THE EFFECT OF EARLY LIFE NUTRITION ON RUMEN MICROBIAL COMMUNITY DEVELOPMENT AND IMPACT ON LIFETIME PERFORMANCE IN RUMINANTS

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Abstract

Manipulation of the rumen microbiota in adult ruminants has been intended to improve animal performance and decrease greenhouse gas emissions, but results have only shown a short- or non-lasting effect after intervention. Changes in the ruminal microbiota during rumen development have recently shown promising results in the short-term. Therefore, the purpose of the present body of work was to determine how dietary management and chemical interventions, during rumen development, modify the ruminal microbial community composition, and whether these changes affect rumen fermentation and development, and consequently, performance in the young ruminants. The objectives of this thesis were to: (i) evaluate the impact of early weaning on rumen development and function in artificially-reared lambs; (ii) characterize the impact of early weaning in lambs on the rumen microbiota in the first 16 weeks of life and examine the relationships between rumen microbiota composition and rumen fermentation profiles, rumen development and blood metabolites; (iii) assess whether contrasting feeding regimes in the first 7 months of life lead to an imprint in the rumen microbial community structure, fermentation profiles and methane emissions in the rumen of calves; (iv) and evaluate the effect of methane inhibitors on the rumen microbial community composition, fermentation pathways, and gas emissions in calves. A series of three experiments were carried out in young ruminants separated from their mothers after colostrum intake, to address the objectives of this thesis. In experiment one, 3-5-day-old lambs were euthanized at weeks 0, 4 and 16 of rearing to investigate objectives (i) and (ii). Early weaning of lambs increased plasma β-hydroxybutyrate at week 4 of rearing, while dry matter intake, fermentation profiles and rumen morphology were similar between groups. Papillae morphology and muscular thickness differed between ruminal sites at 4 and 16 weeks of rearing, but not between treatments. Diversity and relative abundance of ruminal bacteria was affected by feeding management, whilst the archaea community showed few changes. Changes in the proportions of abundant bacteria genera from *Bacteroidetes* and *Firmicutes* were associated with fermentation profiles, rumen morphology and blood metabolites; however, further investigations are required to explain these associations. In experiment two, ~1-week-old calves were reared with two divergent feeding systems and different post-weaning forage quality with a common pasture diet after 7 months of age to investigate objective (iii). Consumption of pre-weaning concentrate compared to forage produced

lower methane yields and greater total short chain fatty acids (SCFA) concentrations and propionate proportions; whist ruminal microbes showed greater proportions of saccharolitic bacteria and *Methanobrevibacter boviskoreani*, but lower hemicellulolytic and cellulolytic bacteria, and *Mbb. gottschalkii*. Post-weaning, high-quality forage produced greater total SCFA concentration and propionate proportions than low-quality forages, while methane yield was similar. Hemicellulolytic bacteria and *Methanosphaera* spp. were greater in high-quality forages, while cellulolytic bacteria and *Methanomassiliicoccales* spp. were greater in low-quality forages. No pre-weaning effect was observed. Finally, the consumption of a common diet after 7 months of age resulted in similar methane emissions, fermentation profiles and microbial communities. In experiment three, ~1-week-old calves fed either concentrate starter diets or starter diets plus methane inhibitors were tested to evaluate objective (iv). Inhibitor intake decreased methane yield, but increased hydrogen yield and the proportion of propionate and had no effect on dry matter intake, total SCFA concentrations or animal growth. Within the abundant bacteria, the proportions of hydrogen utilizing and producing bacteria increased and decreased, respectively. Archaea diversity and proportions were affected during the period of methane inhibitor intake. However, similar gas emissions, fermentation profiles, and microbial communities were observed between groups at 24 and 49 weeks of age. Collectively, these results showed that reducing the age at weaning and introducing the solid feed to lambs at ~1 week of life accelerated some aspects of rumen morphology and function. Dietary management and methanogen inhibitor interventions affected the composition of the ruminal microbiota and fermentation profiles during treatment, however, no permanent changes in the microbial community and resulting ruminal fermentation were observed post-treatment in young ruminants.

List of Publications

Abstracts

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Abbreviations

16S rRNA	16S ribosomal RNA
ADF	Acid detergent fiber
ADG	Average daily gain
d	Day
DMI	Dry matter intake
DNA	Deoxyribonucleic acid
g/d	Grams per day
GHG	Greenhouse gases
LW	Live weight
Mbb.	*Methanobrevibacter*
Mmc.	*Methanomasilliicoccales*
Mph.	*Methanosphaera*
MR	Milk replacer
mRNA	Messenger ribonucleic acid
NDF	Neutral detergent fiber
NA	Not available
OTU	Organizational taxonomic unit
PCR	Polymerase chain reaction
PLSDA	Partial least squares discriminant analysis
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
SCFA	Short chain fatty acids
spp.	Species
TMR	Total mixed ration
wk	Week
yCH_4	Methane yield
yH_2	Hydrogen yield

Definitions

16S rRNA	The component of the 30S small subunit of a prokaryotic ribosome that binds to the Shine-Dalgarno sequence.
Alpha diversity	Species richness (number of taxa) within a single microbial ecosystem.
Amplicon	A piece of DNA or RNA that is the source and/or product of amplification or replication events. It can be formed artificially, using various methods including PCR or ligase chain reactions (LCR), or naturally through gene duplication.
Bacteria biofilm formation	Comprises any syntrophic consortium of microorganisms in which cells stick to each other and often also to a surface.
Beta diversity	Diversity in microbial community between different environments (difference in taxonomic abundance profiles from different samples).
Chao 1	Statistical index used to estimate the microbial richness.
DNA	A molecule composed of two chains that coil around each other to form a double helix carrying the genetic instructions used in the growth, development, functioning and reproduction of all known living organisms and many viruses.
Histomorphometry	The quantitative study of the microscopic organization and structure of a tissue especially by computer-assisted analysis of images formed by a microscope.
Metagenome	The collective genome of microorganisms from an environmental sample used to provide information on the microbial diversity and ecology of a specific environment.

Metagenomics	The analysis of the DNA of microbial communities in their natural environments.
Metatranscriptomics	The study of genes that are transcribed in microbial communities under certain environmental conditions, as measured by the abundance of mRNA transcripts.
Microbiome	The microbiome comprises all of the genetic material within a microbiota (the entire collection of microorganisms in a specific niche, such as the rumen).
Microbiota	A collective term for the micro-organisms that live in and on all multicellular organisms studied to date from plants to animals.
MiSeq Illumina	A next generation platform that performs clonal amplification, genomic DNA sequencing, and data analysis.
mRNA	A large family of RNA molecules that convey genetic information from DNA to the ribosome, where they specify the amino acid sequence of the protein products of gene expression.
Operational taxonomic unit	An operational definition used to classify groups of closely related individuals.
PCR	A technique in molecular biology that permits the analysis of any short sequence of DNA (or RNA) even in samples containing only minute quantities of DNA or RNA.
PLSDA	A derivation of the multiple linear regression and principal components regression methods.
RNA	A polymeric molecule essential in various biological roles in coding, decoding, regulation and expression of genes.

Shine-Dalgarno sequence	A ribosomal binding site in bacterial and archaeal messenger RNA.
Shannon index	A statistical index used to assess the biodiversity in a community.
Shotgun metagenomics	The untargeted ('shotgun') sequencing of all ('meta-') microbial genomes 'genomics' present in a sample.
Total mixed ration	A method of feeding that combines feeds formulated to a specific nutrient content into a single feed mix for a specific physiological stage of an animal.

Preface

Ruminants are the most abundant farmed herbivores (Van Soest, 1994). Global demand for ruminant products is projected to rise by more than 50% by the year 2050, but this predicted trend is also accompanied by a forecasted increase of 60% in methane emissions by the ruminant population (Revell, 2015). Action is therefore needed to increase production while at the same time stabilizing or even reducing methane emissions. One route which has been suggested as a potential method of uncoupling animal performance and reducing methane emissions is manipulation of the rumen microbiota (Grainger and Beauchemin, 2011; Hristov et al., 2013). For example, dietary and chemical interventions have been shown to affect rumen chemistry and alter composition of the microbial community of the rumen (Beauchemin et al., 2008; Benchaar and Greathead, 2011; Knapp et al., 2014). However, in adult ruminants, these manipulations of the rumen microbiota have produced variable results and short-lasting effects (Weimer, 2015). In the last decade, some of the focus has moved to manipulation of the microbial community in the developing rumen, which has been suggested to be more effective and to produce longer-lasting change after interventions (Yáñez-Ruiz et al., 2015).

However, there is little evidence on how a modification of the rumen microbiota in early life influences microbial community diversity and composition that persist when an animals' circumstances change, e.g. in response to changes in rearing management, divergent diets or after exposure to microbial inhibitors. The objective of the present body of work was to determine how dietary management and chemical interventions, during rumen development, modify the ruminal microbial community composition in the short- and long-term, and to determine whether these changes affected rumen fermentation and development, and consequently, performance in young ruminants.

The specific objectives of each chapter in this thesis were:

Chapter 1: Review the existing literature on rumen development and function as well as microbial establishment, manipulation and interaction with the host in ruminants.

Chapter 2: Evaluate the impact of weaning age on rumen development of artificially-reared lambs and compare the histomorphometry of four ruminal sites at 4 and 16 wk of rearing.

Chapter 3: Characterize the rumen microbiota following separation from the mother, the response to two early weaning regimes, and outdoors grazing on ryegrass/clover paddocks, determine the effect of age at weaning on the most abundant rumen bacteria and archaea at 4 and 16 weeks, and explore the associations between abundant rumen microbes and fermentation profiles, rumen morphology and blood metabolites in artificially-reared lambs.

Chapter 4: Determine whether pre-weaning diets rich in concentrate *vs*. forage, and post-weaning diets based on high- *vs*. low-quality forages, lead to an imprint in the rumen microbial community with associated changes in rumen fermentation in artificially-reared calves.

Chapter 5: Assess whether feeding methanogen inhibitors to artificially-reared calves during the first week of life leads to persistent changes in the rumen microbial community and in rumen fermentation, and whether it affects animal live weight.

Chapter 6: A summary and discussion of the main findings of the studies and potential areas for further research.

Chapter 1: Literature review

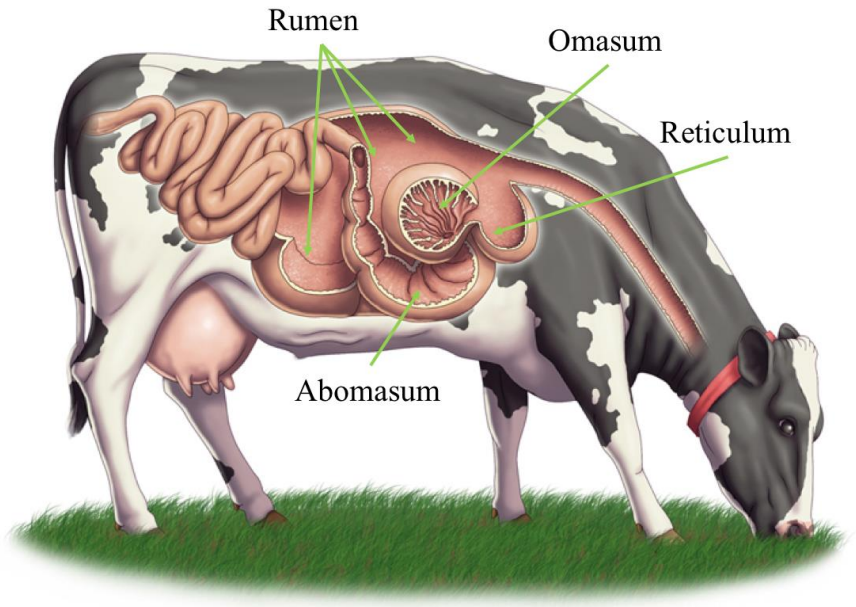
The present chapter reviews some general aspects of the ruminant: from its evolution to the importance of this species in the New Zealand's agricultural sector. It provides a description of how the diet and fermentation end products affect rumen development, morphology and metabolism in the neonate. The chapter describes the main ruminal fermentation pathways, and hydrogen and methane production in the rumen. It summarizes the current knowledge on rumen microbial colonization and establishment, rumen microbial community that integrate the liquid and solid fractions, and rumen epithelium of the rumen, as well as, host-microbial interactions. Finally, the effect of dietary interventions and microbial inhibitors on the rumen microbiota and their fermentation end products is examined.

1.1 Introduction

1.1.1 Ruminants

Ruminants, from the Latin ruminare that means "to chew over again", are mammals classified in order Artiodactyla (even-toed ungulates), and suborder *Ruminantia* (Hernández-Fernández and Vrba, 2005). These animals acquire the majority of their nutrients from the fermentation of ingested plant-based food (Church, 1993; Van Soest, 1994). The fermentation process takes place in the rumen, a specialized pre-stomach (**Figure 1**), through microbial enzymatic actions (Hungate, 1966). This mode of digestion enables ruminants better access to energy from fibrous plant components compared to cecal digesters, e.g. horses and rabbits (Sakaguchi, 2003).

Figure 1.1 Anatomy of the gastrointestinal track in the ruminant.



Adapted from: http://actualidadagropecuaria.com.uy/carne-sin-metano-por-andres-costamagna/.

1.1.2 Ecology and evolution

The first ruminant on earth appeared in the Eocene, and were small, reclusive, forest-dwelling and omnivorous mammals (Webb and Taylor, 1980; Webb, 1998). Van Soest (1994) postulated that "ancient tropical forest browser ruminants, might have developed a predigesting system or pre-stomach that enabled detoxification of secondary plants substances, allowing greater latitude in dietary choice and adaptation".

Modern grazing ruminants (i.e. cattle, sheep and buffalo) and species that are not necessarily cellulose utilizers (i.e. goats and reindeer) have evolved to maximize the utilization of structural carbohydrates (Hofmann and Stewart, 1972; Van Soest, 1981, 1996). Anatomical adaptation of their digestive system, in symbiosis with anaerobic microbes (Hungate, 1966; Hobson and Stewart, 1997), has allowed ruminants to exploit fibrous food resources (Hoppe, 1977; Hofmann and Schnorr, 1982; Hofmann, 1989) and has rendered them relatively free from the need of external sources of essential amino acids and B vitamins (Owens and Basalan, 2016). This specialization has induced metabolic adaptations such as the need for gluconeogenesis and lipogenesis to cover the loss of available carbohydrates (Nafikov and Beitz, 2007). However, during their long association with humans (Zeder, 2008), domestic ruminants species have been selected genotypically and phenotypically (Van Soest, 1994; Phillips, 2009; Alves, 2016). Specialized breeds have been adapted to different environments and diets to cope efficiently with their genetic potential for production (Mignon-Grasteau et al., 2005; Mirkena et al., 2010), making them dependent on humans for their survival (Van Soest, 1994).

1.1.3 Ruminant production

Ruminants inhabit most climates around the world (Van Soest, 1994). Cattle are spread world-wide except in Arctic areas, where reindeer and yaks are utilized by some societies (Phillips, 1961; Van Soest, 1994). Buffalo are reared more regularly in wet tropical areas in Asia and, to a lesser extent, in Africa (Borghese and Mazzi, 2005). As the climate gets drier, relatively more sheep, goats and camels are found in Eurasian and African countries (Faye, 2014; Robinson et al., 2014). Llamas and alpacas are found in relatively large numbers in some South American countries (Wheeler, 1995). Approximately 3.6 billion domestic ruminants were reared around the world in 2011 (1.4 billon cattle, 1.1 billion sheep, 0.9 billion goats and 0.2 billion buffalo), with 25 million

domestic ruminants added to the planet each year over the past 50 years (Ripple et al., 2013). Livestock systems occupy about 30% of the planet's ice-free terrestrial surface area (Steinfeld et al., 2006) with demands for ruminant milk and meat projected to increase about 50% by 2050 (Revell, 2015). Supplying the increased requirements in ruminant commodities will likely involve increases in the number of domestic ruminants and ultimately production of greenhouse gases (GHG).

1.1.4 Global greenhouse emissions

In livestock systems, enteric fermentation and manure production release significant amounts of methane (CH_4) into the atmosphere. This non-carbon dioxide (CO_2) greenhouse gas (GHG), together with nitrous oxide (N_2O), accounted for 69% of GHG from the agriculture sector in 2010 (EPA, 2014), comprising 10 to 12% of global anthropogenic emissions (Tubiello et al., 2013). The livestock sector is projected to increase its total non-CO_2 emissions by 21% between 2010 to 2030 (EPA, 2014). The single largest source of CH_4 comes from fermentation of feed materials in the rumen (Johnson and Johnson, 1995), contributing 32 to 40% of agricultural non-CO_2 emissions (Smith et al., 2014). Globally, livestock is the largest anthropomorphic source of CH_4 emissions, contributing approximately 29% of total global CH_4 emissions in 2010 (EPA, 2014).

1.1.5 New Zealand's ruminant sector and GHG emissions

In New Zealand, ruminant livestock production systems (dairy cattle, beef cattle, sheep and deer) are usually pasture-based, with some farming systems, especially dairy cattle, utilizing preserved forages (hay and silage) or fodder crops during the winter months to supplement slow pasture growth (Hedley et al., 2006; Clark et al., 2007; Morris, 2013). The ruminant sector comprises 6.5 million dairy cattle, 27.5 million sheep, 3.6 million beef cattle and 0.8 million deer (Stats NZ, 2018). This sector accounted for 61% of the total value of exports in primary industries in 2018 (MPI, 2018). By 2019, the dairy sector is forecast to rise 2.1% due to the global dairy commodity prices, while the meat and wool export revenues are expected to decrease 1.3% due to a decline in lamb, mutton and beef production (MPI, 2018). Overall livestock production has grown substantially in response to international demand, and it is likely to continue to do so and thereby significantly increase GHG emissions. A recent report for the Ministry of Primary Industries predicted that livestock GHG emissions would be 22 to 29% above 1990 levels of 30.9 million tons

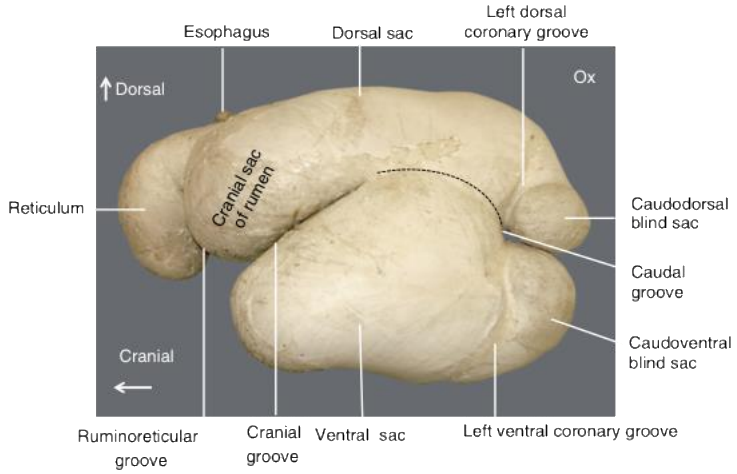
of carbon dioxide equivalent (Mt CO_2 - e; NZAGRC, 2012) by 2030 and 32 to 49% in 2050 (MPI, 2016). This contrasts with the New Zealand government's agreement of reducing GHG emissions (Mt CO_2 - e) from the agricultural sector to targets of 5%, 11% and 50% below 1990 levels by 2020, 2030 and 2050, respectively (New Zealand Gazette, 2011; MFE, 2016, 2017). Increases in CH_4 emissions from the livestock sector will reflect changes in animal numbers and production efficiency of the dairy sector. While there have been major gains in the sheep and beef sector in terms of reduction in emissions intensity, that does not mean that more gains are not required (Beukes et al., 2010; Mackay et al., 2012). It is therefore crucial that in order to meet GHG emission targets, government, industry and researchers are required to make a concerted effort to develop practical new tools to help reduce GHG emissions without curtailing productivity.

