NA	NA	NA
Ruminobacter	<0.01	0.12	1.02	0.05	<0.01	0.18	NA	NA	NA	NA
o. Streptophyta	0.07	0.10	0.06	0.06	<0.01	0.01	NA	NA	NA	NA
Selenomonas	0.03	0.60	0.05	0.07	<0.01	0.10	0.14	0.13	1.00	0.24
Bacteroides	13.60	4.68	0.50	0.36	<0.01	2.31	<0.01	<0.01	1.00	<0.01
Anaerostipes	0.05	<0.01	0.43	0.16	<0.01	0.07	<0.01	<0.01	1.00	0.86
Campylobacter	0.24	0.50	0.23	0.29	<0.01	0.07	0.25	0.58	1.00	<0.01
Corynebacterium	0.09	0.05	0.07	0.14	<0.01	0.02	0.03	0.99	1.00	<0.01
Lactobacillus	0.12	0.84	0.10	0.17	<0.01	0.13	NA	NA	NA	NA
f. Erysipelotrichaceae	0.55	0.28	0.01	0.04	<0.01	0.09	0.02	<0.01	1.00	0.25
Streptococcus	3.53	3.46	0.10	0.05	<0.01	0.75	NA	NA	NA	NA
Butyricimonas	0.37	0.18	0.06	0.05	<0.01	0.06	0.10	0.09	1.00	<0.01
Elusimicrobium	<0.01	0.16	<0.01	0.03	<0.01	0.03	0.78	0.35	1.00	0.62
Faecalibacterium	<0.01	0.77	0.10	0.01	<0.01	0.13	<0.01	0.08	1.00	0.24
Dorea	0.04	0.31	0.04	0.02	<0.01	0.05	0.67	0.01	1.00	<0.01

Actinomyces	4.68	0.21	0.05	0.02	<0.01	0.83	<0.01	0.04	1.00	0.04
f. Neisseriaceae	1.08	0.27	0.13	0.11	<0.01	0.17	NA	NA	NA	NA
Fusobacterium	4.48	0.81	0.02	0.01	<0.01	0.78	<0.01	<0.01	1.00	0.01
Veillonella	3.87	3.57	0.45	0.04	<0.01	0.78	<0.01	<0.01	1.00	0.01
Gallibacterium	3.25	0.32	0.02	0.01	<0.01	0.57	NA	NA	NA	NA
[Ruminococcus]	0.44	0.71	0.09	0.06	<0.01	0.12	0.71	<0.01	1.00	<0.01
Porphyromonas	3.51	0.45	0.02	0.01	<0.01	0.61	<0.01	0.01	1.00	<0.01
Haemophilus	0.37	0.04	<0.01	0.01	<0.01	0.06	NA	NA	NA	NA
Acinetobacter	0.36	0.03	0.02	0.01	<0.01	0.06	<0.01	0.96	1.00	<0.01
Bibersteinia	0.92	0.08	0.02	0.01	<0.01	0.16	NA	NA	NA	NA
Comamonas	1.35	0.03	0.02	0.01	<0.01	0.24	<0.01	0.80	1.00	0.05
Parvimonas	0.63	0.01	<0.01	<0.01	<0.01	0.11	<0.01	0.14	1.00	0.46
Kingella	0.26	0.02	0.02	0.01	<0.01	0.04	NA	NA	NA	NA
Aggregatibacter	0.19	0.01	<0.01	<0.01	<0.01	0.03	NA	NA	NA	NA
Phascolarctobacterium	0.21	0.86	0.19	0.26	<0.01	0.13	NA	NA	NA	NA
Parabacteroides	0.51	0.51	0.08	0.08	<0.01	0.10	0.96	<0.01	1.00	<0.01
Collinsella	0.15	1.16	0.17	0.13	<0.01	0.19	NA	NA	NA	NA
Akkermansia	0.55	0.55	<0.01	<0.01	<0.01	0.12	0.39	<0.01	<0.01	<0.01
Filifactor	0.10	<0.01	<0.01	<0.01	<0.01	0.02	<0.01	<0.01	1.00	<0.01
Peptococcus	0.21	0.02	0.01	<0.01	<0.01	0.04	<0.01	0.14	1.00	0.39
Peptostreptococcus	1.03	0.06	<0.01	<0.01	<0.01	0.18	<0.01	0.03	1.00	0.09
f. Enterobacteriaceae	0.74	0.02	<0.01	<0.01	<0.01	0.13	NA	NA	NA	NA
f. Alcaligenaceae	1.35	0.19	<0.01	0.04	<0.01	0.23	0.26	<0.01	0.45	<0.01
Adlercreutzia	0.21	0.05	<0.01	<0.01	<0.01	0.04	0.03	<0.01	1.00	0.06
Trueperella	0.11	<0.01	<0.01	<0.01	<0.01	0.02	NA	NA	NA	NA
Paludibacter	1.68	<0.01	<0.01	<0.01	<0.01	0.30	<0.01	<0.01	<0.01	<0.01

f. [Weeksellaceae]	0.15	0.02	<0.01	0.01	<0.01	0.03	NA	NA	NA	NA
Helcococcus	0.18	<0.01	<0.01	<0.01	<0.01	0.03	<0.01	0.96	1.00	0.83
Desulfotomaculum	0.27	<0.01	<0.01	<0.01	<0.01	0.05	<0.01	0.37	1.00	0.90
Moraxella	0.43	0.04	0.01	0.01	<0.01	0.07	NA	NA	NA	NA
f. Pasteurellaceae	0.18	0.01	<0.01	<0.01	<0.01	0.03	NA	NA	NA	NA
Rumen Liquid Digesta										
	Mean Abundances						**P-values**			
Genus	**D07**	**D14**	**D21**	**D28**	**D96**	**SEM**	**D7-D14**	**D14-D21**	**D21-D28**	**D28-D96**
Prevotella	9.85	22.90	28.54	32.38	21.75	3.42	0.08	0.86	0.92	0.25
f. Lachnospiraceae	2.86	7.86	2.86	6.36	4.73	0.88	<0.01	0.03	0.51	0.45
Bacteroides	13.23	4.53	1.78	1.20	0.11	2.13	0.02	<0.01	0.99	<0.01
o. Clostridiales	2.70	1.15	3.20	2.80	8.71	1.16	0.07	0.14	0.92	0.01
f. Ruminococcaceae	2.85	4.22	3.30	1.80	5.79	0.60	0.91	0.34	0.92	<0.01
o. Bacteroidales	1.50	1.08	5.34	1.83	5.70	0.89	0.48	0.84	0.92	0.02
Succiniclasticum	1.22	0.63	2.96	2.75	5.09	0.70	0.25	0.13	0.96	0.68
[Prevotella]	9.89	0.19	0.62	1.66	0.22	1.67	NA	NA	NA	NA
Butyrivibrio	0.16	0.21	3.81	3.54	4.53	0.84	0.93	<0.01	0.99	0.98
CF231	7.82	1.12	0.34	0.14	0.24	1.33	NA	NA	NA	NA
Ruminococcus	0.50	0.91	2.60	1.60	3.33	0.47	0.82	0.37	0.92	0.15
Fibrobacter	0.85	0.52	0.61	1.10	1.38	0.14	0.59	0.78	0.86	0.75
Dialister	0.45	0.21	1.80	1.41	0.33	0.29	0.93	0.84	0.01	<0.01
f. Veillonellaceae	0.42	0.49	1.96	0.53	0.72	0.26	0.87	0.03	0.64	0.98
Treponema	0.18	0.13	0.30	0.54	1.52	0.23	0.26	0.84	0.99	0.02
Oscillospira	1.04	0.53	0.37	0.32	0.11	0.14	<0.01	0.02	0.92	<0.01
Desulfovibrio	0.54	0.37	0.64	0.51	0.24	0.06	0.51	0.73	0.99	0.19
Anaerovibrio	0.11	0.76	0.22	0.42	0.15	0.11	0.08	0.01	0.64	0.57