1.2 Rumen

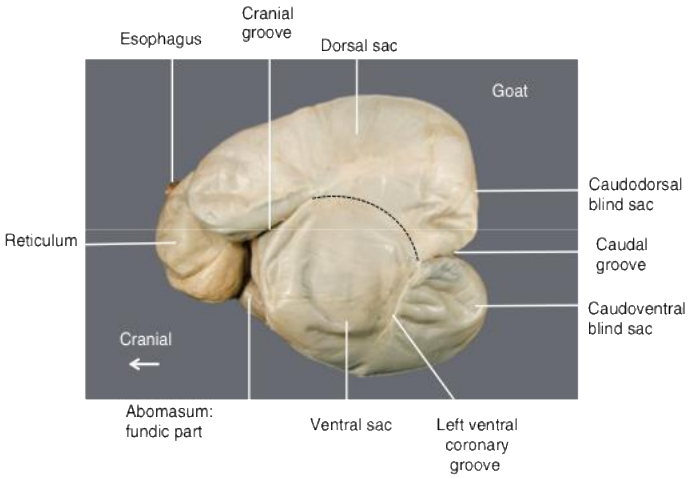
In ruminants, the stomach is composed of four complex compartments: (i) reticulum, (ii) rumen, (iii) omasum, and (iv) abomasum. In adult ruminants, the rumen is the largest compartment and the vat for anaerobic fermentation and absorption of short chain fatty acids (SCFA). The rumen is divided into ventral and dorsal sacs by the longitudinal caudal groove; both the ventral and dorsal sacs have blind sacs demarcated by coronary grooves (Membrive, 2016; Mansour et al., 2017). In cattle, the dorsal and the ventral sacs are similar in size (**Figure 1.2a**), whereas in sheep and goats the caudoventral blind sac extends more caudally than the caudodorsal sac (**Figure 1.2b**) (Mansour et al., 2017).

Figure 1.2 Rumen anatomy. a) Bovine ruminoreticulum: left lateral surface. The dotted line is the left longitudinal groove. The cranial and caudal longitudinal grooves encircle the rumen dividing it into dorsal and ventral sacs. The coronary dorsal and ventral grooves define the caudodorsal and caudoventral blind sacs. b) Goat's ruminoreticulum: left lateral view. Note that the caudoventral blind sac extends more caudally than the caudodorsal blind sac. The dotted line is the left longitudinal groove. Figures were adapted from Mansour et al. (2017).

a)



b)



1.2.1 The neonatal rumen

Neonatal ruminants possess a rudimentary rumen at birth (Warner et al., 1956; Large, 1964). Its size is relatively small compared with the abomasum where milk is digested (Warner et al., 1956; Large, 1964; Church, 1993). The anatomical and physiological development of the rumen at weaning facilitates adequate feed digestion and nutrient absorption, supporting health and productivity of young ruminants (Baldwin et al.,

2004; Khan et al., 2011; Khan et al., 2016). In intensive ruminant systems, the development of the rumen at an early age accelerates the transition from a milk-based diet to a solid feed diet (Baldwin et al., 2004; Khan et al., 2007a; Khan et al., 2011; Stamey et al., 2012; Khan et al., 2016).

Neonatal ruminants reared solely on milk during the first months of life exhibit limited ruminal development with respect to rumen weight, capacity, papillary growth, degree of keratinization and musculature development compared to those with free access to starter concentrates (Warner et al., 1956; Smith, 1961; Gilliland et al., 1962; Tamate et al., 1962; Hamada et al., 1976). Milk generally passes through the oesophageal grove into the abomasum, preventing it from entering into the rumen and being fermented (Membrive, 2016). The amount, method, and quality of the milk fed to the animal affects small intestine development, growth, solid feed intake and metabolic status of the animal (Khan et al., 2007a; Khan et al., 2011; McCoard et al., 2014; Nemati et al., 2015). Slowing down small intestine development negatively affects growth and metabolic status, which indirectly influences the development of the forestomach in young ruminants (Gorka et al., 2011).

1.2.2 Anatomical development of the rumen

At birth, the rumen accounts for 38% of the empty stomach mass, while the abomasum and omasum comprise 49% and 13%, respectively (Membrive, 2016 adapted from Darce, 1977). The development of rumen anatomy is stimulated by solid feed intake and the associated production of fermentation end products (Warner et al., 1956). The ingestion of concentrate diets rich in starch promotes increased density and length of rumen papillae (Stobo et al., 1966; Žitnan et al., 1998; Žitnan et al., 2003; Shen et al., 2004; Golder et al., 2014a) relative to forage-based diets which contain primarily hemicellulose and cellulose. Consumption of bulky forages primarily increases rumen volume (Warner et al., 1956). Highly fermentable diets, such as concentrate diets rich in cereals and legumes, result in rapid production of SCFA, branched-SCFA and ammonia (Van Soest, 1994). The presence of SCFA in the rumen stimulates rumen epithelial cells (Beharka et al., 1998; Shen et al., 2004; Gorka et al., 2009; Moolchand et al., 2013; Gui and Shen, 2016), which react to the physicochemical environment with coordinated changes in cell proliferation, cellular function, and tissue permeability (Penner et al., 2009; Aschenbach et al., 2011; Penner et al., 2011; Yan et al., 2014; Liu et al., 2016; Steele et al., 2016; Wang et al., 2016a; Shen et al., 2017). However, *in vitro* studies have shown that the addition of 10% rumen

fluid from cows inhibits the proliferation of postnatal epithelial cells in culture (Wang and Jiang, 2010). Therefore, increases of intra-ruminal concentrations of SCFA promote epithelial cell proliferation in combination with the release of other components such as hormones and growth factors by the animal (Shen et al., 2004; Zhao and Sun, 2010; Wang et al., 2016a).

1.2.3 Metabolic development

Metabolic adaptations occur during the development of the rumen epithelium including changes of metabolizable substrates and increased production of ketone bodies from butyrate (Baldwin et al., 2004). In neonate ruminants, intra-ruminal infusions of SCFA stimulate epithelial cells to oxidize less glucose and produce more acetoacetate, however, β-hydroxybutyrate production does not differ from non-infused animals (Lane and Jesse, 1997). The time course of rumen metabolic development in neonate ruminants indicates that changes in substrate oxidation from glucose to butyrate does not occur in the absence of solid feed consumption. Whilst the development of rumen ketogenesis occurs in the absence of fermentation end products from solid feed. (Lane et al., 2000; Lane et al., 2002). In conventionally raised ruminants, the rumen epithelium is able to oxidize glucose, lactate and butyrate at high rates within two weeks of birth. By the time of weaning (usually defined as when the young ruminant is consuming enough starter concentrates to be weaned) the capacity for glucose uptake by the rumen epithelium has diminished, and SCFA become the primary oxidative energy source. The capacity of the rumen epithelial cells to produce ketone bodies from SCFA rapidly increases after weaning (Giesecke et al., 1979; Baldwin and Jesse, 1992)

Changes in the mass and/or surface of the rumen epithelium, induced by the level of intake and the composition of the diet, occur simultaneously with metabolic adaptations in epithelial cells (Rémond et al., 1995). In growing and adult ruminants ruminal epithelial development provides the major area for absorption of SCFA (Malhi et al., 2013; Melo et al., 2013). In young ruminants, following a short-term grain challenge, increases in the concentrations of SCFA and reductions of ruminal pH have been associated with rapid adaptations of the ruminal epithelium by increasing ketogenic metabolism and papilla enlargement (Steele et al., 2012). Increased feed intake, associated with greater production of intraruminal SCFA, is associated with increased levels of acetyl-, propionyl- and butyryl-CoA synthetases in the rumen epithelial cells (Harmon et al., 1991). The ruminal

epithelium has an enormous capacity for the absorption and metabolism of SCFA. This not only delivers metabolic energy to the animal but is also an essential regulatory mechanism that stabilizes the intra-ruminal milieu.

Further investigation

Over the last decade, the complete genome of domestic ruminant species have provided new opportunities to investigate the expression of genes related to rumen differentiation and function (Connor et al., 2010; Sun et al., 2018). Recent studies have investigated the effects of dietary manipulation on gene transcription and protein expression of candidate genes important for the normal function of the gastrointestinal tract during the growth and development of ruminants (Naeem et al., 2012; Connor et al., 2014; Sun et al., 2018). Thus, important pathways and mechanisms such as ruminal epithelial cell differentiation, proliferation, function and metabolism have been described using gene expression studies (Connor et al., 2010; Penner et al., 2011; Naeem et al., 2012; Connor et al., 2014; Naeem et al., 2014). However, it has not been clearly established how the microbial community of the rumen (bulk or adherent to the epithelium) affects rumen development and function through direct microbial-host cell signaling.

1.3 Rumen fermentation

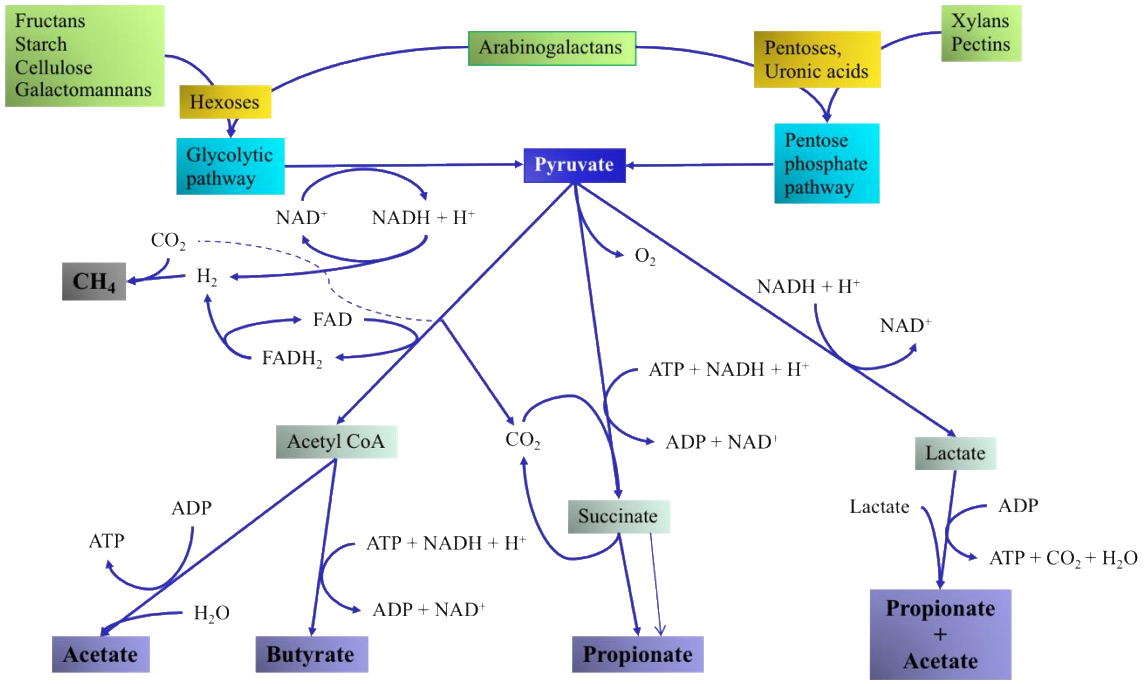
One of the reason ruminants can effectively utilize hemicellulose and cellulose is by virtue of the arrangement and large size of their digestive tract (Church, 1993; Van Soest, 1994). The reticulo-rumen maintains feed for a time period sufficiently long enough for resident microbes to hydrolyze complex cell wall and other polysaccharides to 5- and 6-carbon sugars by the activity of microbial enzymes (Van Soest, 1994; Owens and Basalan, 2016). These hexoses and pentoses are then fermented by the rumen microbes to SCFA and energy is released (Owens and Basalan, 2016).

1.3.1 Short chain fatty acids

The proportions of SCFA vary with substrate type, substrate concentration, and fermentation conditions (Van Soest, 1994). The proportions of SCFA produced dictate the amount and composition of the gases released, energy retention in the fermentation products, and the yield of ATP for microbial growth (Owens and Basalan, 2016). **Figure 1.3** shows the pathways for polysaccharides fermentation and the resultant production of

SCFA and release of CO_2 and CH_4 gases in the rumen. The formation of one mole of butyrate (4 carbons atoms) requires one mole of glucose (6 carbon atoms); in contrast, two moles of either acetate (2 carbon atoms) or propionate (3 carbon atoms) are formed from a single mole of glucose. However, residual carbon from glucose fermentation is lost as gas (CO_2 and CH_4) (Van Soest, 1994; Nagaraja et al., 1997; Owens and Basalan, 2016). Understanding the thermodynamic laws that control the fermentation and the SCFA profiles could enable a more logical approach to manipulate the rumen microbiota to enhance its beneficial aspect while reducing any negative outcome (Nagaraja et al., 1997; Russell and Rychlik, 2001).

Figure 1.3 Schematic representation of the pathways for polysaccharides fermentation by ruminal bacteria. Adapted from Moss et al. (2000); Russell and Rychlik (2001); Nagaraja (2012); Ungerfeld (2013).



1.3.2 Hydrogen and methane production

The formation of acetate and butyrate results in the production of reducing equivalents (NADH; i.e., metabolic hydrogen) for subsequent processing. The excess of hydrogen in the rumen is removed by converting CO_2 and CH_4. Four moles of reducing equivalents are used for each mole of CH_4 formed from CO_2. No CH_4 is generated during propionate production, but unlike acetate and butyrate, some of the excess H_2 generated

during their production is used to form propionate. Thus, the amount of CH_4 produced by the rumen depends on the amount of excess H_2 (NADH) produced during fermentation of carbohydrates. Therefore, the greater the ratio of acetate and butyrate to propionate, the higher total yield of CH_4 from H_2 and CO_2 (Janssen, 2010). The stoichiometry of the main anaerobic fermentation pathways produces reducing equivalents that can be summarized as follows (Hungate, 1966; Czerkawski, 1976; Moss et al., 2000):

2H production reactions:

Glucose → 2 pyruvate + **4H** (Embden-Meyerhof-Parnas pathway)

Pyruvate + H_2O → acetate (C2) + CO_2 + **2H**

2H consumption reactions:

Pyruvate + **4H** → propionate (C3) + H_2O

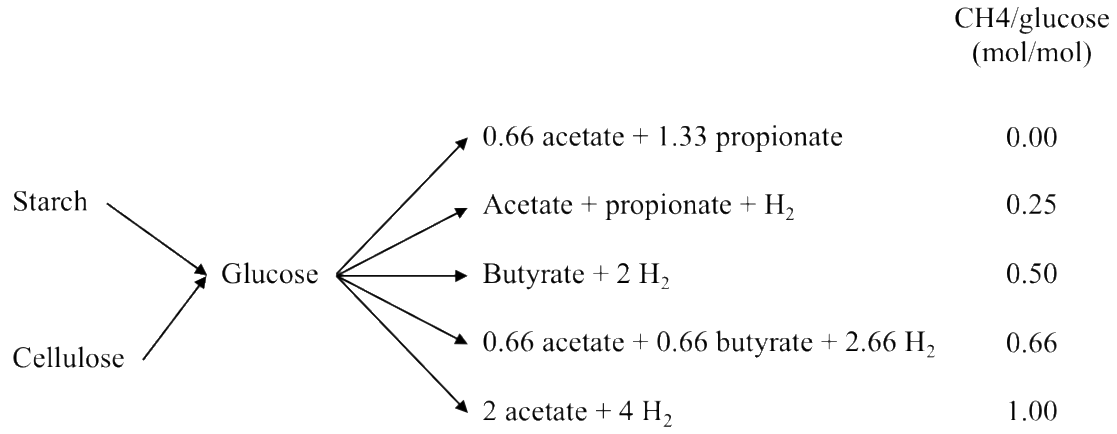
2 C2 + **4H** → butyrate (C4) + $2H_2O$

This metabolic hydrogen is converted to H_2 by hydrogenase-expressing bacterial species, and the H_2 converted to CH_4 by archaea in the combined reaction:

CO_2 + **8H** → methane (CH_4) + $2H_2O$

Hydrogen is a key product of rumen microbial metabolism (**Figure 1.4**) and thermodynamic control of hydrogen partial pressure on individual SCFA produced and associated yield of H_2 and CH_4 cannot be explained without considering NADH oxidation (Van Lingen et al., 2016). Inhibition of CH_4 production results in the redirection of metabolic hydrogen towards propionate and H_2, but not butyrate (Denman et al., 2015; Martinez-Fernandez et al., 2016).

Figure 1.4 Pathways of cellulose and starch fermentation via glucose, to acetate, propionate, butyrate, and H2. CO2, H+, and H2O are not shown for simplicity. Adapted from Janssen (2010).



1.4 The Rumen Microbiota

Herbivore animals lack enzymes capable of degrading cellulose and hemicellulos, the principal components of plant cell walls (Van Soest, 1996). The ability to utilize these structural plant components, as food by the animal, depends on the presence of gastrointestinal microorganisms to degrade them and the capacity of the herbivore host to maintain these microbes and utilize their end-products (Van Soest, 1994; Van Soest, 1996; Hobson and Stewart, 1997; Nagaraja, 2016). The rumen offers the perfect conditions for the development and growth of many of these fermentative microorganisms (Van Soest, 1994; Owens and Basalan, 2016). The retention time of ingested material in the rumen is greater than the generation time of the microbial organisms which allows their maintenance, and prevents microbial wash out from the rumen (Van Soest, 1996; Owens and Basalan, 2016). In the young ruminant, the establishment of an anaerobic microbial ecosystem is essential for the commencement of ingested solid feed fermentation and the development of absorptive mechanisms in the rumen (Baldwin et al., 2004; Malmuthuge and Griebel, 2015; Yáñez-Ruiz et al., 2015; Malmuthuge and Guan, 2017).

1.4.1 Microbial Exposure in early life

Prior to birth, the rumen is free of microorganisms (Malmuthuge and Griebel, 2018). The factors that govern the transfer of microorganisms to young ruminants are not well understood. However, it is likely that maternal transfer and the rearing environment contribute to the establishment of the gastro-intestinal microbial population in young

ruminants (Fonty et al., 1987; Curtis and Sloan, 2004; Fonty et al., 2007; Abecia et al., 2014a; De Barbieri et al., 2015).

Maternal transfer (intrauterine, vaginal, saliva, milk, feces, skin)

In monogastric species, i.e. mice and humans, the microbial colonization of the offspring before, during and after birth plays an important role in the development of the gastrointestinal tract (GIT) (Funkhouser and Bordenstein, 2013). In ruminants, the fetal environment and fetal GIT remain sterile during the third trimester of pregnancy (Malmuthuge and Griebel, 2018). Gastrointestinal microbial colonization likely first occurs during birth when the neonate is in contact with the microorganisms that proliferate in the dam's vagina (Ducluzeau, 1983a; Guzman et al., 2015).

Post-partum, interaction between the offspring and dam is one of the main sources of microbial inoculations, through grooming and licking, and sucking of the teat and ingestion of colostrum and milk (Becker and Hsiung, 1929; Fonty et al., 1987). Instinctively, ruminant dams make physical contact with their offspring, spending much of the first few hours after birth licking the newborn (von Keyserlingk and Weary, 2007). This behavior is vital for stimulating the newborn's physiological activity, and may have important implications in the transfer of microbes through maternal saliva, rich in rumen-like microbiota (Kittelmann et al., 2015), providing an important inoculum for the colonization of the offspring's GIT (Guzman et al., 2015).

The maternal microbial inoculation of the offspring sets in place early life processes for ruminal fermentation of ingested solid feed in ruminants (Dehority and Orpin, 1997). Studies evaluating the effect of rearing system (natural *vs.* artificial) on microbial establishment have indicated that suckled young ruminants had higher concentrations of bacteria, protozoa and fungi in the rumen at weaning than bottle fed contemporaries, which were removed from their dams immediately after birth (Fonty et al., 1987; Abecia et al., 2014a). These differences in microbial colonization may be because the dam's presence increases the number and diversity of microorganisms in the neonatal ruminant's environment (Abecia et al., 2017; Cunningham et al., 2018).

Environmental (soil, feed, water)

Newborn ruminants are constantly exposed to microbes within the environment, some of which may be able to colonize the rumen (Curtis and Sloan, 2004). Although many

rumen microorganisms are obligate anaerobes, it is believed that they are sufficiently resistant to aerobic conditions so as to be transferred by saliva, feed, feces, facilities, and perhaps through aerosols from one animal to another (Fonty et al., 1987; Van Soest, 1994; Dehority and Orpin, 1997). Anaerobic fungi survive in faces for a considerable period of time, and fecal contamination or coprophagy is another means of transfer (Dehority and Orpin, 1997; Hobson and Stewart, 1997). Ruminal protozoa can only be passed from animal to animal by direct transfer of saliva containing the active organisms (Becker and Hsiung, 1929) as there is no aerobic resistant phase or cysts in their life cycle (Strelkov et al., 1933).

1.4.2 Microbial establishment

Microbial communities, composed of bacteria, protozoa, anaerobic fungi, and archaea, play an important role in the nutritional, physiological, immunological and protective function of the host ruminant (Liang et al., 2015; Yáñez-Ruiz et al., 2015; Pitta et al., 2016; Garcia et al., 2017; Malmuthuge and Guan, 2017). In the developing rumen microbial colonization occurs in a defined and progressive sequence (Fonty et al., 1987; Jami et al., 2013; Rey et al., 2014), a process that is affected by diet and contact with older ruminants (Dehority and Orpin, 1997).

During the first hours of life, rumen colonizing bacteria consist of a high abundance of aerobic and facultative anaerobic bacteria (Guzman et al., 2015). However, strict anaerobic bacteria and archaea which predominate in the mature rumen rapidly became the dominant taxa in the rumen one to two days after birth, as reflected by the near disappearance of aerobic and facultative anaerobic taxa (Fonty et al., 1987; Morvan et al., 1994). Prior to the ingestion of solid feed, all major types of rumen bacteria, including proteolytic and cellulolytic species are already present in the rumen (Rey et al., 2014). This has been noted in the neonatal ruminant, in which fermentation activity may be observed as early as the first or second day of life during which milk is the only component of the diet (Rey et al., 2012; Abecia et al., 2014a; Guzman et al., 2015). However, as neonates transition from milk to solid diets and different rearing practices, the bacterial community phyla changes (**Table 1.1**), with reductions of *Proteobacteria* and increases of *Bacteroidetes* and *Firmicutes* (Rey et al., 2014; Kim et al., 2016; Wang et al., 2016c; Abecia et al., 2017; Dias et al., 2017; Saro et al., 2018).

Table 1.1 Ruminal bacteria composition during rumen development in young ruminants. **a)** Abundant bacteria phyla composition in calves at different ages receiving different dietary and rearing managements. **b)** Abundant bacteria phyla in lambs and goat kids at different ages receiving different dietary and rearing managements. Values expressed as range of mean percentages [1, 2].

a)	**Age (days)**									
Phyla	**3**	**7**	**14**	**28**	**35**	**42**	**49**	**56**	**63**	**77**
Proteobacteria	46.6-70.4	16.9-18.7	6.45-16.9	1.8-27.6	4.6-6.2	12.0-27.6	1.9-7.1	5.6-7.4	4.9-7.2	4.2-6.0
Bacteroidetes	13.9-42.6	56.3-56.9	46.0-61.3	49.9-56.3	47.0-60.4	56.3-75.0	18.3-42.8	15.8-28.3	17.2-21.9	21.1-33.9
Firmicutes	5.1-13.9	13.9-17.5	13.9-34.0	13.9-42.1	29.1-39.3	10.0-13.9	40.9-58.6	45.6-48.8	62.6-69.6	44.9-53.7
Actinobacteria	0.1-4.9	0.6-4.9	1.0-4.9	0.3-4.9	2.6-3.4	4.9	4.7-13.9	5.3-8.9	4.9-6.0	6.4-13.5
Spirochaetes	0.0-0.4	0.1-0.4	0.4-2.6	0.4-0.9	0.2-0.7	0.4	0.1	NA	0.1	NA
Fibrobacteres	0.0-0.3	0.0-0.3	0.2-0.3	0.3-1.5	0.1-0.4	0.3-1.6	0.5-0.9	NA	0.0	NA
Tenericutes	0.0	0.8	0.2	0.9	0.3-1.2	1.0	0.2-0.8	NA	1.8-2.8	NA

[1] Data collected from Li et al. (2012b), Jami et al. (2013), Rey et al. (2014), Kim et al. (2016), Meale et al. (2017).
NA – Not available

b)	**Age (days)**								
Phyla	**1**	**10**	**20**	**38**	**41**	**50**	**60**	**98**	**140**
Proteobacteria	70.3	47.1	17.4	2.2	2.6-19.7	13.8	0.3-4.1	0.4	0.8
Bacteroidetes	14.3	29.4	35.6	35.9	27.6-66.0	30	10.7-52.2	61.1	60.1
Firmicutes	12.2	20.9	38.6	55.9	15.0-44.5	41.1	26.6-61.7	28.7	30.1
Actinobacteria	2.5	0.8	0.6	2.9	0.1-1.6	0.9	0.4-0.8	0.4	0.1
Fusobacteria	NA	NA	NA	NA	NA	NA	0	0	0
Spirochaetes	0	0.3	1.5	0.7	1.4-9.6	3	1.1-1.2	1.2	0.1
Fibrobacteres	0.1	0.1	1.3	0.1	0.1-0.8	1.1	0.1-9.4	3.7	3.3
Tenericutes	NA	NA	NA	NA	0.7-1.2	NA	0.3	0.2	0.3

[2] Data collected from Wang et al. (2016c), Wang et al. (2017a), and Saro et al. (2018).
NA – Not available

Methanogenic archaea are found right after birth in the undeveloped rumen of neonates (Guzman et al., 2015). Metabolic active methanogens are identified from one-day of age (Friedman et al., 2017) and reach concentrations equivalent to those in adult animals before the arrival of solid substrate in the rumen (around 10–14 days after birth) (Fonty et al., 1987; Morvan et al., 1994). However, the methanogenic densities become stable as solid feed is introduced and ruminants are weaned (Wang et al., 2017b). The early methanogenic community is characterized by a high activity of methylotrophic methanogenesis, likely performed by members of the order *Methanosarcinales*, found in the underdeveloped rumen. In contrast, higher hydrogenotrophic activity and proportions of hydrogenotrophic, that are similar to that in the mature rumen taxa, are observed after two-weeks of age (Friedman et al., 2017). The establishment of ruminal ciliate protozoa is dependent upon suitable environmental conditions within the rumen. In the ruminant neonate, ruminal pH affects the establishment of protozoa in the newborn (Dehority and Orpin, 1997). A sequence of establishment of protozoan fauna has been described in young ruminants, *Entodinium* first, followed by *Diplodinium* and then the holotrichs (Bryant et al., 1958). Early studies in young ruminants have indicated that *Entodinium* became established at a pH a little above 6.0, and *Diplodinium* and the holotrichs did not develop until the pH reached 6.5 or above (Eadie, 1962). Anaerobic fungi are established in the rumen of neonates within the first 8–10 days after birth, before the entry of solid feed into the rumen (Fonty et al., 1987). These organisms are normally established on forage-based diets, and although anaerobic fungi have amylase activity, it is believed to minimally contribute with starch and glycogen degradation because their population decreases in grain-fed animals (Nagaraja, 2016);however the presence of plant fiber is not essential for their establishment in the pre-rumen (Fonty et al., 1987; Dehority and Orpin, 1997). Obtaining further knowledge on the establishment of the microbial community in the rumen is important to improve the fundamental understanding of the development and functions of this fermentation chamber.

1.4.3 Microbial community in the bulk (liquid and solid content)

The diversity of the rumen microbiota from different sites within the rumen and time after eating showed a high similarity for each individual animal (Li et al., 2009; Söllinger et al., 2018). However, lower similarities were found between the liquid and solid fractions of rumen contents (Wang et al., 2017a; Wang et al., 2017b; O'Callaghan et al., 2018). In the rumen, the liquid fraction is dominated by bacteria (e.g. *Prevotellaceae*) that

degrade soluble nutrients, such as short chain carbohydrates: mono-, di-, tri-saccharides, while the solid fractions composed of long chain polysaccharides such as cellulose, hemicellulose, pectin, have great abundances of cellulolytic bacteria that pioneer biofilm formation[1], together with secondary colonizers such as *Clostridiales, Ruminococcaceae*, *Lachnospiraceae* and *Rikenellaceae* (Henderson et al., 2013; Jiao et al., 2015c; De Mulder et al., 2017; Klevenhusen et al., 2017). In regard to the archaea, the liquid fraction include higher abundances of the *Methanobrevibacter gottschalkii*, while the solid fraction is characterized by a greater abundance of *Methanobrevibacter ruminantium* (Henderson et al., 2013; Jiao et al., 2015c; De Mulder et al., 2017). Contrasting distribution of several members of the ciliate protozoal and fungal communities in the solid or liquid rumen fractions have also been observed (Henderson et al., 2013; Jiao et al., 2015c). However, dietary manipulations, such as changing the forage to concentrate ratio has the potential to alter the microbial composition of the liquid and solid fractions in the rumen (Huo et al., 2014; Jiao et al., 2015c; Ji et al., 2017).

1.4.4 Microbial community attached to the ruminal epithelium

Bacteria attached to the rumen epithelium are small proportion (1 or 2% of the total bacteria) of the total ruminal bacteria (Mueller et al., 1984), but play an important role in an ecosystem where material is constantly being introduced and removed (Church, 1993). Studies using culture-dependent methods and electron microscopy have indicated that epithelial attached or epimural bacterial communities are different from those associated with rumen contents (McCowan et al., 1978; Cheng et al., 1979a; Cheng et al., 1979b; Wallace et al., 1979; McCowan et al., 1980).

Initial studies have suggested that the epimural bacterial community played a role in a range of different functions such as the hydrolysis of urea, the scavenging of oxygen, and the recycling of epithelial cells (Cheng et al., 1979a; Wallace et al., 1979; Dinsdale et al., 1980; Mueller et al., 1984). However, recent studies using polymerase chain reaction (PCR) denaturing gradient gel electrophoresis (PCR-DGGE) (Sadet et al., 2007; Sadet-

[1] A biofilm comprises any syntrophic consortium of microorganisms in which cells stick to each other and often also to a surface (Lopez et al., 2010; Hall-Stoodley et al., 2004). The biofilm formation begins with the attachment of free-floating microorganisms to a surface (O'toole and Kolter, 1998; Watnick and Kolter, 200). The first colonist bacteria of a biofilm may adhere to the surface initially by the weak van der Waals forces and hydrophobic effects (Briandet et al., 2001; Takahashi et al., 2010). If the colonists are not immediately separated from the surface, they can anchor themselves more permanently using cell adhesion structures such as pili.

Bourgeteau et al., 2010) and 16S rRNA sequence analysis (Li et al., 2012a; Petri et al., 2013a; Jiao et al., 2015a) have identified multiple species of epimural bacteria which cannot be cultured, providing a baseline that could give new insights with regards to the functions of these microorganisms in the rumen ecosystem.

Amplicon-pyrosequencing of the 16S rRNA genes has provided a more complete analysis of the taxonomic segregation of bacteria associated with the rumen mucosa. Important differences have been found in the bacterial composition and relative abundance of shared bacterial genera in the rumen digesta and associated with the rumen wall (Li et al., 2012a; Malmuthuge et al., 2014). Sequence analysis has also identified differences at the phylum level in rumen epimural bacterial communities at different stages of rumen development. Neonatal ruminants, the rumen epithelium has high proportions of *Proteobacteria* in the first days of life (Jiao et al., 2015a). During the transition from milk to solid feed intake the proportions of *Proteobacteria* decline and the proportions of *Firmicutes* and *Bacteroidetes* increase (Malmuthuge et al., 2014; Jiao et al., 2015a; Liu et al., 2017a). Finally, once solid feed is stablished the *Firmicutes* became the most abundant bacteria phylum in the rumen walls, independently of the ratio of forage:concentrate, followed by *Proteobacteria* and *Bacteroidetes* (Chen et al., 2011; Li et al., 2012a; Petri et al., 2013a; Jiao et al., 2015a; Liu et al., 2015; Liu et al., 2017a; Shen et al., 2017). The bacterial colonization and establishment of the rumen starts in early life with a distinct segregation of communities between digesta and ruminal surfaces (Malmuthuge et al., 2014). However, there is still little information about the level of variation in epimural communities among individual animals consuming different diets, as well as, the identification at the genus level of uncultured and unidentified epimural bacteria.

1.4.5 Host-microbial interactions

In recent years, it became evident that the study of an individual organism can only partially reveal the molecular processes underlying vital functions and environmental interactions (Gilbert et al., 2012). In fact, it has been postulated that macro-organisms live in mutual symbiosis with micro-organisms such as bacteria, archaea, viruses, protozoa, and fungi (Bosch, 2012). The relationship between a ruminant and its complex microbial community has defined its specialized anatomy, digestive-physiology, feeding behavior, and ultimately determines its evolution (Van Soest, 1994; Kamra, 2005; Puniya et al., 2015). Moreover, the symbiotic microbiota exerts developmental, nutritional, protective

and immunological effects that benefit the host (Hooper, 2004); although, it can also have a detrimental impact on the host as is the case for digestive disturbances such as bloat and acidosis (Pitta et al., 2014b; Mao et al., 2015).

Microbial-host cell signaling

With the development of "omics" methods, an extraordinary wealth of information can be generated by combining rumen microbial metagenomics with the sequenced genomes of cattle and sheep (Wallace et al., 2017). Early studies in mice have shown how the host interacts with its microbial community through the activation of micro RNA (miRNA) mediators in the GIT (Masotti, 2012). The expression of miRNA, during microbial colonization, is initiated as a response to changes in microbial community density and/or composition and is involved in host-microbial cross talk through regulation of mRNA (Liang et al., 2014; Liang et al., 2015). Few studies of microbial-host cell signaling have been carried out in ruminants. Liang et al. (2014) investigated the role of miRNAs in the development of the GIT during the early life of calves. They identified that the expression of miR-129 in the rumen was correlated with total bacteria in the rumen. Based on functional analysis, miR-129 may be involved in the rumen development in response to the increasing bacterial population. However, further studies are required to determine how miRNA expression changes in relation to the host transcriptome and rumen microbiome (metagenome and metatranscriptome), which could provide further evidence of the role of miRNA in mediating host-microbial interactions.

Microbial-host cell metabolism

The coevolution of mammals and their gut bacteria has, in effect, resulted in the outsourcing of developmental signals from animal cells to microbial symbionts (Gilbert et al., 2012). In ruminants, the microbial symbiosis has been expressed through the evolution of their specialized nutritional physiology (Van Soest, 1994). The rumen microbiota provides benefits to the host such as lipid metabolism, xenobiotic detoxification, vitamin synthesis, cellulose digestion, non-nitrogen protein utilization and SCFA production (Hobson and Stewart, 1997; Kamra, 2005; Puniya et al., 2015). From the microbe's perspective, the ruminant offers its symbiont microorganisms an environment where there is SCFA removal, pH regulation, ammonia recycling, constant temperature, and a constant supply of nutrients (Church, 1993; Van Soest, 1994; Owens and Basalan, 2016). High throughput omics-technologies (genomics, transcriptomics, proteomics, and

metabolomics) generating deeper insights into the symbiotic host-microbial metabolic relationship in ruminants (Morgavi et al., 2013; Deusch et al., 2015; Wallace et al., 2017).

1.5 Manipulations of the rumen microbiota

Animal nutrition and rumen microbiology have been studied separately for many decades due to the failure to correlate differences in bacterial populations, functions, or phylogeny to significant responses in the animal. **Figure 1.5** describes how the rumen microbial community is linked to the ruminant nutrition field, which describes the diet's ability to meet animal requirements by measuring performance, voluntary intake, fermentation parameters, passage rate, diet digestibility, and nitrogen metabolism.

1.5.1 Diet

The microbes that inhabit the rumen are the main agents for the degradation of complex carbohydrates, lipids and proteins ingested in the diet (Church, 1993; Van Soest, 1994). The biology and ecology of the microbial community is similar within ruminant species, but different across species (Henderson et al., 2015). However, the specification and adaptation of these respective populations are determined mainly by the diet's chemical composition and turnover time as opposed to the species of host (Van Soest, 1994; Henderson et al., 2015).

Under grazing conditions, the rumen microbial community continuously adapts to changing dietary composition, nutrient density and environmental conditions. The rumen microbial diversity of animals fed forages with high contents of structural carbohydrates and proteins is more diverse than that observed in animals fed forages with more soluble nutrients (Kong et al., 2010; Pitta et al., 2010). Variations in the rumen microbial ecosystems are attributed to the physicochemical composition of the forage diet, where greater bacterial diversity may be required to degrade forages rich in structural carbohydrates compared to forages rich in soluble carbohydrates.