f. [Mogibacteriaceae]	0.37	0.30	0.17	0.35	0.34	0.03	0.30	0.84	0.71	0.88
f. [Paraprevotellaceae]	0.17	0.16	0.32	0.22	0.43	0.05	0.95	0.73	0.70	0.81
Succinivibrio	0.05	0.72	6.10	9.45	1.25	1.63	<0.01	0.26	0.71	<0.01
Catenibacterium	0.01	6.30	3.16	5.82	0.37	1.18	<0.01	0.84	0.92	<0.01
Megasphaera	0.01	5.60	4.10	3.21	1.94	0.85	<0.01	0.73	0.92	0.17
f. Coriobacteriaceae	0.08	1.27	2.92	3.96	3.89	0.68	<0.01	0.74	0.92	0.98
Roseburia	0.01	4.30	1.47	2.13	0.53	0.67	<0.01	0.25	0.92	0.03
Bifidobacterium	0.01	5.16	1.33	1.07	0.35	0.83	<0.01	0.19	0.94	0.57
Lachnospira	0.03	2.23	1.28	1.13	0.60	0.33	<0.01	0.29	0.99	0.30
Acidaminococcus	0.01	0.57	1.63	1.49	0.91	0.27	<0.01	0.35	0.92	0.05
Blautia	0.41	1.49	1.12	0.42	0.04	0.24	0.37	0.30	0.82	<0.01
Campylobacter	1.18	0.90	0.77	0.47	0.04	0.17	0.85	0.89	0.94	<0.01
Parabacteroides	1.52	0.93	0.33	0.29	0.01	0.24	0.30	0.03	0.99	<0.01
Sphaerochaeta	0.09	0.10	0.70	0.69	0.54	0.12	0.99	0.31	0.92	0.89
Mitsuokella	<0.01	0.14	0.62	0.29	0.66	0.12	<0.01	0.49	0.99	0.93
Phascolarctobacterium	0.42	0.57	0.29	0.30	<0.01	0.08	0.07	<0.01	0.04	<0.01
Clostridium	0.09	0.21	0.78	0.39	0.12	0.11	0.85	0.24	0.64	0.36
Pseudoramibacter_Eubacterium	0.01	0.35	0.26	0.23	0.15	0.05	<0.01	0.95	0.92	0.04
[Eubacterium]	0.01	0.24	0.26	0.17	0.23	0.04	<0.01	0.96	0.99	0.99
Mogibacterium	0.09	0.10	0.15	0.22	0.12	0.02	0.77	0.48	0.99	0.55
o. YS2	<0.01	<0.01	1.58	1.03	4.50	0.74	0.40	<0.01	0.95	0.08
f. Succinivibrionaceae	0.02	0.23	0.09	0.14	4.79	0.84	<0.01	<0.01	0.70	<0.01
f. Neisseriaceae	4.01	0.80	0.35	0.05	0.01	0.67	0.02	0.58	0.78	0.02
Veillonella	1.01	3.02	0.27	0.04	<0.01	0.51	0.59	<0.01	0.47	0.03
f. RFP12	<0.01	<0.01	0.39	1.50	0.94	0.26	0.99	0.30	0.70	0.42
o. RF39	0.21	0.09	0.03	0.17	1.87	0.31	0.09	0.03	0.39	<0.01

Sharpea	0.01	0.01	0.31	0.11	1.77	0.30	0.88	0.03	0.99	0.36
f. S24-7	0.01	0.01	0.46	0.80	0.89	0.17	0.85	<0.01	0.82	0.89
Butyricimonas	1.37	0.30	0.28	0.08	0.02	0.22	<0.01	0.58	0.85	0.02
Coprococcus	0.20	0.09	0.11	0.02	1.03	0.17	0.17	0.48	0.51	<0.01
[Ruminococcus]	0.39	0.79	0.14	0.07	<0.01	0.13	0.64	<0.01	0.82	<0.01
Lactobacillus	0.05	1.01	0.14	0.13	0.02	0.17	<0.01	<0.01	0.96	0.03
Bulleidia	<0.01	0.04	0.32	0.49	0.40	0.09	<0.01	0.19	0.92	0.72
Collinsella	0.06	0.47	0.19	0.21	<0.01	0.07	0.08	0.17	0.82	<0.01
p-75-a5	0.12	0.27	0.41	0.05	0.01	0.07	0.09	0.81	0.92	0.09
Pseudobutyrivibrio	0.07	0.20	0.11	0.02	0.25	0.04	0.14	<0.01	0.58	<0.01
Corynebacterium	0.15	0.04	0.11	0.23	0.02	0.03	<0.01	0.84	0.51	<0.01
f. Pirellulaceae	0.01	<0.01	0.12	0.11	0.12	0.02	0.20	0.06	0.92	0.05
Akkermansia	3.06	2.96	<0.01	0.01	<0.01	0.66	0.51	<0.01	0.82	0.43
Streptococcus	1.49	4.38	0.07	0.06	0.01	0.75	0.08	<0.01	0.94	0.01
Gallibacterium	5.30	0.20	0.02	0.01	<0.01	0.94	<0.01	<0.01	0.82	0.01
Porphyromonas	4.62	0.27	0.02	0.02	<0.01	0.81	<0.01	<0.01	0.99	<0.01
Actinomyces	2.23	0.68	0.07	0.01	<0.01	0.38	<0.01	<0.01	0.70	0.12
Fusobacterium	2.43	0.19	0.01	0.01	<0.01	0.43	<0.01	<0.01	0.92	0.07
f. Alcaligenaceae	2.21	0.20	<0.01	0.02	<0.01	0.39	NA	NA	NA	NA
Ruminobacter	<0.01	0.05	2.12	<0.01	0.15	0.37	0.89	0.28	<0.01	<0.01
Pyramidobacter	0.07	0.54	0.53	0.09	0.10	0.10	NA	NA	NA	NA
Anaerostipes	0.03	<0.01	0.82	0.29	0.09	0.14	<0.01	<0.01	0.82	0.02
Faecalibacterium	<0.01	0.79	0.29	0.03	0.01	0.14	NA	NA	NA	NA
o. RF32	<0.01	0.09	0.10	0.02	0.87	0.15	<0.01	0.63	0.82	<0.01
BF311	0.04	<0.01	0.16	0.03	0.42	0.07	0.26	0.95	0.99	0.08
Elusimicrobium	<0.01	0.51	<0.01	0.11	0.03	0.09	NA	NA	NA	NA