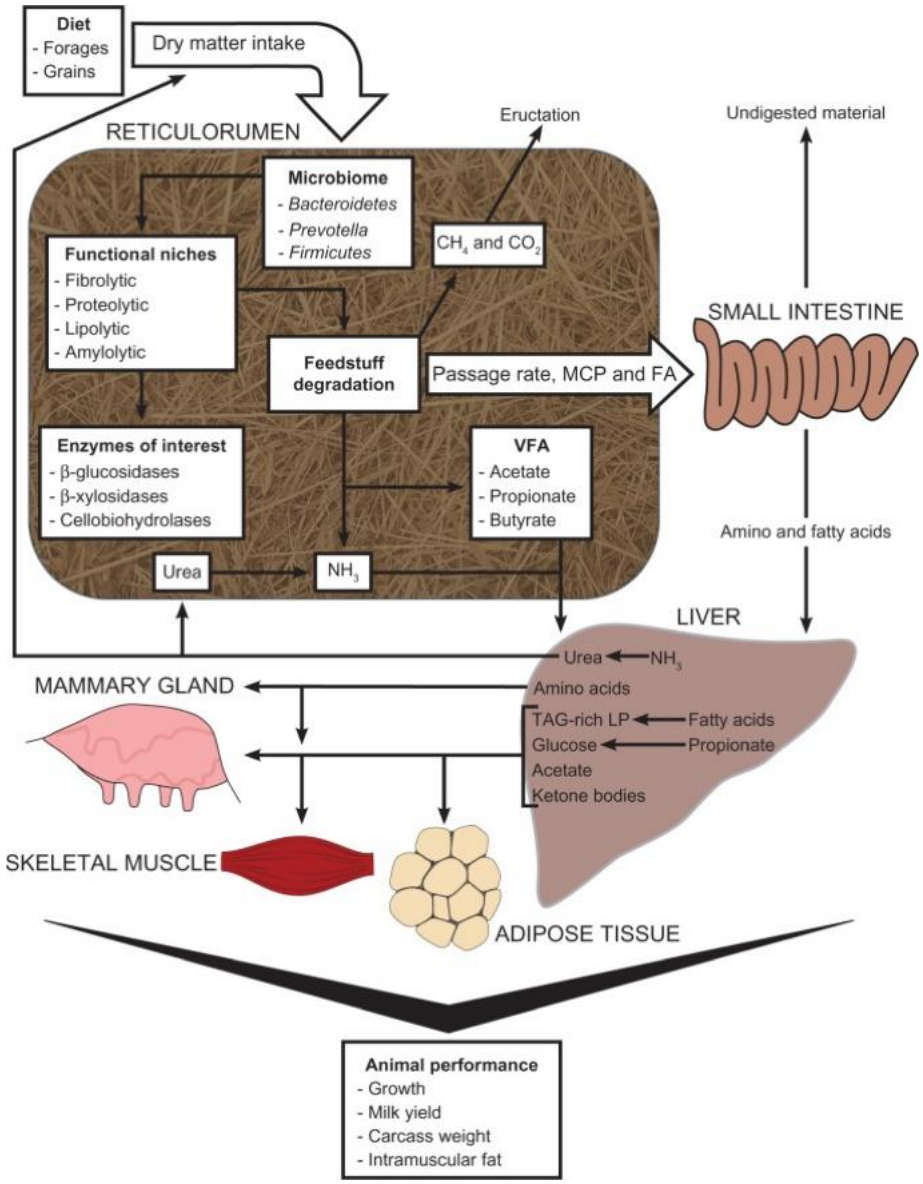
The transition of ruminants from forage diets to highly-digestible diets results in a decrease in ruminal pH (Slyter, 1976; Nocek, 1997; Marchesini et al., 2013), which can results in decreased animal performance (Owens et al., 1998). During the adaptation to highly fermentable diets, significant changes in the rumen environment and in the structure if the bacterial community are observed (Tajima et al., 2000; Fernando et al., 2010; Petri et

al., 2013b; Golder et al., 2014b). The rumen microbial community has lower diversity in grain-fed compared to forage-fed animals (Fernando et al., 2010; Petri et al., 2013b). There is a reduction in microbiota diversity and a reduction the proportions of cellulolytic bacteria, while saccharolitic genera from *Bacteroidetes* and *Firmicutes* increase (Fernando et al., 2010; Petri et al., 2013b; Golder et al., 2014b). Increases in the abundance of saccharolytic bacteria from the phylum *Bacteroidetes* predominate when there is a step-up transition from forage to concentrate diets, while the abrupt increases of starch in the diet favors the increase from the *Firmicutes* which tolerate low ruminal pH.

1.5.2 Inhibitors

Rumen fermentation and nutrient outflow from the rumen can be manipulated by adjusting the microbial activity and population structure with chemical agents that modulate selective pathways of microbial metabolism (Chalupa, 1977). The ingestion of methanogen inhibitors, e.g. chloroform, anthraquinone, bromochloromethane, has produced reductions in the number of archaea with corresponding reductions in CH_4 emissions and increases in intra-ruminal H_2. These changes then result in alterations in the composition of the bacterial populations and increases in the proportions of propionate (Kung et al., 2003; Abecia et al., 2014b; Denman et al., 2015; Martinez-Fernandez et al., 2016). In adult ruminants, the inhibition of specific group of ruminal microorganisms produces temporary changes in ruminal fermentation and CH_4. and H_2. emissions (Weimer, 2015). However, these differences in the archaeal community composition do not persist after treatment ceases; although, there are longer term changes in some less abundant groups of archaea species that remained different between treated and control animals (Abecia et al., 2014b).

Figure 1.5 The link between nutrition, metabolism and rumen microbiology (Figure adapted from McCann et al., 2014).



1.6 Rationale and research questions

The international demands for ruminant products coupled with pressures on reducing GHG emissions on the livestock pastoral-systems in New Zealand are leading to changes in the rearing management of ruminants. Whilst, it is well known that ruminal microbes digest the plant material in the rumen and produce GHG, there is still limited research into the structure and dynamics of the rumen microbiota, especially during rumen development and dietary management transitions of growing animals. Furthermore, it has not been elucidated whether different management interventions during the early life may lead to a ruminal microbiome imprint that could persist during adulthood. Additionally,

there is a need for understanding whether or not changes in the rumen microbiome composition are linked to the animal performance/environmental impact. This knowledge may represent an important step in the analysis of the rumen microbiota and should guide efforts in the formulation of rearing strategies to improve animal performance and mitigate GHG emissions.

In summary, there is a need to understand how dietary management or chemical interventions during the early life of neonate ruminants affects: a) the development of the rumen morphology and function; and b) the establishment, ecology and metabolism of the ruminal microbiota that inhabits the rumen contents. Additionally, it is important to explore how changes in microbial community affect: a) the rumen fermentation and gas production; and b) the rumen epithelia development and host phenotype. Therefore, this program of research is based on the existing knowledge gaps in the current literature, and intends to address and test the following research questions and hypothesis:

1. Studies in lambs have shown that abrupt weaning at an early age and before the ingestion of solid feeds produce growth check due to the undeveloped rumen at weaning. However, the implementation of gradual (step-down) weaning in calves has shown to improve rumen developemnet during the transition from milk to solid feeds.

   Based on the current knowledge, the following questions were formulated: How does weaning off milk at different ages through a step-down weaning procedure affect rumen development of artificially-reared lambs? and 2) Is the histomorphometry of four ruminal sites equally affected by the diet offered at 4 and 16 wk of rearing?

   It is hypothesized that: 1) early rumen development will be achieved in lambs weaned at wk 4 compared to those weaned at wk 6 using the same step-down weaning procedure, and 2) rumen histomorphology will differ across rumen sites within and between wk 4 and wk 16.

2. Studies in young ruminants have showed that the weaning method does not affect the establishment of the microbiota in the rumen. However, it has not been indicated whether the age at weaning off milk may affect the rumen microbial establishment. Additionally, there are no reports of the rumen microbiota composition in lambs

throughout different rearing management transitions like: after separation from the mother, when step weaned at different ages with free access to starter concentrates and fiber, and during grazing of mixed-sward pastures.

The following research questions were generated based on these knowledge gaps: Is the rumen microbiota affected by the rearing management applied to the lambs? Does the age at weaning affect the microbial establishment at weeks 4 and 16, when lambs are fed concentrates/fiber and mixed swards, respectively? Are changes in rumen microbiota associated with ruminal fermentation profiles?

It is hypothesized that: 1) the rearing management imposed affects the microbial composition in young lambs. 2) The age at weaning affects the microbial composition if the dry matter intake is compromised, and the diet feed to the lamb by the time of sampling will drive the microbial composition. Finally, changes in the rumen microbiota affect the ruminal fermentation profiles.

3. In preweaned calves the microbial community established in the developing rumen is affected by diet. However, there is limited information about the effect that the diet offered during early life has on the microbial community composition when animals transition between different diets through different stages of growth.

   Based on the existing literature the following question was formulated: Do contrasting feeding regimes during the pre- and post-weaning of calves imprint the rumen microbial community with associated changes in rumen fermentation?

   It is hypothesized that: 1) the use of contrasting feeding regimes, in pre- and post-weaned calves, will imprint the rumen microbial community and produce changes in rumen fermentation.

4. In adult ruminants, the use of methanogen inhibitors produces temporal changes in the rumen microbial community and fermentation of food. Additionally, the intake of inhibitors in young ruminants, lambs and goat kids, have shown middle term lasting changes in methane production with contrasting effects on growth. Whilst there is no evidence of the effects of feeding methanogen inhibitors during early-

life on rumen microbial establishment, rumen function and performance in dairy calves.

Therefore, the following question was formulated: Does the intake of microbial inhibitors during early life lead to an imprint on the rumen microbial community and changes in fermentation pathways, with associated gas emissions and growth alterations in calves?

It is hypothesized that: 1) the intake of microbial inhibitors during early life may lead to an imprint on the rumen microbial community and changes in fermentation pathways, with associated gas emissions and growth alterations in calves.

The target outcome of this research program is to contribute to a better understanding of the effect that dietary and chemical interventions, during rumen development, have on the establishment and metabolic activity of rumen microorganisms. These studies establish a baseline for future work, leading potential microbial interventions that could modulate the rumen microbiota, thereby providing new insight on feeding strategies to improve animal performance and reduce methane emissions.

Chapter 2: Impact of weaning age on rumen development in artificially-reared lambs

General overview of the chapter:

Rumen development facilitates the transition from milk to a solid diet. Rumen development is stimulated by the intake and fermentation of solid feed, but these can be affected by the milk rearing management. This chapter aims to determine the effect of weaning at 4 and 6 weeks from milk on rumen development and function in lambs at 4 and 16 weeks of artificial rearing. Additionally, show the histomorphometry development of the four ruminal sites at 4 and 16 wk of rearing. The information generated will contribute to gain insight into the development of artificial rearing management options for commercial farming practices (e.g. rearing of orphan lambs and dairy-sheep systems), and to plan further studies evaluating the impact of different diets on rumen development of lambs.

Chapter 2: O. Cristobal-Carballo, M. A. Khan, F. W. Knol, S. J. Lewis, D. Stevens, R. A. Laven, S. A. McCoard. 2019. Impact of weaning age on rumen development in artificially-reared lambs. Journal of Animal Science (accepted).

The format has been adjusted to the general format of the thesis. Table and figure numbers were kept as in publication.

2.1 Abstract

This study examined the impact of weaning age (4 *vs.* 6 wk) on rumen development, morphology and ontogeny in artificially-reared lambs. Thirty-two mixed-sex lambs (3-5 d old) were randomly allocated to one of two weaning groups: early weaning (EW; 4 wk) and control (Ctrl; 6 wk). Lambs were individually penned and fed milk replacer (MR; 24% CP and 25% fat, DM basis) at 20% of their corresponding initial live weight (LW). Weaning was achieved by gradual reduction of milk replacer (MR) allowance over a period of 3 wk using a step-down procedure. Concentrate and chopped meadow hay were offered *ad libitum* from 1 d of study until 6 wk, when lambs were transferred to a mixed sward pasture. At wk 4, individual intakes were recorded, and blood samples collected to measure β-hydroxybutyrate (BHBA). Eight animals per group were euthanized at wk 4 and 16 to evaluate short chain fatty acids (SCFA) and rumen wall histomorphometry of the dorsal (DS), ventral (VS), dorsal blind (DBS) and ventral blind (VBS) sacs. Hay intake tended to be lower in EW than Ctrl lambs at wk 4 ($P = 0.07$), while no differences in concentrate and total solid feed intake were detected ($P > 0.05$). SCFA profiles were similar between groups ($P > 0.05$) at wk 4 and 16. Plasma BHBA concentrations was 65% higher ($P = 0.01$) in EW than in Ctrl lambs at wk 4. No effect of weaning age on rumen weight (full or empty) and histomorphometry at any of the four rumen sites was found ($P > 0.05$) at either wk 4 or wk 16, except for papillae epithelium thickness in the DBS which was greater ($P = 0.02$) at wk 4 in EW than Ctrl lambs. Rumen morphology differed across the four sites at wk 4 and wk 16 ($P < 0.05$), except for papillae density and surface area ratio at 4 wk ($P > 0.05$). Ontogenic changes (between 4 and 16 wk; $P < 0.05$) were observed for the rumen histomorphometry parameters, except for papillae epithelial thickness that did not differ ($P > 0.05$). The results of this study indicate that morphological and physiological development of the rumen can be accelerated to support weaning of artificially-reared lambs at 4 wk, using a grain/fiber-assist step-down weaning system. Morphological differences between rumen sacs indicate that future studies in lambs evaluating the impact of different diets should involve representative sampling across the rumen rather than a single site to more accurately study rumen development and ontogenic changes.

2.2 Introduction

In ruminants, the transition from milk to solid diets requires the development of a functional rumen (Khan et al., 2016). Artificial lamb rearing systems can be expensive due to the high costs of milk replacers and labor costs during the milk-feeding period (Bimczok et al., 2005). Strategies to reduce these costs include restricting the intake of milk (Owen et al., 1969) and/or reducing the age of weaning (Heaney et al., 1984). These approaches may affect lamb post-weaning performance if key changes in the structure and functionality of the rumen are not achieved prior to weaning (Baldwin et al., 2004). In artificially reared lambs, weaning off milk can be introduced as early as 14 d of age; however, these lambs might undergo weight loss until the rumen becomes functional (Lane et al., 1986). Rumen development is primarily driven by the intake and fermentation of solid feed (Warner et al., 1956; Jesse, 2005), which can be affected by rearing practices including the amount of milk consumed, age at weaning, method of weaning and the time of introduction to solid diets (Khan et al., 2016). In calves, gradual (step-down) weaning has been shown to improve ruminal papillae growth and function during the transition from milk to solid feeds (Khan et al., 2007a). Step-down weaning is a method that gradually reduces the amount of milk fed over time. This allows a smooth transition from liquid to solid feed, which favors the consumption and digestion of sufficient solid feed to support rumen development, and decreases the animals' stress and intestinal damage (Khan et al., 2007a; Meale et al., 2015). However, there is no evidence on the effect that step-down weaning off milk at an early age has on rumen development and function in lambs. Feeding different starter diets have also shown changes in development across anatomical sites in the rumen (Lesmeister et al., 2004). However, there have been no published studies describing the rumen histomorphology and its ontogenic changes in lambs when a gradual weaning system is applied at different ages. Additionally, studies on rumen histomorphometry with lambs have only looked at one site, which may not account for variations within the rumen as indicated by Lesmeister et al (2004). The objectives of this study were 1) to evaluate the impact of weaning age on rumen development of artificially-reared lambs, and 2) to compare the histomorphometry of four ruminal sites at 4 and 16 wk of rearing. It is hypothesized that: 1) early rumen development will be achieved in lambs weaned at wk 4 compared to those weaned at wk 6 using the same step-down weaning procedure, and 2) rumen histomorphology will differ across rumen sites within and between wk 4 and wk 16.

2.3 Materials and methods

This study (AE13233) was approved by the Animal Ethics Committee of AgResearch Grasslands, Palmerston North, New Zealand, in compliance with the institutional Code of Ethical Conduct for the Use of Animals in Research Testing and Teaching, as prescribed in the Animal Welfare Act of 1999 and its amendments (New Zealand).

2.3.1 Experimental design, feeding management, feed intake and live weight measure.

Thirty-two mixed-sex twin-born lambs from Romney ewes, one twin per ewe, were sourced from a commercial farm on the same day at 3-5d of age, having allowed sufficient time for colostrum intake from their dams. The study was carried out in a 2x2 factorial design with two different weaning groups and two slaughtering times. Lambs were randomly allocated, following a stratified randomization procedure balanced for sex and LW, to one of two weaning groups: 4 wk (early weaning; EW) and 6 wk (control; Ctrl). Slaughter timepoints were established at 4 and 16 wk of rearing to compare rumen development between weaning groups. The dairy sheep sector in New Zealand is currently weaning lambs at 6 wk of age (Stevens and Bibiloni, 2014; Peterson and Prichard, 2015); therefore, for the purpose of this study lambs weaned at wk 6 were designated as control's, while lambs weaned at wk 4 were designated as the early weaning group. The lambs were housed in individual pens (1.2 x 1.2 m) with openings on the 4 sides to allow visual and physical interactions with neighboring lambs.

Lambs were individually fed milk replacer (MR; 24% CP and 25% fat, DM basis; Anlamb, Auckland, NZ) at 20% of initial LW (40 g of DM/kg of LW/d). The MR was reconstituted at 200 g/L of water and offered at a temperature of ~37ºC. Gradual weaning off MR was achieved over a three-week period reducing MR allowance by 25% per wk prior to completely removing MR by wk 4 or 6 of the experiment. Concentrate and chopped meadow hay were offered separately *ad libitum*. At the end of wk 6, lambs were moved outdoors onto a ryegrass/white clover pasture. The transition to an all pasture diet was completed by wk 10 through a 10% daily reduction in the allowance of the solid feeds as determined by solid feed intake measured during the first three d of wk 8. Weekly samples of MR, concentrate and hay (one batch used per diet) were collected and pooled into two replicates for chemical compositional analysis by Hill Laboratories Ltd (Hamilton, New

Zealand). The compositional analyses (**Table 2.1**) were determined by wet chemistry methods according to the Association of Official Analytical Chemists (AOAC, 1990, 2010, 2012). Weekly, mixed sward pasture samples were hand-plucked from at least 15 random sites scattered over the grazing paddocks and pooled into 3 representative samples. Pasture samples were analyzed by near infrared reflectance spectroscopy (NIRS) at Grasslands, AgResearch (Palmerston North, New Zealand), in accordance with the methods of Corson et al. (1999). Fresh water was offered *ad libitum* throughout the trial. Individual intakes of concentrate and hay were recorded in wk 4 over a seven-day period to determine daily DMI (g/d) during the transition from MR to solid feed of EW lambs.

2.3.2 Plasma β-hydroxybutyrate (BHBA)

All lambs were fed in pairs at two-minute intervals to enable timed collection of the blood samples using jugular venipuncture 2h post MR feeding on wk 4 of the trial. Blood was collected into potassium-EDTA containing vacutainers (BD Vacutainers, NJ, USA), put on ice for 40 to 60 minutes and then centrifuged for 15 minutes at 1000 x g, and plasma stored at -20°C. BHBA and NEFA concentrations were analyzed by the New Zealand Veterinary Pathology Laboratory (Palmerston North, New Zealand) using the Ranbut kit (kit no. RB 1007 and kit no. FA 115, Randox laboratories Ltd., Crumlin, UK) on a Modular-P800 automatic biochemistry analyzer (Roche Diagnostics, Mannheim, Germany).

2.3.3 Rumen short chain fatty acids (SCFA)

Eight animals per weaning group were randomly selected (using stratified randomization) and euthanized by captive bolt stunning and exsanguination at wk 4 (on last day of MR feeding for the EW lambs) and at wk 16. Rumen contents were collected, thoroughly mixed and a subsample collected to evaluate SCFA concentrations. Samples for SCFA analysis were frozen and stored at -20°C. Samples were prepared as described by Guyader et al. (2016) and SCFA were determined as described by Attwood et al. (1998) gas chromatography using a Hewlett-Packard 6890 equipped with an auto-sampler fitted with a Zebron ZB-FFAP 30.0m x 0.53mm I.D. x 1µm film column and a flame ionization detector (Tavendale et al., 2005).

Table 2.1 Chemical composition (% of DM) of the milk replacer (MR; casein based), concentrate, and hay fed to lambs during the first 6wk of rearing, and of the mixed sward grazed during the post-weaning rearing.

	MR	Concentrate	Hay	Mixed sward[10]
Dry matter[1]	94.9	88.9	88.2	12.7
Crude protein[2]	24.0	20.1	11.1	31.7
ADF[3]	--	6.4	35.9	18.1
NDF[4]	--	15.7	55.2	40.5
Organic matter[5]	94.5	88.9	91.0	87.0
Soluble sugars[6]	40.5	5.8	5.4	7.3
Starch[7]	--	35.6	--	--
Ether extract[8]	25.0	2.7	1.6	5.7
Ash[9]	5.5	11.1	9.1	10.9

[1] Method 945.15; AOAC, 2010.

[2] Method 992.15; AOAC, 2010.

[3] Acid detergent fiber; Method 7.074; AOAC, 1990

[4] Neutral detergent fiber; Method 7.074; AOAC, 1990

[5] Method 942.05; AOAC, 2010.

[6] Paul, A.A and Southgate, D.A. The Composition of Foods. 4th Edition, 1978.

[7] Method 996.11; AOAC, 2010.

[8] Total Fat* Subcontracted test, Cawthron Institute, Nelson

[9] Method 942.05; AOAC, 2012

[10] The chemical composition analyzed by Near Infrared Reflectance Spectroscopy in accordance with the methods of Corson et al. (1999).

2.3.4 Rumen morphology

The rumen was collected post-mortem, and full (tied off with strings) and empty (washed with tap water and dried with paper towels) weights were recorded. Approximately 4 x 4 cm of tissue was dissected from four sites: the ventral sac (VS), dorsal sac (DS), ventral blind sac (VBS) and dorsal blind sac (DBS). These were then rinsed in phosphate buffered saline (PBS) and fixed in 4% formaldehyde. A square centimeter section was dissected from each sample to measure papillae density using a dissecting microscope (Stereozoom, Bausch & Lomb, Rochester, NY) fitted with a measuring eyepiece at 11.25x magnification. One photo was captured and ImageJ 1.36b (Wayne Rasband, National Institutes of Health, USA) used to enumerate total papillae number.

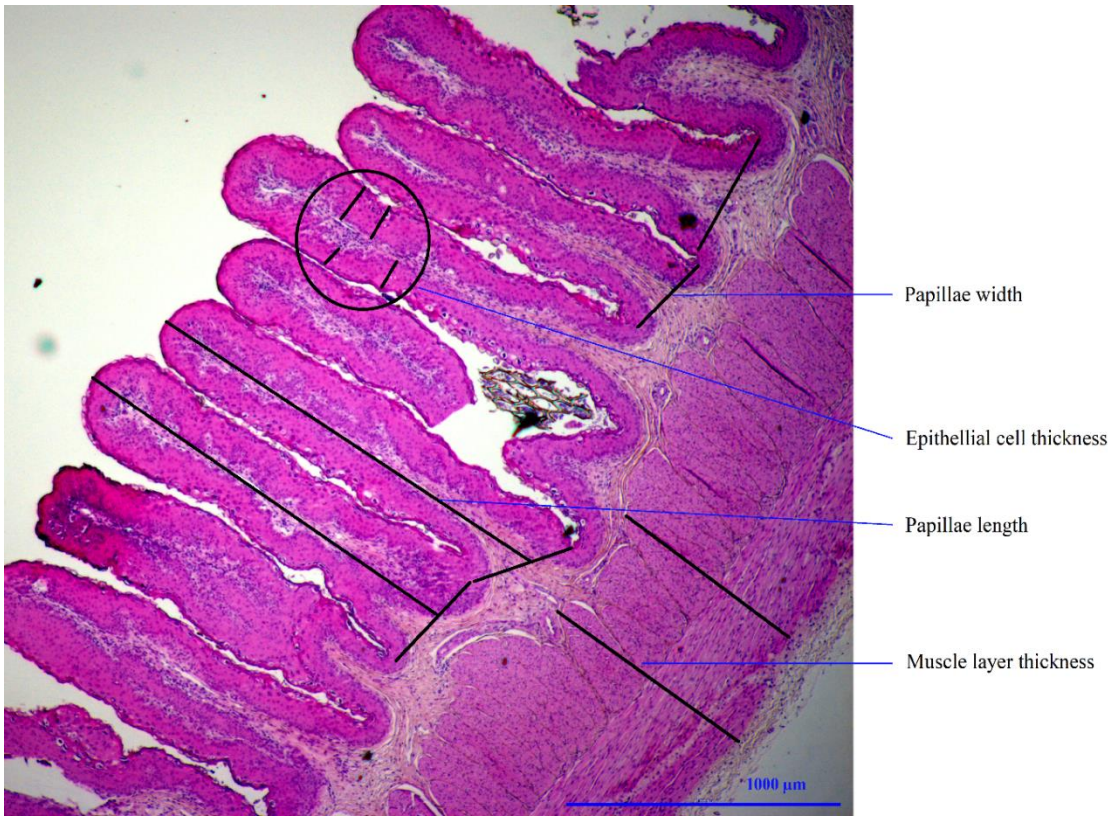
Histology slices were prepared by the Histopathology Laboratory of the School of Veterinary Sciences at Massey University (Palmerston North, New Zealand). Three to five segments (approximately 5 x 15mm with five mm separation between each selected segment) were trimmed from each rumen site using a sagittal cut through the papillae. The segments were dehydrated overnight through graded levels of alcohol (70%, 95% and absolute alcohol) at ambient temperature, cleared in xylene and impregnated with Histosec pastilles (Merck, Darmstadt, Germany) under pressure at 60°C (Excelsior ES Tissue Processor, ThermoFisher Scientific, MA, USA). The segments were then embedded in wax (HistoStar Embedding, ThermoFisher Scientific, MA, USA), sectioned to four-micrometer slices (Rotary Microtome, microTec, Duisburg, Germany; and RM2235 Rotary Microtome, Leica Microsystems, Wetzlar, Germany), and mounted on a slide to create three to five replicate tissue samples per site on each slide. The cut sections were stained with a Mayer's-Harris hematoxylin mixture and Eosin stain (Autostainer XL, Leica Microsystems, Wetzlar, Germany).

Photomicrograph sections were taken of each slide (ProgRes C14 camera, Jenoptik/ Jena, Germany) with the 1x and 2.5x objectives (Olympus BH-2 microscope, Tokyo, Japan). Image-Pro Plus v. 7.0 (2009 Media Cybernetics, MD, USA) was used to measure papilla length (PL) and width (PW), muscular layer thickness (MLT), and mucosal epithelium thickness (PET) of each rumen site (**Figure 2.1**). Twenty complete papillae were chosen from areas selected randomly across the three to five representative segments of each rumen site per animal and measured for PL and PW as described by Lesmeister et al. (2004). For the muscular layer, 20 measurements were performed for each rumen site per animal. For the mucosal epithelium thickness, 20 measurements were undertaken using five complete papillae chosen randomly across the three to five representative segments per slide. The surface area ratio (SAR) was measured using the procedures of Hill et al. (2005) to determine the surface area of papillae per square centimeter of each ruminal section.

2.3.5 Statistical Analysis

Dry matter intake was adjusted to percentage of BW. After checking for normality, DMI and rumen histomorphometry data were transformed using natural logarithm, except for relative rumen weight, SCFA, BHBA and NEFA.

Figure 2.1 Histomorphometric measurements performed in the rumen of lambs at wk 4 and 16 of the trial. Twenty measurements for each variable (papillae base width and length, epithelial cell thickness and muscle layer thickness) were recorded on three to five sections per animal from the dorsal, ventral, dorsal blind and ventral blind sacs of the rumen. Ruminal sections were stained with a mixture of hematoxylin and eosin.



All analysis were performed using a linear mixed effect (LME) model via the restricted maximum likelihood (REML) framework as implemented in the NLME package in R (Pinheiro et al., 2015; R CoreTeam, 2016).

To determine whether an interaction between treatment (weaning age) and slaughter time points was evident, a 2 x 2 analysis of variance (ANOVA), two treatments (EW and Ctrl) and two slaughter timepoints (4 and 16 wk), was undertaken using an LME model. The results from the factorial arrangement did not show any interaction between treatments and slaughter timepoints. Therefore, treatments and slaughter timepoints (ontogeny) were analyzed separately as described below.

The effect of weaning age at each slaughter timepoint wase compared for feed intake, concentrations and proportions of SCFA, rumen full and empty weight, rumen histomorphometry from the four rumen sections (DS, VS, DBS and VBS), and plasma BHBA and NEFA concentrations using an LME model with treatment and sex as fixed

effects, and animal as a random effect. The histomorphology of different ruminal sites was compared at each slaughter timepoint (4 wk and 16 wk) using an LME model with rumen site as fixed effect, and animal as a random effect. Ontogenic changes on rumen weight, histomorphometry and SCFA were evaluated by comparing slaughter timepoints (4 wk vs. 16 wk) using an LME model with slaughter timepoints as fixed effect, and animal as random effect.

Effects from the different models were assessed using analysis of variance (ANOVA) and predicted means, together with estimates of the standard errors of the means (SEM) were obtained, back transformed and post-hoc compared (Tukey's test) using the predicmeans package of R (Luo et al., 2014a). Values of $P \leq 0.01$ were considered highly significant, $P \leq 0.05$ considered significant, $P \leq 0.10$ considered a trend and $P > 0.10$ not significant.

2.4 Results

2.4.1 Impact of early weaning on DMI, rumen fermentation and development

In wk 4, the average daily solid feed DMI (concentrate and hay) per animal did not differ between EW and Ctrl lambs (135 *vs*. 134 g/d, respectively; P = 0.79). The average DMI adjusted to LW of concentrate, hay and solid feed (concentrate + hay) in both groups of lambs during wk 4 of rearing is shown in **Table 2.2**. Lambs from the EW group tended (P = 0.06) to consume less hay (95% CI = 0.35, 1.26) compared to the Ctrl group (95% CI = 0.86, 3.21). Hay constituted 7% and 17% of the DMI of solid feed (concentrate and hay) in EW and Ctrl lambs, respectively. Concentrate intake was numerically greater in EW (95% CI = 10.75, 20.49) than Ctrl (95% CI = 8.48, 16.5) lambs, but were not significant (P = 0.32).

Table 2.2 Average dry matter intake (g) of concentrate, hay and solid feed (concentrate + hay) per kilogram of LW in early weaning (EW) and control (Ctrl) lamb at wk 4 of rearing[1].

	Ctrl	EW	P
Concentrate	11.8±2.70	14.8±3.39	0.32
Hay	1.7±0.77	0.67±0.31	0.06
Solid feed	15.1±2.92	15.9±3.08	0.79

[1] DMI were averaged from 7 d of feed intakes recorded at wk 4. Data presented are back transformed means, confidence intervals at 95% and P-values (P; Tukey's test).

Total SCFA concentrations were numerically greater in EW than Ctrl lambs at both 4 and 16 wks respectively, but these differences were not statistically significant (P = 0.24 and P = 0.27, respectively). Proportions of acetate, propionate, butyrate and minor SCFA did not differ (P > 0.10) between EW and Ctrl lambs at either wk 4 or 16 of rearing (**Figure 2.2**). Mean plasma concentrations of BHBA were 69% higher (P = 0.03) in EW (0.18 mmol/L; 95% CI = 0.144, 0.227) compared to Ctrl (0.12 mmol/L; 95% CI = 0.098, 0.157) lambs at wk 4. (**Figure 2.3a**). Mean plasma concentrations of NEFA did not differ (P = 0.95) between groups at wk 4 (**Figure 2.3b**).

Figure 2.2 Ruminal fermentation profiles of the early weaning (EW) and control (Ctrl) lamb groups at wk 4 and 16 of rearing. Short chain fatty acids (SCFA) total concentration and individual proportions were analyzed in the rumen at wk 4 and 16 of rearing. Lambs at 4 wk, the EW group was consuming 25% of their milk allowance and *ad libitum* concentrate, while the Ctrl group was consuming 75% of their milk allowance and *ad libitum* concentrate. At 16 wk, all lambs were grazing ryegrass/white clover pasture. Columns and error bars are predicted means and SEM. Different letter for each SCFA among columns within rearing periods represent significant difference (P < 0.05).

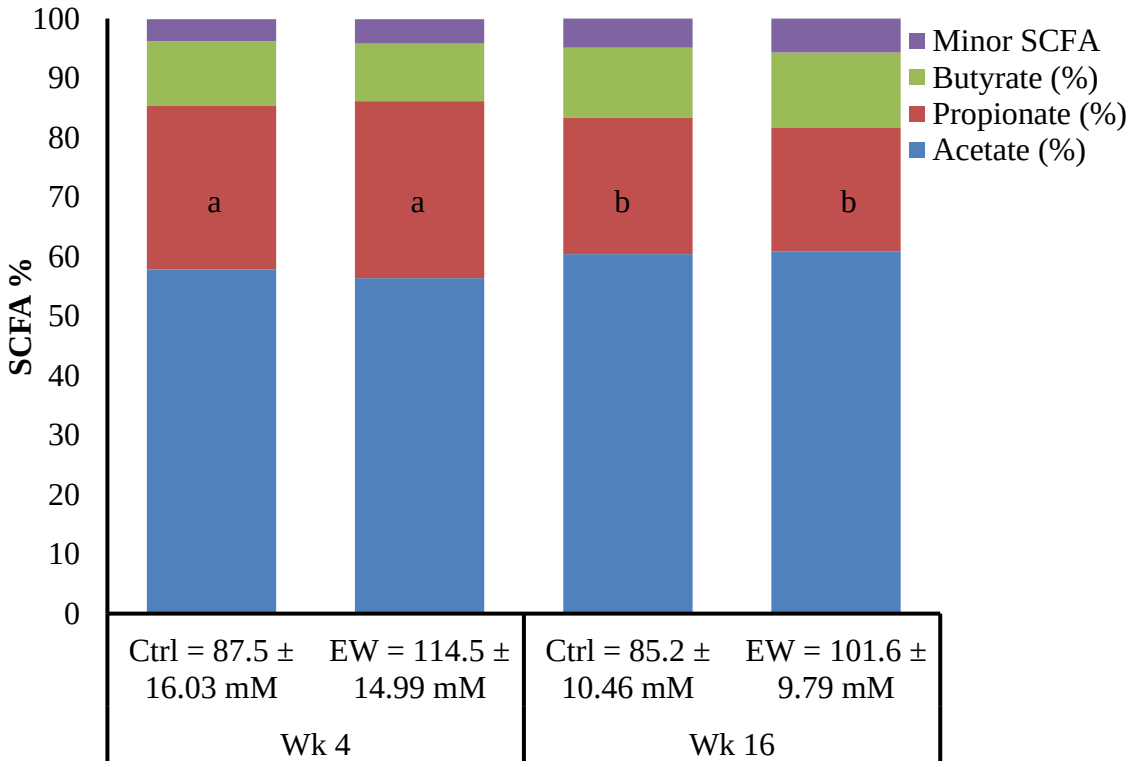
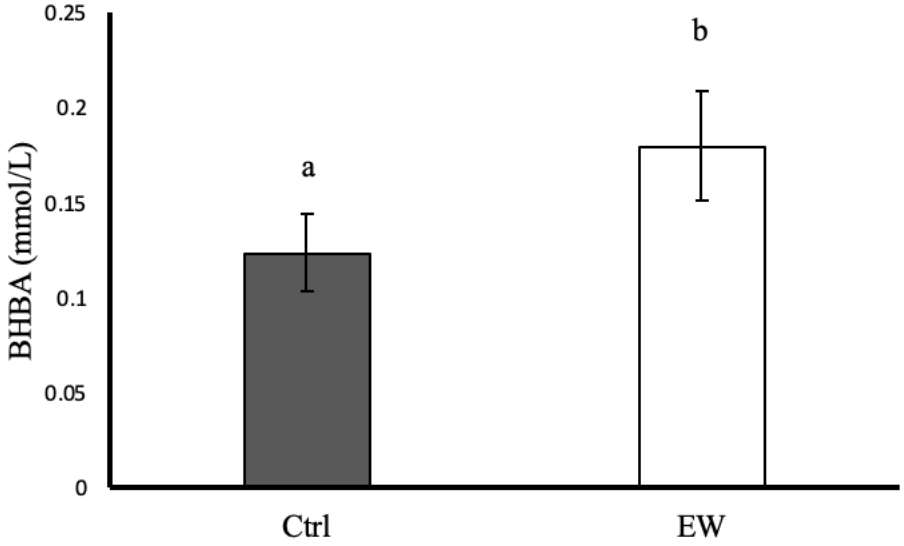
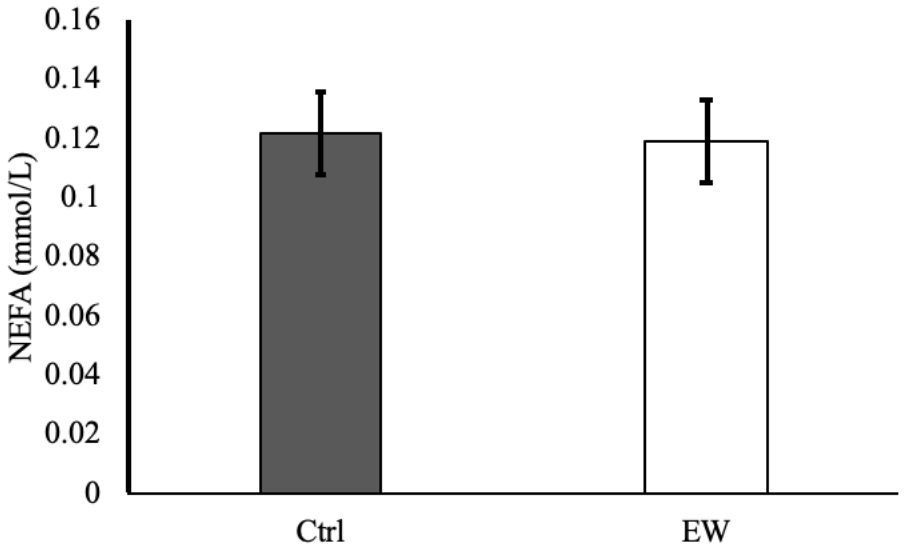


Figure 2.3 Effect of weaning at 4 wk (early weaning, EW) versus 6 wk (control, Ctrl) on: a) plasma β-hydroxybutyrate (BHBA mmol/L) and b) non-esterified fatty acids (NEFA mmol/L) at 4 wk of the trial. Columns are back transformed means and bars are SE. Different letter among columns represent significant difference (P < 0.05).

a)



b)



Rumen morphology of EW and Ctrl lambs at wk 4 and 16 of rearing is presented in **Table 2.3**. Lambs in the EW group tended (P = 0.09) to have a heavier full rumen than Ctrl lambs at wk 4, but no differences (P = 0.38) between groups were observed for empty rumen weight. At wk 16, full and empty weight of the rumen did not differ (P > 0.10) between EW and Ctrl lambs. Lambs in the EW group had 15% thicker rumen papillae epithelium in the DBS only (P = 0.02) compared to Ctrl lambs at wk 4. No treatment effects were observed at wk 4 (P > 0.10) for any of the other morphological traits. At wk 16, EW lambs tended (P = 0.08) to have greater papillae density in the VS only compared to Ctrl lambs. No other morphological trait differences (P > 0.10) were observed between groups.