f. Erysipelotrichaceae	0.32	0.24	0.01	0.05	0.03	0.06	NA	NA	NA	NA
c. Alphaproteobacteria	0.12	<0.01	0.03	0.01	0.18	0.03	0.02	0.81	0.78	<0.01
Comamonas	3.55	0.04	0.05	0.02	<0.01	0.63	NA	NA	NA	NA
Shuttleworthia	0.01	0.02	0.02	0.02	1.18	0.21	0.14	0.05	0.92	<0.01
YRC22	<0.01	0.10	0.02	0.06	1.00	0.17	0.02	0.30	0.92	0.02
Paludibacter	1.17	<0.01	<0.01	<0.01	<0.01	0.21	<0.01	<0.01	<0.01	0.68
Selenomonas	0.03	0.61	0.08	0.07	0.05	0.10	NA	NA	NA	NA
Bibersteinia	0.57	0.08	0.01	0.02	<0.01	0.10	<0.01	<0.01	0.92	<0.01
Acinetobacter	0.46	0.04	0.07	0.03	<0.01	0.08	<0.01	0.70	0.99	<0.01
Dorea	0.08	0.25	0.04	0.08	0.01	0.04	NA	NA	NA	NA
Peptostreptococcus	0.42	0.03	<0.01	<0.01	<0.01	0.07	<0.01	<0.01	0.96	0.62
f. Enterobacteriaceae	0.39	0.02	0.01	0.01	<0.01	0.07	NA	NA	NA	NA
f. F16	<0.01	<0.01	<0.01	<0.01	0.41	0.07	0.66	0.92	0.96	<0.01
Moraxella	0.34	0.04	0.01	0.01	<0.01	0.06	<0.01	<0.01	0.99	<0.01
f. RF16	<0.01	<0.01	0.04	<0.01	0.32	0.06	<0.01	<0.01	<0.01	<0.01
RFN20	0.03	0.06	0.07	0.04	0.13	0.02	0.14	0.62	0.92	0.43
Haemophilus	0.26	0.03	0.01	0.01	<0.01	0.04	<0.01	<0.01	0.99	<0.01
Parvimonas	0.28	<0.01	<0.01	<0.01	<0.01	0.05	<0.01	0.19	0.99	0.68
f. Elusimicrobiaceae	<0.01	<0.01	0.02	<0.01	0.25	0.04	<0.01	<0.01	<0.01	<0.01
Kingella	0.21	0.02	0.03	0.01	<0.01	0.04	<0.01	0.54	0.82	<0.01
f. Christensenellaceae	0.02	<0.01	<0.01	<0.01	0.23	0.04	<0.01	0.96	0.99	<0.01
p. WPS-2	<0.01	<0.01	0.02	<0.01	0.18	0.03	<0.01	0.05	<0.01	<0.01
Enterococcus	0.05	0.13	<0.01	0.02	<0.01	0.02	NA	NA	NA	NA
f. [Weeksellaceae]	0.16	0.02	0.01	<0.01	<0.01	0.03	<0.01	<0.01	0.94	<0.01
Adlercreutzia	0.10	0.08	<0.01	<0.01	<0.01	0.02	0.47	<0.01	0.92	0.01
Filifactor	0.18	<0.01	<0.01	<0.01	<0.01	0.03	<0.01	<0.01	<0.01	0.60

f. Pasteurellaceae	0.17	<0.01	<0.01	<0.01	<0.01	0.03	<0.01	0.22	0.78	0.43
o. ML615J-28	<0.01	<0.01	0.04	<0.01	0.11	0.02	<0.01	<0.01	0.39	<0.01
Peptococcus	0.13	0.01	<0.01	<0.01	<0.01	0.02	<0.01	0.11	0.82	0.38

Appendix D: Archaeal species abundance in the rumen digesta during early life. Values presented are percentages of the total archaeal population at each timepoint. FDR-corrected *P*-values were obtained from temporally adjacent contrasts in DeSeq2.

Rumen Solid Digesta										
	Mean Abundances						**P-values**			
Genus	**D07**	**D14**	**D21**	**D28**	**D96**	**SEM**	**D7-D14**	**D14-D21**	**D21-D28**	**D28-D96**
Methanobrevibacter boviskoreani clade	0.76	0.69	0.03	0.05	22.86	4.02	1.00	0.12	1.00	<0.01
Methanobrevibacter gottschalkii clade	20.83	49.82	29.73	17.96	8.67	6.23	1.00	1.00	1.00	0.61
Methanobrevibacter ruminantium clade	53.13	14.09	10.61	25.32	43.53	7.39	0.66	0.30	1.00	1.00
Methanobrevibacter smithii	18.28	33.22	27.82	9.23	4.70	4.82	1.00	1.00	1.00	1.00
Methanobrevibacter wolinii clade	3.46	0.05	9.25	19.65	5.81	3.00	NA	NA	NA	NA
Methanosphaera sp. A4	0.32	<0.01	4.58	5.63	4.22	1.04	0.26	<0.01	1.00	1.00
Methanosphaera sp. Group5	1.25	2.02	2.22	0.22	0.58	0.35	NA	NA	NA	NA
Methanosphaera sp. ISO3-F5	0.01	0.05	1.75	4.76	1.59	0.77	1.00	<0.01	1.00	0.33
Methanomassiliicoccaceae Group10 sp.	ND	ND	0.84	10.46	0.34	2.08	NA	NA	NA	NA
Candidatus Methanomethylophilus alvus	ND	ND	2.57	<0.01	5.55	1.01	0.20	<0.01	1.00	0.71
Methanomassiliicoccaceae Group12 sp. ISO4-H5	ND	ND	0.50	4.51	1.86	0.74	NA	NA	NA	NA
Methanomassiliicoccaceae Group4 sp. MpT1	ND	ND	0.29	1.45	0.12	0.26	1.00	<0.01	1.00	1.00
Methanomassiliicoccaceae Group9 sp. CH1270	ND	ND	4.21	0.09	<0.01	0.88	0.26	<0.01	0.72	0.51
Methanomassiliicoccaceae Group9 sp. ISO4-G1	ND	ND	5.22	0.05	<0.01	1.10	NA	NA	NA	NA
Methanobrevibacter boviskoreani clade	0.76	0.69	0.03	0.05	22.86	4.02	1.00	0.12	1.00	<0.01
Rumen Liquid Digesta										
	Mean Abundances						**P-values**			
Species	D7	D14	D21	D28	D96	SEM	D7 vs D14	D14 vs D21	D21 vs D28	D28 vs D96
Methanobrevibacter boviskoreani clade	0.79	0.67	0.02	0.05	22.93	4.04	0.73	1.00	1.00	<0.01

Methanobrevibacter gottschalkii clade	26.58	57.20	25.51	23.87	22.75	5.85	0.16	1.00	1.00	0.08
Methanobrevibacter ruminantium clade	35.35	10.41	12.06	21.91	33.39	4.64	NA	NA	NA	NA
Methanobrevibacter smithii	30.98	29.34	34.16	10.78	0.30	5.92	1.00	1.00	1.00	0.78
Methanobrevibacter wolinii clade	2.44	0.06	2.11	16.86	7.71	2.71	NA	NA	NA	NA
Methanosphaera sp. A4	0.38	ND	8.48	2.39	2.90	1.34	0.73	<0.01	1.00	1.00
Methanosphaera sp. Group5	0.05	2.11	0.14	0.08	0.33	0.35	0.50	1.00	1.00	1.00
Methanosphaera sp. ISO3-F5	0.21	0.08	0.88	3.76	1.33	0.60	NA	NA	NA	NA
Methanomassiliicoccaceae Group10 sp.	ND	ND	<0.01	9.73	0.43	2.01	<0.01	<0.01	0.03	0.60
Candidatus Methanomethylophilus alvus	ND	ND	7.06	<0.01	0.49	1.44	<0.01	<0.01	1.00	1.00
Methanomassiliicoccaceae Group12 sp. ISO4-H5	<0.01	<0.01	<0.01	5.41	5.03	1.14	NA	NA	NA	NA
Methanomassiliicoccaceae Group4 sp. MpT1	ND	ND	0.13	1.03	0.35	0.17	NA	NA	NA	NA
Methanomassiliicoccaceae Group9 sp. CH1270	ND	ND	9.10	0.80	0.02	1.84	<0.01	0.21	1.00	<0.01
Methanomassiliicoccaceae Group9 sp. ISO4-G1	ND	0.07	ND	0.08	0.01	0.01	0.24	1.00	0.28	0.08

Appendix E: Taxonomic profiles of rumen and hindgut microbiota in calves fed milk replacer with or without sodium butyrate. Values presented are a percentage of total community abundance at phylum or genus level within each tissue/treatment group as appropriate. FDR-corrected *P*-values were obtained using DeSeq2.

	Colon						
Taxa							
	Mean Overall	**CON**		**SB**			
Phylum		**Mean**	**SD**	**Mean**	**SD**	**P-value**	**FDR**
Firmicutes	63.23	66.29	9.41	60.17	10.24	0.45	0.89
Bacteroidetes	20.28	15.33	5.64	25.24	9.11	0.02	0.44
Actinobacteria	3.80	3.95	2.29	3.66	1.80	0.83	0.98
Tenericutes	3.11	3.72	2.10	2.50	1.62	0.32	0.89
Euryarchaeota	2.40	2.93	1.78	1.86	1.15	0.27	0.89
Verrucomicrobia	2.00	1.89	3.48	2.12	2.38	0.81	0.98
Proteobacteria	1.61	2.13	2.05	1.09	0.26	0.41	0.89
Spirochaetes	1.58	1.78	3.48	1.38	1.30	0.62	0.98
Cyanobacteria	1.22	1.01	0.57	1.43	1.13	0.38	0.89
Fibrobacteres	0.36	0.27	0.29	0.44	0.68	0.96	0.98
Elusimicrobia	0.32	0.55	1.21	0.08	0.08	0.98	0.98
Fusobacteria	0.08	0.14	0.24	0.03	0.03	0.17	0.89
	Mean Overall						
Genus		**Mean**	**SD**	**Mean**	**SD**	**P-value**	**FDR**
[Eubacterium]	0.24	0.27	0.26	0.21	0.11	0.75	0.97
[Prevotella]	1.68	1.55	2.28	1.82	1.10	0.32	0.97
[Ruminococcus]	0.02	0.02	0.03	0.03	0.02	0.19	0.97
5-7N15	0.12	0.05	0.10	0.19	0.31	0.31	0.97
Acidaminococcus	0.01	0.01	0.01	0.01	0.01	0.38	0.97
Akkermansia	1.36	1.78	3.66	0.93	1.55	0.81	0.97
Anaeroplasma	0.80	0.64	0.34	0.96	1.48	0.16	0.97

Anaerostipes	0.37	0.55	0.87	0.18	0.17	0.92	0.97
Anaerovibrio	0.04	0.03	0.02	0.06	0.08	0.29	0.97
Bacillus	0.64	0.44	0.56	0.85	1.15	0.42	0.97
Bacteroides	4.01	4.13	1.14	3.89	3.04	0.67	0.97
Blautia	0.36	0.25	0.11	0.46	0.29	0.06	0.89
Bulleidia	0.33	0.25	0.12	0.41	0.39	0.27	0.97
Butyricimonas	0.04	0.04	0.05	0.03	0.03	0.95	0.97
Butyrivibrio	0.30	0.46	0.20	0.14	0.09	0.01	0.49
Campylobacter	0.17	0.23	0.26	0.12	0.25	0.69	0.97
Catenibacterium	0.58	0.51	0.41	0.65	0.89	0.44	0.97
CF231	0.95	0.96	1.05	0.94	1.15	0.96	0.97
Clostridium	2.89	3.07	1.04	2.71	0.71	0.88	0.97
Coprobacillus	0.06	0.05	0.05	0.07	0.07	0.41	0.97
Coprococcus	1.47	1.28	0.67	1.67	0.82	0.35	0.97
Corynebacterium	0.02	0.03	0.06	0.01	0.01	0.69	0.97
Desulfovibrio	0.02	0.02	0.04	0.02	0.01	0.48	0.97
Dietzia	0.01	0.01	0.02	<0.01	<0.01	0.26	0.97
Dorea	0.95	1.01	0.54	0.88	0.33	0.91	0.97
Elusimicrobium	0.01	0.01	0.03	<0.01	<0.01	0.44	0.97
Epulopiscium	0.01	0.02	0.03	0.01	0.01	0.66	0.97
Faecalibacterium	0.34	0.19	0.29	0.50	0.50	0.09	0.94
Fibrobacter	0.36	0.28	0.30	0.44	0.69	0.48	0.97
Fusobacterium	0.08	0.14	0.24	0.03	0.03	0.21	0.97
Helicobacter	0.02	0.03	0.07	<0.01	<0.01	0.64	0.97
Lachnospira	0.09	0.06	0.05	0.12	0.09	0.23	0.97
Megasphaera	0.15	0.22	0.30	0.08	0.10	0.16	0.97
Methanobrevibacter	1.66	2.16	1.50	1.15	0.75	0.20	0.97
Methanosphaera	0.78	0.84	0.48	0.73	0.42	0.95	0.97
Mitsuokella	0.02	0.02	0.04	0.01	<0.01	0.13	0.97
Mogibacterium	0.16	0.22	0.14	0.10	0.07	0.09	0.94
Odoribacter	0.16	0.21	0.08	0.12	0.06	0.35	0.97