2.4.2 Rumen morphology between sampling sites

Histomorphometry of four anatomical sites of the rumen (DBS, DS, VBS, and VS) at 4 and 16 wk of rearing is presented in **Figure 2.4**. At wk 4, papillae density did not differ (P = 0.60) between anatomical sites, while SAR tended (P = 0.09) to be greater in the DS compared to the VBS. At wk 16, papillae density (P < 0.01) and SAR (P < 0.01) were 17-25% and 17-24% greater in the VS, respectively, compared to the other rumen sites (**Figure 2.4a** and **2.4b**). At 4 wk papillae length and width in the DS were 11-19% (P < 0.01) and 12-16% (P < 0.01) greater, respectively, than in the other sites. Papillae length at wk 16 was 10-37% greater (P < 0.01) in the DBS compared to all other rumen sites, while width was 6% and 16% greater (P < 0.05) in the DS than in the VBS and DBS, respectively (**Figure 2.4c** and **2.4d**). Muscular layer thickness in the DS was 15-32% (P < 0.01) and 10-39% (P < 0.01) thicker at 4 and 16 wk, respectively, than in the other sites (**Figure 2.4e**). Papillae epithelium thickness at wk 4 was ~10% greater (P < 0.01) in the VS than in the other sites, while at wk 16 papillae epithelium thickness (**Figure 2.4f**) in the DS was only greater (P < 0.05) than the VBS (5%) and DBS (7%).

Table 2.3 Rumen morphology[1] of early weaning (EW) and control (Ctrl) lamb groups at wk 4 and 16 of rearing. Data presented are predicted means plus standard error of the mean and P-values (P).

	4 wk			16 wk		
	Ctrl	EW	P	Ctrl	EW	P
Rumen weight						
Full	752±103	986±135	0.094	4803±344	4994±358	0.740
Empty	121±12	135±14	0.375	805±27	823±27	0.869
Papillae density						
DS	241±33.6	240±33.5	0.970	64±5.0	70±5.5	0.136
VS	268±45.3	239±40.5	0.579	75±6.2	86±7.1	0.084
DBS	271±44.2	242±39.4	0.700	62±6.8	57±6.2	0.561
VBS	259±43.1	202±33.7	0.195	58±4.8	62±5.2	0.303
Surface Area Ratio						
DS	6.1±1.51	5.5±1.35	0.722	3.2±0.39	3.6±0.45	0.238
VS	4.5±1.11	4.5±1.11	0.983	3.7±0.54	4.4±0.65	0.231
DBS	5.0±0.81	5.0±0.82	0.706	3.5±0.45	3.3±0.42	0.607
VBS	4.5±1.02	3.6±0.82	0.350	2.9±0.44	3.2±0.49	0.461
Papillae length						
DS	1344±299.7	174.9±262.1	0.596	2319±380.1	2557±419.0	0.585
VS	1071±114.8	1075±115.3	0.988	2112±207.0	1983±194.4	0.461
DBS	1146±194.1	1173±198.7	0.822	3276±374.0	3155±360.3	0.707
VBS	1022±176.1	11423±196.8	0.551	2741±340.0	2926±362.9	0.673
Papillae base width						
DS	527±62.8	526±62.7	0.992	578±38.4	563±37.4	0.621
VS	429±54.3	479±60.6	0.462	565±25.1	548±24.4	0.432
DBS	466±38.3	489±42.0	0.359	492±27.7	519±29.3	0.446
VBS	462±35.1	434±32.9	0.343	549±27.5	535±26.8	0.669
Muscle layer thickness						
DS	1336±129.6	1373±133.2	0.845	1912±105.7	1878±103.8	0.715
VS	1171±128.8	1142±125.7	0.816	1554±132.6	1805±154.0	0.167
DBS	1067±137.1	1110±142.7	0.907	1284±248.5	1289±249.4	0.955
VBS	912±71.3	938±73.3	0.670	1063±104.5	1243±122.2	0.141
Epithelial cell thickness						
DS	126±13.7	120±13.0	0.640	136±9.4	132±9.2	0.797
VS	128±13.0	140±14.2	0.458	134±6.5	133±6.5	0.960
DBS	115±6.4	133±7.1	0.023	126±5.4	128±5.5	0.722
VBS	123±13.9	122±13.8	0.918	122±8.0	130±8.6	0.269

[1] Rumen weight (full and empty; g), papillae density (no./cm2), surface area ratio (cm2/cm2), papillae length (µm), papillae base width (µm), muscle layer thickness (µm), epithelial cell thickness (µm) in the dorsal (DS), ventral (VS), dorsal blind (DBS) and ventral blind (VBS) sacs of the rumen of lambs at wk 4 and 16.

2.4.3 Ontogenic changes of the rumen

The full and empty weight of the rumen at 16 wk increased 4.5 ($P < 0.01$) and 5.0 ($P < 0.01$) times, respectively, compared to lambs at 4 wk. Changes in histomorphometry of four anatomical sites of the rumen (DBS, DS, VBS, and VS) between 4 and 16 wk are illustrated in **Figure 2.5**. Papillae density (**Figure 2.5a**) in each rumen site decreased 69-77% ($P < 0.01$), while SAR (**Figure 2.5b**) decreased between 4 and 16 wk in the DS (45%; $P < 0.01$) and DBS (33%; $P = 0.02$) only. Papillae length (**Figure 2.5c**) increased across all sites (from 94-181%; $P < 0.01$), while papillae width (**Figure 2.5d**) increased only in the VS (19%; $P = 0.03$) and VBS (21%; $P < 0.01$). Rumen muscular layer thickness (**Figure 2.5e**) increased 38%, 46% and 26% ($P < 0.01$) in the DS, VS and VBS, respectively, with a similar numerical trend in the DBS (20%; $P = 0.15$). Epithelial cell thickness (**Figure 2.5f**) did not differ for any site between 4 wk and 16 wk old lambs ($P > 0.10$).

2.5 Discussion

In neonatal ruminants, the rumen is rudimentary (Warner et al., 1956) and metabolically non-functional with respect to ketogenic capacity (Lane et al., 2000; Lane et al., 2002). The anatomical and physiological development of the rumen is one of the most important events during weaning transition in young ruminant (Baldwin et al., 2004). In artificially-reared ruminants, the pre-weaning development of the rumen influences the adaptation from milk to solid diets and can affect post-weaning growth performance (Khan et al., 2011). This study has shown that when a step-down weaning regime is used, weaning at 4 wk rather than 6 wk increased concentrations of plasma BHBA which could signify an earlier metabolic development of the rumen wall, but rumen morphology and fermentation pattern did not differ. While solid feed intake increased over time in all lambs irrespective of weaning age, and there was no difference in solid feed intake, papillae development and SCFA concentrations at 4 wks. These observations indicate that anatomical development and establishment of fermentation was similar in both groups despite the greater restriction of MR intake in the EW than Ctrl group. Early intake of solid feed in both groups of lambs was also associated with establishment of adult-like rumen fermentation patterns (Jonker et al., 2014; Jonker et al., 2016a) by 4 wk of rearing.

Figure 2.4 Histomorphometric measurements illustrating the effect of the anatomical site of the rumen and age irrespective of treatment group. Histomorphometric measurements: **a)** papillae density (PD; no/cm^2); **b)** surface area ratio (SAR; cm^2/cm^2); **c)** papillae length (PL; µm); **d)** papillae width (PW; µm); **e)** muscular layer thickness (MLT; µm); and **f)** papillae epithelium thickness (PET; µm). Anatomical site of the rumen: dorsal blind (DBS), dorsal (DS), ventral blind (VBS) and ventral (VS) sac. Lamb's age: 4 wk ⬚ and 16 wk ⬚. Columns and error bars are predicted means and SEM. Different letter among columns represent significant difference between rumen sites ($P < 0.05$).

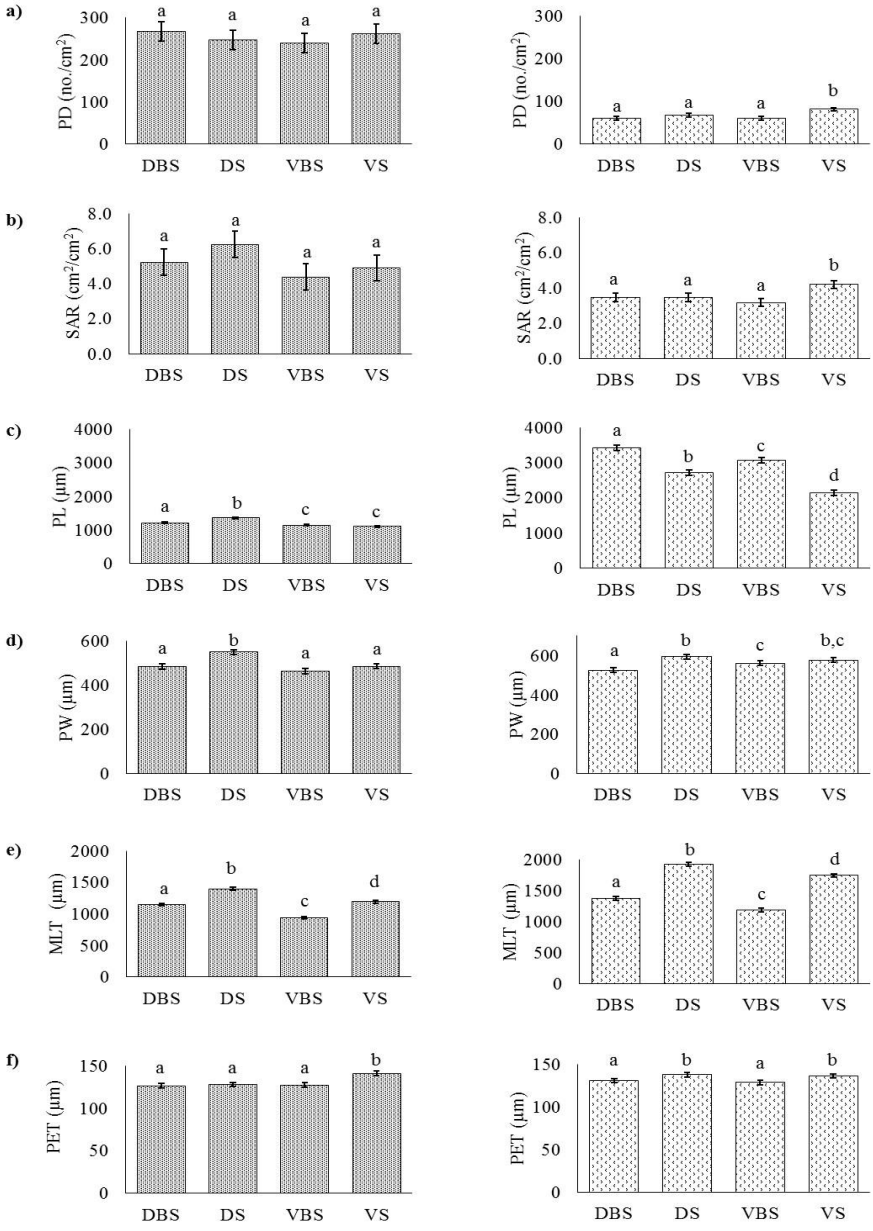
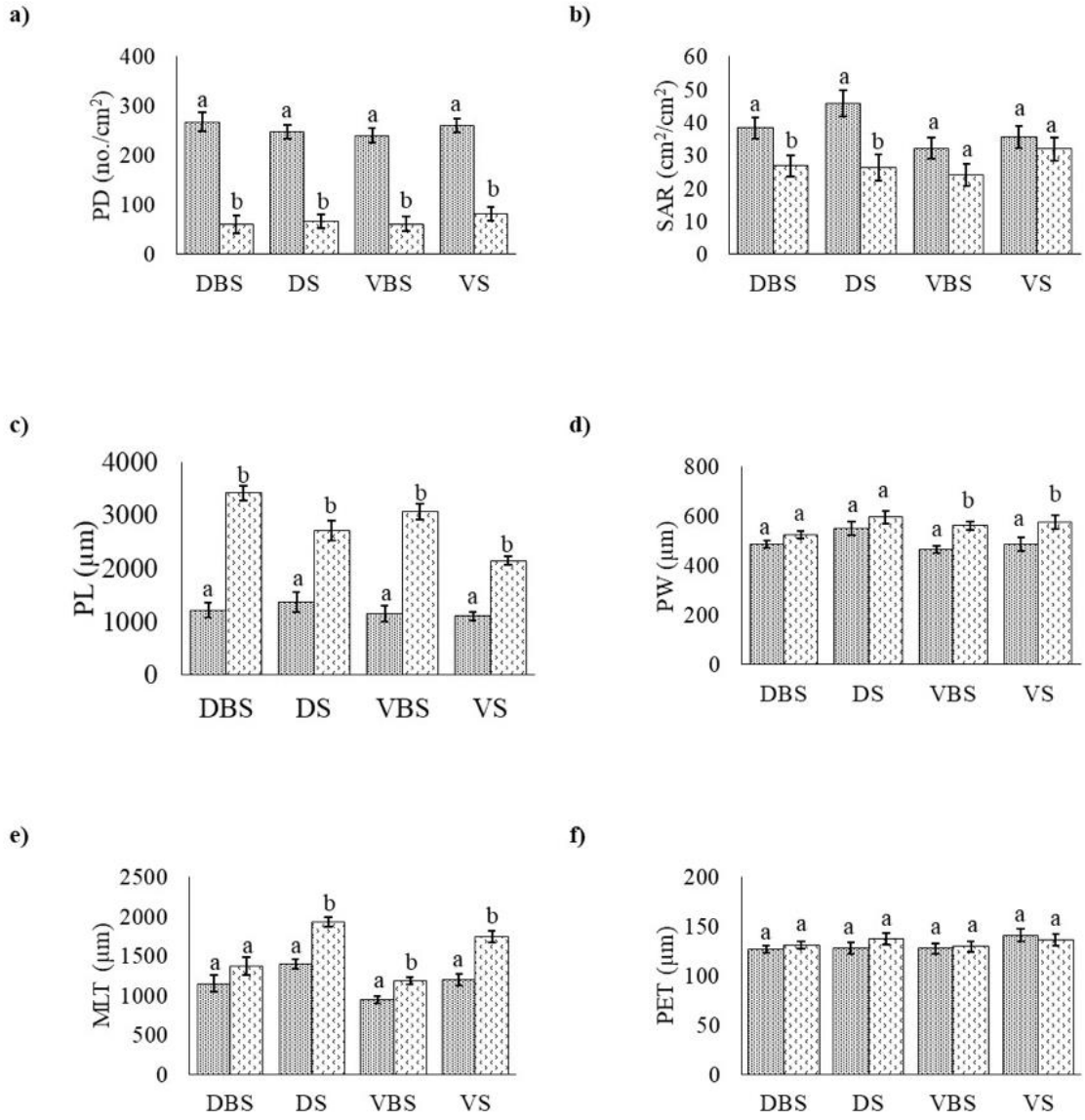


Figure 2.5 Histomorphometric measurements illustrating ontogenic changes of the anatomical site of the rumen by age irrespective of treatment group. Histomorphometric measurements: **a)** papillae density (PD; no/cm^2); **b)** surface area ratio (SAR; cm^2/cm^2); **c)** papillae length (PL; µm); **d)** papillae width (PW; µm); **e)** muscular layer thickness (MLT; µm); and **f)** papillae epithelium thickness (PET; µm). Anatomical site of the rumen: dorsal blind (DBS), dorsal (DS), ventral blind (VBS) and ventral (VS) sac. Lamb's age: 4 wk and 16 wk. Columns and error bars are predicted means and SEM. Different letter between columns represent significant difference between 4 and 16 wk ($P < 0.05$).



Physical development of the rumen can be partitioned into two aspects: increases in mass (which includes muscle) and growth of papillae (Baldwin et al., 2004). Ruminal mass is stimulated by the amount and physical structure of the diet consumed by the animal (Žitnan et al., 1998). In the current study, the trend for heavier full rumen weight in EW lambs at wk 4 may correspond to a greater gut fill as a result of the numerical increase in concentrate DMI since the empty rumen weight was similar. Small changes in DMI per animal or kg of LW^{-1}, especially fiber DMI, appeared to have little impact on the weight of the empty rumen or musculature. The difference in the physical structure of the diet consumed between groups, i.e. 7% rather than 17% of the diet as hay and 35% more concentrate in EW lambs compared to Ctrl, was not associated with detectable changes in rumen weight or muscle layer thickness. Our findings agree with those of Norouzian et al. (2011), who reported that the inclusion of 7.5 or 15% of coarse alfalfa hay in the concentrate diet of 3 wk old lambs resulted in similar rumen weight and muscular layer thickness development in both groups at 9 wk. The total solid feed intake in the current study was not affected by the fiber intake as previously shown in lambs (Norouzian et al., 2011) and calves (Khan et al., 2007b; Terré et al., 2013). The inclusion of coarse fiber into a concentrate diet establishes a floating mat that stimulates rumination activity in ruminants (Van Soest, 1994; Castells et al., 2012). The results from our study suggest that in the EW lambs consuming ~7% of the total solid DMI in wk 4 of rearing as hay may have provided enough physical stimulus to support development of the rumen wall (Greenwood et al., 1997; Baldwin et al., 2004).