Oscillospira	1.10	1.08	0.42	1.11	0.22	0.31	0.97
p-75-a5	0.21	0.22	0.14	0.21	0.13	0.76	0.97
Paludibacter	0.22	0.09	0.11	0.36	0.51	0.24	0.97
Parabacteroides	0.38	0.32	0.15	0.44	0.18	0.11	0.97
Peptococcus	0.02	0.03	0.05	0.02	0.04	0.85	0.97
Phascolarctobacterium	1.19	0.94	0.54	1.44	0.37	0.03	0.89
Prevotella	7.37	3.01	1.30	11.72	7.45	<0.01	0.02
Pseudoramibacter_Eubacterium	0.29	0.33	0.15	0.25	0.11	0.63	0.97
Rc4-4	0.31	0.31	0.12	0.31	0.15	0.54	0.97
Roseburia	0.11	0.11	0.07	0.10	0.09	0.62	0.97
Ruminococcus	4.06	3.23	3.19	4.88	2.77	0.31	0.97
Sharpea	0.84	1.41	1.57	0.26	0.24	<0.01	0.26
Shuttleworthia	0.01	0.01	0.02	<0.01	<0.01	0.09	0.94
Sphaerochaeta	0.06	0.06	0.06	0.06	0.03	0.56	0.97
Succiniclasticum	0.03	0.05	0.08	0.01	0.02	0.16	0.97
Succinivibrio	0.66	1.12	2.23	0.21	0.08	0.53	0.97
Sutterella	0.41	0.48	0.25	0.34	0.16	0.54	0.97
Treponema	1.54	1.75	3.52	1.34	1.30	0.71	0.97
Turicibacter	0.13	0.14	0.29	0.12	0.14	0.96	0.97
Und. (c) Alphaproteobacteria	0.02	0.01	0.01	0.04	0.06	0.45	0.97
Und. (f) [Barnesiellaceae]	0.02	0.03	0.04	0.01	<0.01	0.06	0.89
Und. (f) [Mogibacteriaceae]	0.62	0.83	0.41	0.40	0.12	0.08	0.94
Und. (f) Anaeroplasmataceae	0.05	0.08	0.08	0.02	0.02	0.04	0.89
Und. (f) Bifidobacteriaceae	0.02	0.02	0.02	0.02	0.02	0.58	0.97
Und. (f) Christensenellaceae	0.01	0.01	0.01	0.01	0.01	0.89	0.97
Und. (f) Clostridiaceae	1.10	1.15	0.67	1.05	0.84	0.92	0.97
Und. (f) Coriobacteriaceae	3.80	3.96	2.30	3.64	1.81	0.86	0.97
Und. (f) Dehalobacteriaceae	0.01	0.01	0.01	0.02	0.02	0.24	0.97
Und. (f) Desulfovibrionaceae	0.05	0.05	0.03	0.05	0.04	0.76	0.97
Und. (f) Elusimicrobiaceae	0.32	0.56	1.27	0.08	0.09	0.44	0.97
Und. (f) Erysipelotrichaceae	0.57	0.82	0.71	0.32	0.18	0.06	0.89

Und. (f) Erysipelotrichaceae gut	0.13	0.13	0.15	0.12	0.22	0.57	0.97
Und. (f) Lachnospiraceae	16.45	18.03	7.29	14.88	6.03	0.78	0.97
Und. (f) p-2534-18B5	0.06	0.07	0.17	0.04	0.09	0.52	0.97
Und. (f) Peptostreptococcaceae	0.45	0.34	0.61	0.56	0.44	0.53	0.97
Und. (f) RF16	0.59	0.67	0.49	0.52	0.44	0.75	0.97
Und. (f) RFP12	0.68	0.16	0.18	1.20	1.93	0.04	0.89
Und. (f) Rikenellaceae	0.60	0.78	0.73	0.42	0.32	0.46	0.97
Und. (f) Ruminococcaceae	16.37	16.92	6.70	15.81	5.39	0.76	0.97
Und. (f) S24-7	2.83	2.37	1.81	3.29	2.08	0.24	0.97
Und. (f) Succinivibrionaceae	0.01	0.01	0.01	0.01	0.02	0.93	0.97
Und. (f) Veillonellaceae	0.06	0.05	0.04	0.07	0.03	0.24	0.97
Und. (o) Bacteroidales	1.40	1.29	1.30	1.51	1.33	0.44	0.97
Und. (o) Clostridiales	9.41	10.30	4.68	8.51	3.17	0.92	0.97
Und. (o) ML615J-28	0.23	0.16	0.20	0.30	0.37	0.16	0.97
Und. (o) Natranaerobiales	0.03	0.03	0.07	0.03	0.04	0.95	0.97
Und. (o) RF32	0.24	0.20	0.18	0.29	0.20	0.31	0.97
Und. (o) RF39	2.09	2.93	1.93	1.25	0.63	0.17	0.97
Und. (o) YS2	1.23	1.03	0.57	1.44	1.15	0.15	0.97
YRC22	0.21	0.13	0.10	0.28	0.20	0.08	0.94
Taxa	**Cecum**						
	Mean Overall	**CON**		**SB**			
Phylum		**Mean**	**SD**	**Mean**	**SD**	**P-value**	**FDR**
Firmicutes	64.98	65.95	0.057	0.64	0.062	0.99	1.00
Bacteroidetes	16.82	13.05	0.046	0.206	0.049	0.01	0.07
Actinobacteria	6.04	8.09	0.027	0.04	0.019	<0.01	0.05
Euryarchaeota	4.99	6.29	0.025	0.037	0.021	0.07	0.33
Tenericutes	2.35	1.83	0.014	0.029	0.019	0.16	0.61
Verrucomicrobia	1.15	1.32	0.016	0.01	<0.018	0.56	0.95
Proteobacteria	1.04	1.14	0.01	<0.019	<0.013	0.59	0.95
Spirochaetes	1.04	0.90	0.015	0.012	0.013	0.61	0.95

Cyanobacteria	1.03	0.70	<0.013	0.014	<0.015	0.01	0.05
Fibrobacteres	0.30	0.29	<0.014	<0.013	<0.015	0.95	1.00
Elusimicrobia	0.18	0.30	<0.017	<0.011	<0.011	0.26	0.62
Fusobacteria	0.07	0.12	<0.012	ND	ND	0.98	1.00
Deferribacteres	0.01	<0.01	ND	0.02	ND	0.69	0.95
	Mean Overall	**CON**		**SB**			
Genus		**Mean**	**SD**	**Mean**	**SD**	**P-value**	**FDR**
[Eubacterium]	0.28	0.26	0.15	0.29	0.15	0.94	0.97
[Prevotella]	1.90	1.72	2.21	2.08	2.46	0.75	0.97
[Ruminococcus]	0.03	0.02	0.03	0.03	0.03	0.52	0.91
5-7N15	0.05	0.03	0.06	0.08	0.18	0.88	NA
Acidaminococcus	0.03	0.05	0.11	0.01	0.01	0.96	NA
Adlercreutzia	0.01	0.01	0.01	0.01	0.01	0.55	NA
Akkermansia	0.77	0.97	1.53	0.57	0.68	0.35	0.85
Anaeroplasma	0.49	0.24	0.10	0.74	0.95	0.03	0.17
Anaerostipes	0.31	0.35	0.46	0.27	0.33	0.62	0.97
Anaerovibrio	0.08	0.07	0.06	0.09	0.12	0.71	0.97
Bacillus	0.20	0.17	0.22	0.24	0.40	0.83	0.97
Bacteroides	2.83	2.41	0.91	3.26	1.77	0.41	0.85
Bifidobacterium	0.01	0.02	0.05	<0.01	<0.01	0.31	NA
Blautia	0.40	0.29	0.14	0.51	0.25	0.12	0.46
Bulleidia	0.49	0.31	0.15	0.67	0.58	0.12	0.45
Butyricimonas	0.02	0.01	0.02	0.02	0.04	0.51	0.91
Butyrivibrio	0.47	0.81	0.43	0.13	0.08	<0.01	<0.01
Campylobacter	0.13	0.12	0.19	0.15	0.27	0.84	0.97
Catenibacterium	0.67	0.86	0.50	0.48	0.58	0.25	0.73
CF231	0.86	0.65	0.69	1.07	0.92	0.54	0.94
Clostridium	4.21	4.27	1.95	4.15	2.00	0.81	0.97
Coprobacillus	0.10	0.06	0.07	0.14	0.13	0.36	0.85
Coprococcus	1.38	0.96	0.30	1.81	1.10	0.09	0.44

Corynebacterium	0.04	0.06	0.08	0.02	0.02	0.12	0.45
Desulfovibrio	0.02	0.02	0.02	0.01	0.01	0.47	NA
Dialister	0.01	0.02	0.02	0.01	0.01	0.39	NA
Dietzia	0.01	0.02	0.03	0.01	0.01	0.39	NA
Dorea	1.05	1.05	0.48	1.05	0.34	0.85	0.97
Epulopiscium	0.02	0.02	0.05	0.02	0.02	0.92	0.97
Faecalibacterium	0.41	0.30	0.46	0.51	0.52	0.48	0.91
Fibrobacter	0.31	0.29	0.39	0.32	0.47	0.89	0.97
Fusobacterium	0.07	0.12	0.24	0.02	0.02	0.94	0.97
Lachnospira	0.07	0.02	0.02	0.13	0.07	<0.01	0.04
Megasphaera	0.56	0.72	0.72	0.40	0.72	0.31	0.84
Methanobrevibacter	3.13	4.18	2.16	2.07	1.19	0.03	0.17
Methanosphaera	2.00	2.33	0.71	1.67	1.17	0.19	0.62
Mitsuokella	0.03	0.04	0.07	0.03	0.03	0.72	0.97
Mobiluncus	0.01	0.01	0.02	<0.01	0.01	0.34	NA
Mogibacterium	0.19	0.26	0.14	0.12	0.06	0.02	0.11
Mucispirillum	0.01	<0.01	<0.01	0.02	0.05	0.68	NA
Odoribacter	0.10	0.12	0.07	0.09	0.06	0.46	0.91
Oscillospira	1.21	1.14	0.31	1.28	0.33	0.77	0.97
p-75-a5	0.21	0.11	0.05	0.31	0.23	0.01	0.11
Paludibacter	0.07	0.04	0.05	0.10	0.20	0.52	0.91
Parabacteroides	0.33	0.31	0.19	0.36	0.14	0.70	0.97
Peptococcus	0.02	0.03	0.03	0.02	0.03	0.64	0.97
Phascolarctobacterium	1.03	0.66	0.26	1.40	0.34	0.01	0.11
Prevotella	6.90	4.31	2.25	9.48	5.38	0.04	0.23
Pseudoramibacter_Eubacterium	0.38	0.45	0.18	0.32	0.08	0.19	0.62
rc4-4	0.43	0.41	0.22	0.45	0.21	0.90	0.97
Roseburia	0.11	0.10	0.09	0.11	0.07	0.98	0.98
Ruminococcus	3.02	2.08	1.53	3.95	1.64	0.11	0.45
Sharpea	0.70	1.09	0.98	0.32	0.42	<0.01	0.01
Shuttleworthia	0.03	0.06	0.10	0.01	0.01	<0.01	0.04