The consumption of high milk volumes can suppress the pre-weaning intake of solid feed in young ruminants (Jasper and Weary, 2002). This was not observed in the current study, likely due to the restricted level of milk offered to lambs. In the rumen, the production of SCFA is related to the total amount of organic matter digested by ruminal microorganisms (Weston and Hogan, 1968), while ruminal SCFA concentrations are regulated by a balance between production and absorption (Giesecke, 1970). Production of SCFA in the developing rumen starts with the ingestion of solid feed, which provides substrate for anaerobic fermentation (Žitñan et al., 1993). Steady increases of solid feed intake correspond to increases in the concentration of ruminal SCFA, attaining adult like-level concentrations at a very young age (Baldwin et al., 2004). In our study, total concentrations of SCFA at wk 4 and 16, irrespective of weaning age, were in the range of adult sheep grazed on ryegrass and in high starch diets (Jonker et al., 2014; Jonker et al.,

2016a), indicating early onset of adult-like fermentation. Our observations differ from those of Liu et al. (2016), who reported that lambs reared indoors with restricted access to their dams and with early (1 wk) or late inclusion of starter diets (6 wk) reached adult-like SCFA concentrations by 6 and 8 wk of age, respectively. Collectively, these results indicate that restriction of milk feeding to stimulate solid feed intake is a key driver in the early establishment of rumen fermentation. Notably, this study shows that early weaning using a step-down weaned method combined with early access to a highly digestible starter solid feeds can establish adult-like rumen fermentation in lambs by wk 4 of artificial rearing.

In young ruminants, transitioning from milk to solid diets is facilitated by the development of the absorptive epithelium or enlargement of the papillae in the rumen (Khan et al., 2016). Papillae development is dependent on the intra-ruminal concentration of SCFA (Sakata and Tamate, 1978; Lane and Jesse, 1997), and the timing and period of exposure of the rumen epithelium to SCFA (Lane et al., 2000). In the current study, as there were only a few morphological differences in papillae development between treatments indicated that the numerical 31% and 19% increase in total SCFA concentrations in the rumen of EW lambs at 4 and 16 wk, respectively, did not influence rumen papillae development compared to Ctrl lambs. Long periods of exposure to elevated SCFA are required to induce genes responsible for rumen epithelium enlargement (Lane et al., 2000; Naeem et al., 2012; Naeem et al., 2014). The 2.3 times larger papillae observed in all lambs at 16 compared to 4 wk is consistent with this notion. In summary, our results together with previous studies in lambs indicate that the age and period of exposure to elevated concentrations of SCFA stimulates the morphological development of the ruminal papillae in lambs.

A fully developed and functional rumen is characterized by the production of ketones from ruminal butyrate, which differs from the pre-ruminant rumen in which levels are negligible (Baldwin et al., 2004). As lambs transition from liquid to solid feed, the metabolism of rumen epithelial cells changes from glucose to SCFA as the oxidizing substrate (Lane et al., 2000). The ketogenic capacity of the epithelial cells, i.e. their ability to produce BHBA, has been associated with rumen development (Lane et al., 2002). As such, delaying the initiation of solid feed intake limits rumen metabolic development (Lane et al., 2000). Circulating BHBA concentrations may also be a meaningful indicator of intra-ruminal uptake and metabolism of SCFA in the developing rumen (Quigley et al., 1991).

In the present study, the increase in BHBA concentrations in EW lambs is consistent with the reports of Steele et al. (2012), where mean BHBA concentrations increased in 14 wk old lambs given a short term grain challenge (12 d) which increased rumen epithelial ketogenesis. When animals are in negative energy balance, elevated blood BHBA concentrations can result from BHBA production by the liver (Katz and Bergman, 1969). However, this is unlikely in the current study since plasma NEFA concentrations did not differ between EW and Ctrl lambs. Therefore, it is likely that the increased mean plasma BHBA concentrations of EW lambs reflects increased absorption of SCFA and ketogenic activity of the rumen epithelium following milk removal as observed previously in lambs and other ruminants (Steele et al., 2012; Deelen et al., 2016; Kern et al., 2016; Liu et al., 2016). These results indicate accelerated metabolic development of the rumen in EW compared to Ctrl lambs, which presented similar papillae histomorphology development, as an adaptation mechanism to enable absorption of increased SCFA concentrations from the rumen.

The decrease in the number of papillae per square centimeter of the four sites of the rumen wall, as rumen mass increased, is consistent with the previous observations in the ventral sac of the rumen of 12 wk old lambs artificially-reared on only MR until slaughter or weaned from ~7 wk onto solid feed (Lane et al., 2000). Decreased papillae density with increasing age was associated with increased ruminal volume. In wild ruminants, changes in papillae density have been observed during seasonal changes, e.g. in spring, the rumen expansion decreases the papillae density, while in winter, reductions of the rumen volume increases the papillae density (Forsyth and Fraser, 1999; Mathiesen et al., 2000). Collectively, these indicate that papillae number is set early in life in lambs, by at least 4 wk of age.

The differences in rumen histomorphometry seen in the current study between the different ruminal sacs at both 4 and 16 wk of age, is to the authors' knowledge the first time such differences have been characterized in growing lambs. While Norouzian et al. (2011) implied differences in morphological characteristic between ruminal sacs, results were not described. Differences in the morphology of the DS and VS at wk 4 and 16 in the present study agree with those reported by Álvarez-Rodríguez et al. (2012), who showed that histology measurements between the DS and VS of the rumen differed among feeding. Consistent with our observations, differences in rumen morphology between the different

ruminal sacs has been reported in calves fed on different dietary treatments (Lesmeister et al., 2004), in red deer calves at 4, 8 and 12 wk of age grazing with their dams (Thompson et al., 2008) and in adult Himalayan Tahr and Norwegian reindeer as a result of seasonal changes in composition and abundance of the diet (Forsyth and Fraser, 1999; Mathiesen et al., 2000). In ruminants, differences in papillae development across ruminal sacs have been suggested as an anatomical adaptation resulting from the stratification of the rumen contents whereby browsing ruminants have a more homogenous rumen content and thereby more even papillae development , while grazing species have a more uneven papillae development across ruminal sacs as a result of a greater heterogeneity of rumen contents (Clauss et al., 2009). In our study, the diversity of rumen morphology within and between lambs at 4 and 16 wk was not related to the age at weaning. The observed changes may reflect the diet consumed, with concentrates and small component of dietary fiber at 4 wk supporting a more homogenous papillae histomorphology compared to 16 wk when lambs were consuming a mixed sward, which may have resulted in greater diet stratification (Evans et al., 1973; Van Soest, 1994).

In conclusion, the results of this study indicate that weaning at 4 rather than 6 wk of age using step-down weaning off milk system improves some aspects of the morphological and functional development of the rumen to support the weaning transition in artificially-reared lambs. These findings from the step-down weaning off milk into slid diets will contribute to the development of artificial rearing management options for commercial farming practices (e.g. rearing of orphan lambs and dairy-sheep systems). Additionally, future studies evaluating the impact of different diets on rumen development of lambs should include samples taken from multiple ruminal sites areas for a more representative description of rumen growth and development.

Chapter 3: Rumen microbial establishment and its interactions with rumen fermentation profiles in lambs.

General overview of the chapter:

Differences in rumen function in lambs weaned at 4 weeks, compared to lambs weaned at 6 weeks, were identified in Chapter 2. However, similar dry matter intake and rumen morphology were observed by week 4 between lambs weaned at different ages. This chapter characterizes the impacts of age at weaning in lambs from Chapter 2 on the rumen microbiota in the first 16 weeks of life and to examine the relationships between rumen microbiota composition and rumen fermentation profiles.

3.1 Abstract

The aims of the present study were to characterize the effect of rearing management on the rumen microbiota of lambs, and to explore the relationships between rumen microbes and rumen fermentation profiles. Thirty-seven twin-born lambs, one twin per ewe, were separated from their respective dams at 3-5 days of age. Five lambs were slaughtered after separation from the ewe (Wk00). The other thirty-two lambs were randomly allocated to one of two weaning groups: early weaning (EW; 4 weeks) and control (Ctrl; 6 weeks). Lambs were individually fed reconstituted milk replacer (MR, 200 g/L) at 20% of their initial LW until weaning when they were step-down weaned. Starter concentrate and meadow hay were offered *ad libitum* until wk 6. A mixed sward of ryegrass and clover was grazed from wk 6 to 16. At wk 4, starter intakes were recorded. Rumen fermentation profiles were measured after slaughter of half of each group at wk 4 (Wk04) and the other half at wk 16 (Wk16). The rumen microbial community was analyzed in Wk00, Wk04 and Wk16 samples. Analysis of the 16S rRNA genes showed a diverse microbial community with Wk16 lambs-like proportions of bacteria and archaea in Wk00 lambs. The diversity and relative abundance of bacterial taxa (i.e. cellulolytic and hemicellulolytic bacteria) decreased during artificial rearing in Wk04 lambs relative to Wk00, but increased when grazing in Wk16 lambs. Neither age at weaning nor its interaction with sampling time had a significant effect on the rumen microbiota, although sampling time had a considerable impact on the abundance of specific bacterial taxa. These results suggest that neonatal lambs are colonized early by a complex and diverse microbial community, which decreases in diversity during artificial rearing in individual pens and in response to access to concentrates, but increases again when animals are returned to a group system and fed pasture. The correlation analysis showed high levels of *Bacteroidetes* taxa were positively correlated with total short chain fatty acids (SCFA) concentrations and relative proportions of propionate; while *Firmicutes* taxa had positive correlations with proportions of acetate, butyrate, isobutyrate and isovalerate. These results showed how predominant rumen microbial taxa, i.e. *Bacteroidetes* and *Firmicutes*, are associated with the production of SCFA in the rumen. Therefore, further studies are necessary to elucidate whether differences in rumen microbial communities during dietary interventions may affect rumen morphology through the production of SCFA. Additionally, further experiments are required to explain the relationship between these specific microbial

groups and their fermentation end-products during rumen development, and whether these effects are of biological importance.

3.2 Introduction

Ruminants at birth are functionally monogastric animals with a rudimentary rumen, and thus are dependent on the nutrients from milk being absorbed in the small intestine (Baldwin et al., 2004; Steele et al., 2016). Prior to birth, the rumen is a microbe-free milieu (Malmuthuge and Griebel, 2018), which is potentially colonized during the parturition process by the acquisition of microbes from the mother and the environment (Dehority and Orpin, 1997; Guzman et al., 2015). Further changes in the microbial composition of the rumen occur as the young ruminant transitions from a milk-based to a solid diet (Jami et al., 2013; Rey et al., 2014). As the fermentative process of the rumen commences with the ingestion of solid feed, primary microbial colonizers, i.e. aerobic and facultative anaerobes, are gradually replaced by exclusively anaerobic taxa (Rey et al., 2014; Jiao et al., 2015c). The composition of the starter diet consumed by the neonate affects the rumen microbial richness and diversity during and after weaning (Jiao et al., 2015c; Kim et al., 2016). Furthermore, as the animal grows, its solid feed intake increases, resulting in further changes in the microbial community structure (Rey et al., 2014). Weaning strategy (i.e. abrupt or gradual) has been shown to have no effect on the development of the rumen microbiota when solid feeds are consumed prior to weaning (Meale et al., 2016). However, analysis of the rumen microbiota throughout rearing management transitions like separation from the mother, step weaning at different ages with free access to starter concentrates and fiber, and browse of mixed sward pastures has not been investigated.

In adult animals, despite the high microbial complexity in the rumen (Weimer, 2015), it has been suggested that the abundance of specific microbial genotypes within the rumen can depend significantly on the host phenotype (Guan et al., 2008; Kittelmann et al., 2014; Kamke et al., 2016). In young ruminants, prokaryotic microorganisms are known to colonize the rumen soon after birth and contribute to carbon and nitrogen metabolism through their fermentation (Baldwin et al., 2004; Khan et al., 2016). End products of the fermentation process stimulate the proliferation and differentiation of ruminal epithelial cells (Lane and Jesse, 1997; Gui and Shen, 2016). The development of the ruminal epithelium results in major shifts in the pattern of nutrients being delivered to the intestines and liver, and thus to the peripheral tissues of the animal (Baldwin et al., 2004). Rumen development has been divided in three phases: non-rumination phase (0–3 wks); transition

phase (3 to 8 wks); and rumination phase (from 8 wks onwards) (Jiao et al., 2015b). In the last few decades, several studies have been undertaken in young ruminants to explore the microbial colonization of the rumen (Fonty et al., 1987; Skillman et al., 2004; Jami et al., 2013; Rey et al., 2014; Guzman et al., 2015). However, there is still little information available as to how the composition of rumen microbiota is related to the fermentation profiles, especially during the transition from a milk to a solid feed-based diet and during early rumination.

The aims of the present study were to: (1) characterize the rumen microbiota during wks 0 (Wk00), 4 (Wk04) and 16 (Wk16) that correspond to different rearing managements, (2) determine the effects of weaning age at Wk04 and Wk16 of rearing on the predominant bacteria and archaea in the rumen microbiota, and (3) explore the association between the predominant bacteria and archaea in the rumen microbiota and rumen fermentation profiles in the developing rumen of lambs.

3.3 Materials and methods

All of the experimental procedures in this study were reviewed and approved by the Animal Ethics Committee of AgResearch Grasslands, Palmerston North, New Zealand (Approval number 13233), in agreement with Institutional Code of Ethical Conduct for the Use of Animals in Research Testing and Teaching, as prescribed in the Animal Welfare Act of 1999 and its amendments.

3.3.1 Experimental design and animal management

Lambs from Romney crossbred ewes were sourced from a commercial farm. Thirty-seven mixed-sex twin-born lambs, one twin per ewe, were separated from their dams at three to five days of age to facilitate sufficient time for colostrum intake. Five lambs were randomly selected from thirty-seven and slaughtered at arrival to the rearing facility (Wk00) at AgResearch Grasslands. The other thirty-two lambs were randomly allocated to one of two weaning groups: 4 wks (early weaning; EW) and 6 wks (control; Ctrl). Lamb groups were balanced for live weight (LW) and sex.

Lambs were housed indoors in individual pens (1.2 x 1.2 m) from arrival for six wks. Pens had openings on the four sides to allow visual and physical interactions with

neighboring lambs. Grazing on paddocks with a shelter provided was initiated from wk 6 and maintained through to wk 16.

3.3.2 Feeding management

Reconstituted milk replacer (MR, 200 g/L) was individually fed to lambs at 20% of their initial LW (40 g of DM/kg of LW). Lambs were gradually weaned off MR over a three-week period, by reducing 25% of allowance per wk with complete removal of MR by wk 4 or 6 of rearing, depending on weaning group. Concentrate and chopped meadow hay (**Table 2.1 in Chapter 2**) were offered separately *ad libitum* during indoors. From wk 6 to 16, both groups were transferred outdoors and grazed on ryegrass/clover pastures. Starter diets were offered to support the transition onto pastures and gradually removed by reducing 20% of allowance every two days from wk 9 to 10. Fresh water was offered *ad libitum* throughout the trial.

3.3.3 Dry matter intake

Concentrate and hay intake of individual lambs of was recorded during wk 4. Daily dry matter intake (DMI, g/d) was averaged over a seven-day period of measurement, which coincided with the wk prior to transitioning from MR to solid feed in EW lambs.

3.3.4 Rumen content – sampling

Rumen contents samples were obtained at slaughter. Euthanasia was performed by captive bolt stunning and exsanguination. Five animals were randomly euthanized at Wk00 (arrival), eight animals per weaning group were euthanized at Wk04 (last day of MR feeding for the EW lambs) and Wk16 of the trial. From each animal at each slaughter, rumen contents were thoroughly mixed, ruminal pH taken, and a subsample of 1.8 and 0.9 ml collected to evaluate short chain fatty acid (SCFA) profiles and microbial community composition, respectively. Subsamples were composed of liquid and solid phases of the rumen contents. At Wk00, rumen contents were obtained from three of the five lambs; the other two lambs had an empty rumen. Samples for SCFA and DNA extractions were frozen and stored at -20°C.

3.3.5 Rumen fermentation analyses

Ruminal pH was measured using a portable pH meter (EZDO-7011 waterproof tester; GOnDO Electronic CO. Ltd, Taipei, Taiwan) immediately after sample collection

at Wk16. Ruminal pH was not measured in Wk00 and Wk04 lambs due to the limited amount of rumen contents available. Fermentation profiles were only analyzed in Wk04 and Wk16 lambs. Collected rumen samples were prepared as described by Guyader et al. (2016) and SCFA were determined as described by Attwood et al. (1998) using a Hewlett-Packard 6890 series gas chromatograph equipped with an auto-sampler fitted with a Zebron ZB-FFAP 30.0m x 0.53mm I.D. x 1um film column and a flame ionization detector Tavendale et al. (2005).

3.3.6 Extraction, amplification and purification of microbial nucleic acids

Rumen samples were thawed and nucleic acids (DNA) were extracted from 200 µl of the DNA samples using the phenol-chloroform, bead beating with filtration kit for purification II (PCQI) method (Henderson et al., 2013). Primers (**Table A.1, Appendix A**) and amplification reactions (30 and 35 cycles, respectively), used for PCR of bacterial and archaeal V3-V4 region of the 16S rRNA genes, were prepared in triplicate as described by Kittelmann et al. (2013). PCR products were pooled, and the correct sizes and the absence of signal from negative controls were verified by agarose gel electrophoresis and quantified by fluorescence using the Quant-iT dsDNA BR assay kit (Invitrogen, Carlsbad, CA, USA). A 150ng of each amplicon from the same target gene and region (i.e., all bacteria and archaea amplicons) were pooled. Pooled samples were concentrated, and the final PCR product concentration was determined using Quant-iT dsDNA HS assay kit (Invitrogen, Carlsbad, CA, USA). Pools were purified using the NucleoMag NGS kit (Macherey-Nagel, Dueren, Germany). The final purification of amplicons was done using the QIAquick PCR Purification kit (QiaGen, Valencia, CA, USA) and the DNA concentration quantified using Quant-iT dsDNA HS assay kit (Invitrogen Carlsbad, CA, USA). Both pools were diluted to $6.0x10^9$ copies µl and combined at a bacteria to archaea ratio of 5:1 (Kittelmann et al., 2013). Amplicons were sequenced using an Illumina MiSeq system (Massey University, Palmerston North, NZ). The libraries were quality control (QC) checked using PerinElmer GX Touch HT Instrument using Bioanalyzer DNA High Sensitivity Assay. Pooled library was run on one Illumina MiSeq 2 x 250 base PE run version 2 chemistry (Reagent Kit v2, 500 cycles; Invitrogen, Carlsbad, CA, USA). Each Illumina MiSeq 2 x 250 base PE. An

Illumina prepared PhiX control library was loaded onto the Illumina MiSeq run at 20% volume as control for the run[2]. Sequence reads were provided in fastq format.

3.3.7 Phylogenetic analysis of sequencing data

Sequencing reads were quality-filtered using the DynamicTrim function of SolexaQA (Cox et al., 2010). Reads were then processed and analyzed using the QIIME software package 1.8 (Caporaso et al., 2010). Sequencing reads were grouped into operational taxonomic units (OTUs) sharing over 97 and 99% UCLUST similarity for bacteria and archaea, respectively (Edgar, 2010). Sequences were assigned to phylogenetic groups using BLAST (Altschul et al., 1990). Bacterial 16S rRNA genes were assigned using SILVA 123 (Henderson et al., 2019) and archaeal 16S rRNA genes using RIM-DB (Seedorf et al., 2014). QIIME generated OTU-tables were used for downstream statistical analysis.

3.3.8 Statistical analyses

Multivariate analyses were used to find meaning in the whole dataset from the metagenome with the aim of identifying differences in the microbiota community between treatments and the correlation of the individual microorganisms with parameters such as rumen fermentation, rumen development and plasma metabolites.