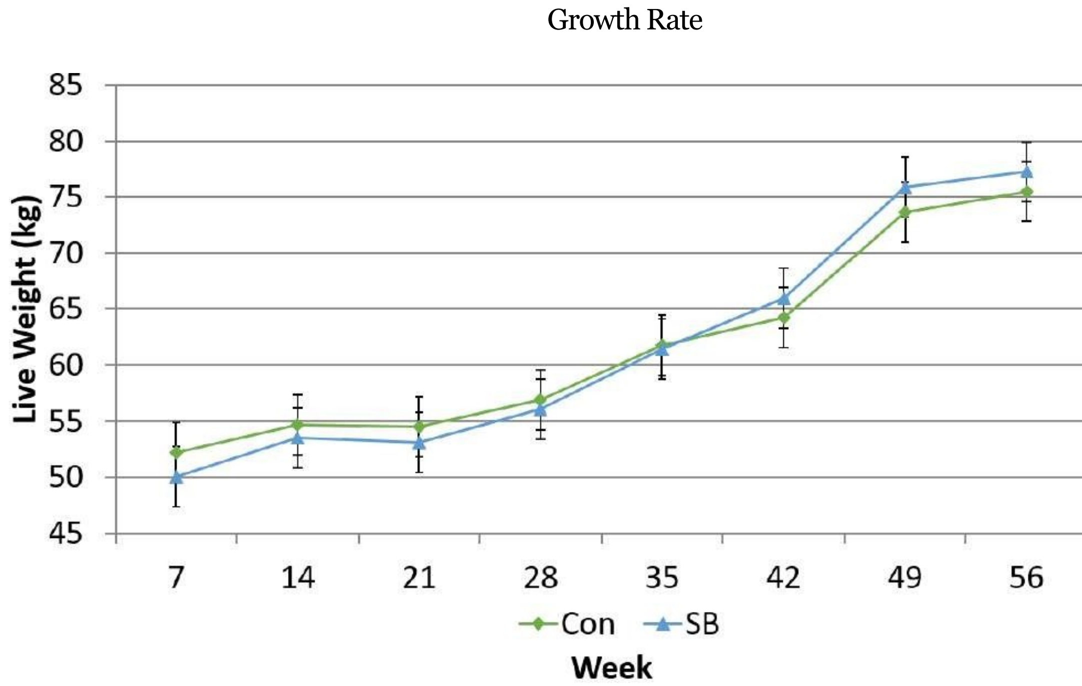
Sphaerochaeta	0.14	0.13	0.14	0.14	0.15	0.91	0.97
Streptococcus	0.01	0.01	0.01	0.02	0.02	0.34	NA
Succiniclasticum	0.08	0.15	0.33	0.02	0.01	0.97	NA
Succinivibrio	0.26	0.34	0.53	0.17	0.10	0.30	0.84
Sutterella	0.34	0.39	0.33	0.30	0.12	0.40	0.85
Treponema	0.91	0.79	1.44	1.04	1.30	0.75	0.97
Turicibacter	0.14	0.15	0.29	0.13	0.13	0.88	0.97
Und. (c) Alphaproteobacteria	0.01	<0.01	<0.01	0.03	0.04	0.02	NA
Und. (f) [Barnesiellaceae]	0.01	0.02	0.04	<0.01	<0.01	0.04	NA
Und. (f) [Mogibacteriaceae]	1.11	1.56	0.84	0.65	0.42	0.01	0.11
Und. (f) Anaeroplasmataceae	0.04	0.05	0.07	0.02	0.03	0.37	0.85
Und. (f) Bifidobacteriaceae	0.04	0.05	0.06	0.03	0.03	0.36	0.85
Und. (f) Christensenellaceae	0.01	0.02	0.01	0.01	0.01	0.67	NA
Und. (f) Clostridiaceae	1.35	1.34	1.17	1.35	0.92	0.87	0.97
Und. (f) Coriobacteriaceae	6.06	8.17	2.80	3.96	1.94	0.01	0.11
Und. (f) Dehalobacteriaceae	0.02	0.02	0.02	0.01	0.02	0.72	NA
Und. (f) Desulfovibrionaceae	0.05	0.06	0.04	0.04	0.03	0.40	0.85
Und. (f) Elusimicrobiaceae	0.19	0.31	0.68	0.06	0.12	0.39	0.85
Und. (f) Erysipelotrichaceae	0.56	0.79	0.96	0.34	0.21	0.09	0.44
Und. (f) Erysipelotrichaceae gut	0.08	0.08	0.12	0.07	0.09	0.89	0.97
Und. (f) Lachnospiraceae	19.03	20.71	7.22	17.34	9.90	0.51	0.91
Und. (f) p-2534-18B5	0.02	0.01	0.03	0.03	0.07	0.63	NA
Und. (f) Peptostreptococcaceae	0.66	0.57	1.14	0.75	0.59	0.73	0.97
Und. (f) RF16	0.32	0.43	0.31	0.21	0.25	0.20	0.62
Und. (f) RFP12	0.41	0.39	0.87	0.43	0.59	0.90	0.97
Und. (f) Rikenellaceae	0.41	0.35	0.26	0.47	0.41	0.64	0.97
Und. (f) Ruminococcaceae	15.55	15.29	4.78	15.82	5.75	0.96	0.97
Und. (f) S24-7	2.16	2.06	0.66	2.27	0.94	0.92	0.97
Und. (f) Succinivibrionaceae	0.06	0.11	0.24	0.01	0.02	0.95	NA
Und. (f) Veillonellaceae	0.11	0.12	0.16	0.10	0.04	0.56	0.95
Und. (o) Bacteroidales	0.97	0.74	0.44	1.21	0.95	0.34	0.85

Und. (o) Clostridiales	7.26	7.03	2.51	7.50	1.69	0.95	0.97
Und. (o) ML615J-28	0.11	0.04	0.03	0.19	0.24	0.01	0.11
Und. (o) Natranaerobiales	0.02	0.01	0.03	0.02	0.03	0.82	NA
Und. (o) RF32	0.15	0.09	0.08	0.21	0.20	0.18	0.62
Und. (o) RF39	1.76	1.56	1.40	1.97	1.77	0.64	0.97
Und. (o) Streptophyta	0.06	0.09	0.21	0.02	0.02	0.70	NA
Und. (o) YS2	0.99	0.63	0.31	1.36	0.46	0.02	0.11
YRC22	0.19	0.19	0.13	0.19	0.08	0.88	0.97
Taxa	**Rumen**						
	Overall	**CON**		**SB**			
Phylum	**Mean**	**Mean**	**SD**	**Mean**	**SD**	**P-Value**	**FDR**
Firmicutes	49.42	50.72	5.48	48.12	10.53	0.74	0.91
Bacteroidetes	24.62	23.44	3.52	25.81	11.71	0.51	0.91
Proteobacteria	15.62	15.92	3.43	15.32	7.62	0.68	0.91
Actinobacteria	3.81	2.85	1.04	4.76	4.18	0.14	0.83
Cyanobacteria	3.62	4.90	3.52	2.34	1.95	0.28	0.83
Euryarchaeota	2.03	1.22	0.67	2.84	3.57	0.35	0.87
Tenericutes	0.51	0.63	0.59	0.39	0.22	0.57	0.91
Fibrobacteres	0.13	0.05	0.06	0.22	0.50	0.96	0.96
Spirochaetes	0.11	0.14	0.12	0.09	0.10	0.74	0.91
Synergistetes	0.05	0.07	0.04	0.03	0.02	0.23	0.83
TM7	0.04	0.03	0.02	0.05	0.07	0.30	0.83
Verrucomicrobia	0.01	0.01	0.01	0.02	0.02	0.20	0.83
	Overall	**CON**		**SB**			
Genus	**Mean**	**Mean**	**SD**	**Mean**	**SD**	**P-value**	**FDR**
[Eubacterium]	0.15	0.08	0.04	0.23	0.39	0.77	0.97
[Prevotella]	0.10	0.11	0.12	0.08	0.18	0.60	0.97
Acidaminococcus	1.81	1.63	0.74	1.99	0.82	0.61	0.97
Bifidobacterium	0.04	0.02	0.04	0.05	0.07	0.28	0.97

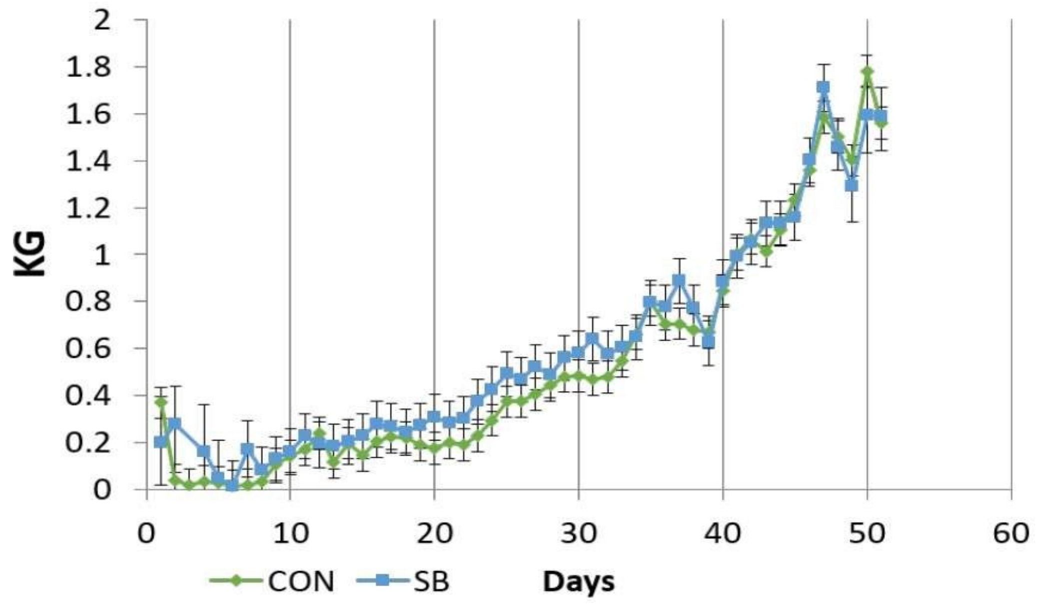
Bulleidia	0.43	0.14	0.09	0.72	0.91	<0.01	0.46
Butyrivibrio	4.91	5.30	3.69	4.52	4.64	0.86	0.98
Campylobacter	0.02	0.02	0.01	0.02	0.02	0.91	0.99
Catenibacterium	0.60	0.40	0.39	0.79	1.25	0.34	0.97
Clostridium	0.02	0.01	0.01	0.03	0.01	0.23	0.97
Coprococcus	0.57	0.30	0.17	0.85	0.83	0.06	0.97
Corynebacterium	0.01	0.01	0.02	0.01	0.01	0.29	0.97
Desulfovibrio	0.07	0.06	0.02	0.07	0.03	0.78	0.97
Dialister	1.26	0.97	0.39	1.54	0.85	0.32	0.97
Fibrobacter	0.13	0.05	0.06	0.22	0.50	0.31	0.97
Lachnospira	0.39	0.42	0.53	0.36	0.65	0.82	0.97
Megasphaera	0.74	0.55	0.38	0.94	0.59	0.25	0.97
Methanobrevibacter	1.47	0.96	0.54	1.98	2.24	0.19	0.97
Methanosphaera	0.53	0.22	0.09	0.83	1.35	0.42	0.97
Mitsuokella	0.36	0.27	0.15	0.45	0.17	0.14	0.97
Mogibacterium	0.10	0.09	0.04	0.11	0.07	0.72	0.97
Oscillospira	0.04	0.04	0.01	0.05	0.03	0.75	0.97
Peptococcus	0.01	0.01	<0.01	0.01	0.01	0.30	0.97
Prevotella	24.19	22.92	3.63	25.46	11.53	0.78	0.97
Pseudoramibacter_Eubacterium	0.52	0.30	0.11	0.75	0.86	0.06	0.97
Pyramidobacter	0.02	0.03	0.02	0.02	0.01	0.26	0.97
RFN20	0.04	0.07	0.07	0.01	0.01	0.01	0.47
Ruminococcus	0.14	0.17	0.14	0.10	0.10	0.22	0.97
Sharpea	4.48	4.43	2.01	4.53	3.08	0.87	0.98
Shuttleworthia	4.24	5.68	2.45	2.80	2.68	0.31	0.97
Sphaerochaeta	0.06	0.09	0.08	0.03	0.03	0.14	0.97
Succiniclasticum	2.85	3.79	2.89	1.90	2.23	0.36	0.97
Succinivibrio	1.21	1.05	1.02	1.38	2.15	0.65	0.97
Treponema	0.05	0.05	0.06	0.06	0.09	0.95	0.99
Turicibacter	0.05	<0.01	<0.01	0.10	0.25	0.64	0.97
Und. (f) [Mogibacteriaceae]	0.78	0.69	0.37	0.86	0.68	0.69	0.97

Und. (f) [Paraprevotellaceae]	0.17	0.17	0.06	0.17	0.13	0.88	0.99
Und. (f) Alcaligenaceae	0.01	<0.01	<0.01	0.01	0.03	0.16	0.97
Und. (f) Bifidobacteriaceae	0.09	0.01	0.01	0.18	0.39	0.09	0.97
Und. (f) Christensenellaceae	0.02	0.02	0.02	0.01	0.01	0.48	0.97
Und. (f) Clostridiaceae	0.39	<0.01	<0.01	0.78	1.91	0.66	0.97
Und. (f) Coriobacteriaceae	3.67	2.82	1.07	4.52	4.20	0.30	0.97
Und. (f) Dehalobacteriaceae	0.01	0.01	0.01	0.02	0.03	0.17	0.97
Und. (f) Erysipelotrichaceae	0.13	0.15	0.10	0.11	0.06	0.70	0.97
Und. (f) F16	0.04	0.03	0.02	0.05	0.07	0.59	0.97
Und. (f) Lachnospiraceae	13.92	13.53	4.33	14.32	10.02	0.89	0.99
Und. (f) p-2534-18B5	0.02	0.03	0.04	0.01	0.01	0.18	0.97
Und. (f) Peptostreptococcaceae	0.07	<0.01	<0.01	0.15	0.36	0.24	0.97
Und. (f) RF16	0.01	0.01	0.03	<0.01	<0.01	<0.01	0.22
Und. (f) RFP12	0.01	0.01	<0.01	0.01	0.02	0.62	0.97
Und. (f) Ruminococcaceae	1.16	0.98	0.53	1.34	1.56	0.72	0.97
Und. (f) S24-7	0.19	0.21	0.25	0.17	0.30	0.71	0.97
Und. (f) Succinivibrionaceae	14.33	14.80	3.08	13.86	7.38	0.99	0.99
Und. (f) Veillonellaceae	3.78	4.26	2.52	3.30	1.69	0.72	0.97
Und. (o) Bacteroidales	0.07	0.09	0.07	0.04	0.05	0.23	0.97
Und. (o) Clostridiales	5.11	6.07	2.11	4.16	2.34	0.21	0.97
Und. (o) RF39	0.51	0.63	0.60	0.39	0.22	0.33	0.97
Und. (o) Streptophyta	0.01	0.01	<0.01	0.01	0.01	0.76	0.97
Und. (o) YS2	3.64	4.93	3.56	2.34	1.97	0.22	0.97
vadinCA11	0.04	0.05	0.06	0.03	0.05	0.68	0.97
YRC22	0.03	0.05	0.12	<0.01	0.01	0.76	0.97

Appendix F: Performance data of dairy calves fed milk replacer with or without sodium butyrate during preweaning.



Solid Feed Intake



Milk Replacer Intake