The alpha diversity of the rumen microbiota in lambs at different rearing management (Wk00, Wk04 and Wk16) was analyzed using the Shannon index in the VEGAN package of R (Oksanen et al., 2017). The beta diversity was analyzed using the partial least squares discriminant analysis (PLSDA) in the MixOmics package of R (Lê Cao et al., 2016). This is a supervised multivariate linear regression method aiming to sharpen the maximum separation between classes, and to understand which variables carry the class separating information (Gonzalez et al., 2012; Henderson et al., 2015). For the beta diversity analyses, classes were arranged by combining treatment groups (EW and Ctrl lambs) and rearing managements (Wk04 and Wk16). From these group (classes) of lambs

[2] Based on Illumina PhiX control library at supported cluster densities (865-965 k/mm^2 clusters passing filter for v2 chemistry and 1200-1400 k/mm^2 clusters passing filter v3 chemistry). Actual performance parameters may vary on sample type, sample quality, and clusters passing filter; https://sapac.illumina.com/systems/sequencing-platforms/miseq/specifications.htm

the rumen microbiota (bacteria and archaea) was analyzed using a PLSDA to the entire microbial community (ALL) and to only the abundant (MinReads) microbial community. The aim was to observe if the abundant microbial community showed a similar behavior pattern like the entire microbiota. The abundant bacteria genus and archaea species were selected at an average of ≥ 0.5% and ≥ 1.0%, respectively, of relative abundance across samples. The aim was to identify the impact of rearing management on rumen microbial communities. Association scores were visualized via clustered image maps (CIM, heatmaps) representing the first two dimensions. Canonical correlation analysis (CCA) was used to explore potential associations between the microbial community and fermentation profiles (Henderson et al., 2016). All statistical analyzes were performed using R software version 3.3.2 (R CoreTeam, 2016).

Univariate analyses were used to determine the effect of the three rearing managements (Wk00, Wk04 and Wk16), as well as the effect of weaning age (EW and Ctrl), rearing management at Wk04 and Wk16, and their interactions on the abundant microbial community. After checking for normality, bacteria (phyla and genera) and archaea (species) community data were transformed using natural logarithm, except for SCFA. All the analyses were performed using a linear mixed effect (LME) model via the restricted maximum likelihood (REML) framework as implemented in the NLME package in R (Pinheiro et al., 2015; R CoreTeam, 2016).

The effect of rearing management at Wk00, Wk04 and Wk16 was compared for the abundant rumen microbiota using rearing management as fixed effect, and animal as a random effect. Additionally, a 2 x 2 factorial analysis was done to determine whether an interaction between weaning age (EW and Ctrl) and rearing management (only at Wk04 and Wk16) was evident for the abundant microbial taxa and total concentrations and individual proportions of SCFA . The analyses these variables, the model was fitted with weaning age and rearing management as fixed effects, and animal as random effect.

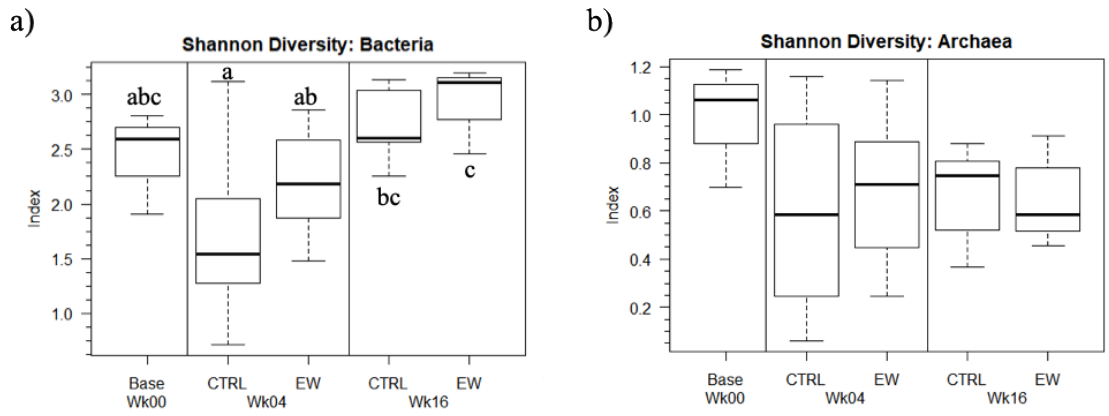
The LME models were analyzed using an analysis of variance (ANOVA). Predicted means from the model, together with estimates of the standard error of the mean and pairwise comparisons (Benjamini-Hochberg' test) were obtained, and back transformed (for the analyses of the microbial community composition only) using the PREDICTMEANS package of R (Luo et al., 2014b).

3.4 Results

A total of 2,428,748 sequences were obtained from the 35 samples, using Illumina MiSeq sequencing, with 54,797 bacteria and 20,462 archaea sequences per sample. The number of organizational taxonomic units (OTUs) were 1,664 and 262 for bacteria and archaea respectively. A total of 174 bacteria and 16 archaea taxa were analyzed among all the rumen samples after keeping representative that were: a) present in one sample with a relative abundance >0.01; b) present in >2% of the samples with a relative abundance >0.01%; and c) present in 5% of the samples at any abundance level.Rumen microbiota – Alpha diversity

The bacterial Shannon index in the Ctrl group at Wk04 had the lowest diversity compared to the other groups, but the overall variability was higher in Wk04 especially in the Ctrl group (**Figure 3.1**). The EW group at Wk16 had the highest diversity index and was ($P < 0.05$) higher than that in either weaning group at Wk04. The diversity index for young lambs Wk00 did not differ from those of Ctrl and EW lambs at either Wk04 or Wk16 ($P > 0.05$) (**Figure 3.1a**). There was no effect ($P > 0.05$) of rearing management on the Shannon diversity index for the archaea community (**Figure 3.1b**).

Figure 3.1 Shannon diversity index of rumen microbes in lambs at wk 0, 4 and 16. Week 0 (Wk00) corresponds to when the lambs were separated from their dams and euthanized prior to entry to the rearing facility (base); wk 4 (Wk04) corresponds to the indoor rearing phase when lambs had differing milk intake and free access to concentrate and fiber; and wk 16 (Wk16) corresponds to the end of the outdoor rearing period when both groups were weaned off milk (by wk 4 (EW) or 6 (Ctrl)) and starter diets (by wk 10) and had free access to ryegrass/clover. **a)** Shannon diversity for bacteria for different rearing groups of lambs; and **b)** Shannon diversity for archaea for different rearing groups of lambs. Boxplots represent the 25th and 75th percentiles, lines within boxes are the medians and the whiskers extend to the most extreme data points. Boxplots with different letters indicate significant difference ($P < 0.05$) between groups of lambs.



Rumen microbiota – Beta diversity

The PLSDA of all bacterial genera (**Figure 3.2a**) showed a clear clustering of the lambs by Wk04 and Wk16, whereas a very weak clustering was observed for the treatments Ctrl and EW. The PLSDA of the abundant bacteria showed a similar clustering by Wk04 and Wk16 and for Ctrl and EW (**Figure 3.2b**).

The PLSDA of the whole and abundant archaea species in Ctrl and EW lambs at wk 4 and 16 of rearing are presented in **Figure 3.3**. No clear separation of either sampling time (Wk04 and Wk16) or the weaning regimen (Ctrl and EW) were observed for either the whole or the abundant archaea microbiota.

Figure 3.2 Effect of weaning age at two sampling times on rumen bacteria community of lambs. Lambs were allocated to early weaning (EW; weaning at wk 4) and control (Ctrl; weaning at wk 6) groups, and rumen sampled at wk 4 (Wk04; blue) and 16 (Wk16; red) of rearing. **a)** The partial least discriminant analysis (PLSDA) of the 193 bacteria or whole bacteria genus (Bacteria – ALL). **b)** The PLSDA of the 31 most abundant bacteria genera (Bacteria – MinReads – ALL).

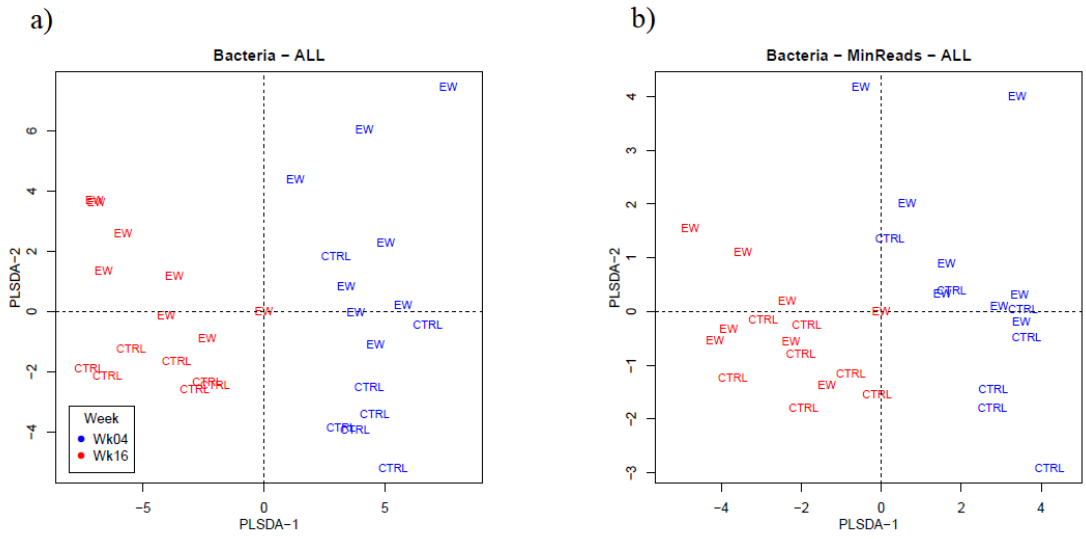
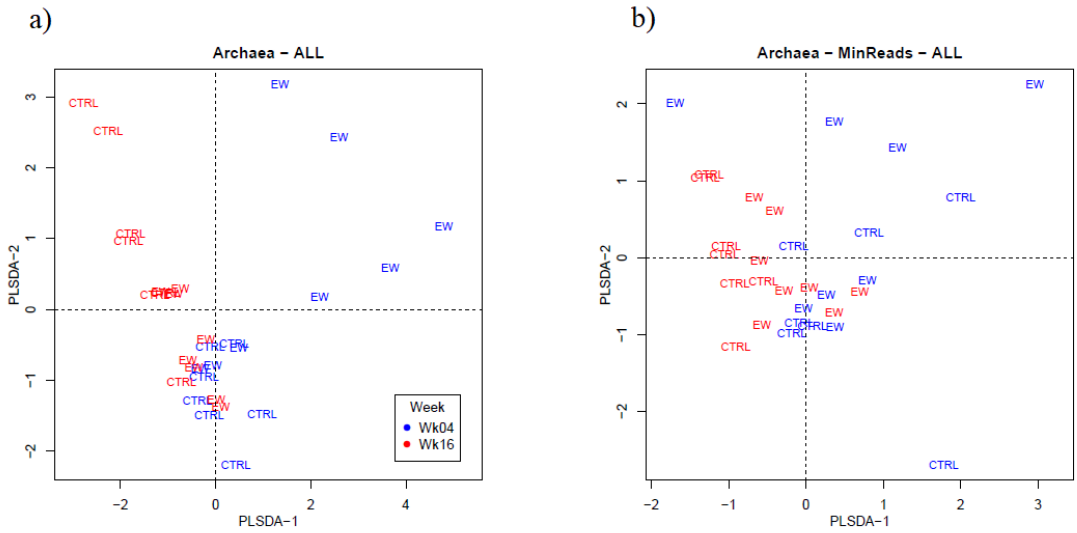


Figure 3.3 Effect of weaning age at two sampling times on the rumen archaea community of lambs. Lambs were allocated to early weaning (EW; weaning at wk 4) and control (Ctrl; weaning at wk 6) groups, and rumen sampled at wk 4 (Wk04; blue) and 16 (Wk16; red) of rearing. **a**) The partial least discriminant analysis (PLSDA) of the 16 archaea species (Archaea – ALL). **b**) The PLSDA of the 7 abundant archaea species (Archaea – MinReads – ALL).



3.4.1 Bacteria phyla composition

Using a cut off of more than 12 reads, the bacterial community was represented by 17 phyla. Comparison of the bacterial phyla at Wk00 and in each rearing group at Wk04 and Wk16 are shown in **Table 3.1**. Lambs at Wk00 had higher proportions of *Fibrobacteres*, *Saccharibacteria* and SR1 (*Absconditabacteria*) compared to lambs from the Ctrl and EW group at Wk04 and Wk16. Otherwise on a phylum level the communities of the weaning groups at wk 4 and 16 were similar. A more detailed analysis is found in **Table B.1 (Appendix B)**

Table 3.1 Analysis of variance of the bacteria phylum[1] in different groups of lambs[2] at three rearing periods[3]. Results of relative abundance of bacteria phyla (%) are natural log back transformed means and standard error of the differences (SED), P-value (P-val). Results in row with different letter indicate significant difference ($P < 0.05$) between groups of lambs.

	Wk00	Wk04		Wk16			
Bacteria phylum	**Baseline**	**Ctrl**	**EW**	**Ctrl**	**EW**	**SED**	**P-val**
Actinobacteria	0.11	0.34	0.37	0.31	0.59	0.199	0.296
Armatimonadetes	0.02	0.01	0.01	0.01	0.02	0.012	0.584
Bacteroidetes	65.12	52.87	54.25	41.64	44.69	9.107	0.190
Chloroflexi	0.09	0.16	0.04	0.02	0.02	0.104	0.547
Cyanobacteria	0.51	0.11	0.17	0.10	0.22	0.120	0.063
Elusimicrobia	0.13	0.04	0.17	0.09	0.13	0.097	0.650
Fibrobacteres	3.37^{a}	0.72^{b}	0.55^{b}	0.22^{b}	0.34^{b}	0.638	0.003
Firmicutes	23.75	42.25	39.51	50.93	48.54	9.227	0.137
Omnitrophica	0.05	0.01	0.11	0.02	0.08	0.047	0.123
Planctomycetes	0.02	0.04	0.03	0.01	0.06	0.036	0.621
Proteobacteria	3.47	1.85	2.00	2.23	1.40	0.940	0.457
Saccharibacteria	0.42^{a}	0.04^{b}	0.11^{b}	0.01^{b}	0.15^{b}	0.110	0.039
Spirochaetae	0.49	0.34	0.32	2.80	1.60	1.063	0.069
SR1 (Absconditabacteria)	0.15^{a}	0.01^{b}	0.04^{b}	0.05^{b}	0.03^{b}	0.034	0.020
Synergistetes	0.04	0.07	0.05	0.09	0.07	0.033	0.584
Tenericutes	0.73	0.22	0.33	0.27	0.53	0.171	0.080
Verrucomicrobia	0.00	0.00	0.28	0.04	0.14	0.208	0.551
Other	1.55	0.93	1.68	1.16	1.39	0.396	0.274

[1] The bacteria community corresponded to 17 phyla.

[2] Group of lambs corresponded to baseline (at arrival; wk 0), early weaning (EW; weaning at wk 4) and control (Ctrl; weaning at wk 6).

[3] Rearing periods corresponded to lambs separated from their dams at wk 0 (Wk00), reared in pens and with access to concentrates and hay at wk 4 (Wk04) and grazed in paddocks in a sward of mixed forages at wk 16 (Wk16) of rearing.

3.4.2 Bacteria genus composition

Analysis of 16S rRNA gene sequencing data indicated the presence of 193 bacterial genera. Of these 193 genera (**Table B.2, Appendix B**), 31 had a relative abundance ≥ 0.5% across the complete dataset and accounted for 87.8 ± 5.28% of the total bacterial community; this group represents the abundant bacteria. Cellulolytic bacteria genera from the *Bacteroidetes* and *Firmicutes* phyla were more abundant at Wk16 compared to Wk04 (**Table 3.2**). No differences were observed for pairwise comparison between lambs at Wk00, and Ctrl and EW lambs at Wk04. A tendency for greater proportions of *Fibrobacter* was observed in lambs at Wk00 compared to group of lambs at Wk04 and Wk16. The analysis of weaning groups at Wk04 and Wk16 only showed differences in the genus *Butyrivbrio* 2, which was higher in the EW compared to the Ctrl lambs. A more detailed analysis is found in **Table B.3 (Appendix B).**

3.4.3 Archaea species composition

Analysis of 16rRNA genes sequencing data from 33 samples indicated the presence of 16 species of archaea at a cut off of ≥ 21 16S rRNA reads per OTU (**Table B.4, Appendix B**). Of these 16 species, seven were relatively abundant (species that had a relative abundance of ≥ 1.00% across samples; **Figure 3.4)**. No differences ($P>0.05$) in the abundant *Methanobrevibacter* spp. were observed between rearing management groups (**Table 3.3)**. Within the less abundant groups, *Methanomassiliicoccales* Group9 sp. was more abundant ($P<0.03$) in Wk00 and Wk04 lambs than in Wk16 lambs. While, *Methanosphaera* sp. ISO3-F5 was more abundant ($P<0.02$) in Wk00 compared to the two groups at wk4 and 16. Weaning age and rearing management (Wk04 and Wk16) interactions were only observed for *Methanosphaera* sp. ISO3-F5 between Ctrl lambs at Wk04 and Wk16 with a 4.2-fold increase ($P<0.04$) in relative abundance(**Table B.5, Appendix B).**

Table 3.2 Analysis of variance of the bacteria genus[1] in different groups of lambs[2] at three rearing periods[3]. Results of relative abundance of bacteria genera (%) are natural log back transformed means and standard error of the differences (SED), P-value (P-val). Results in row with different letter indicate significant difference ($P < 0.05$) between groups of lambs.

	Wk00	Wk04		Wk16			
Bacteria genus	**Baseline**	**Ctrl**	**EW**	**Ctrl**	**EW**	**SED**	**P-val**
Bacteroidetes							
Prevotella 1	50.00	48.62	40.40	32.65	29.74	10.84	0.256
Bacteroidales S24-7 group	0.28^{ab}	0.23^{a}	0.86^{ab}	3.64^{c}	2.74^{bc}	1.06	0.005
Rikenellaceae RC9 gut group	6.48	2.50	7.67	2.78	3.29	3.08	0.265
Mucilaginibacter	0.68^{ab}	0.56^{b}	1.20^{abc}	1.73^{bc}	1.90^{c}	0.50	0.035
Prevotella 7	2.73	1.79	1.46	1.58	0.84	0.80	0.368
Prevotellaceae UCG-003	0.69	0.41	0.39	1.22	2.23	0.97	0.220
Tannerella	0.13^{a}	0.08^{a}	0.11^{a}	1.19^{b}	1.39^{b}	0.35	0.000
Alloprevotella	0.81	0.96	1.28	0.50	0.50	0.39	0.140
Fluviicola	0.07	1.67	2.95	0.00	0.02	2.45	0.588
Fibrobacteres							
Fibrobacter	3.37^{a}	1.26^{b}	1.30^{b}	0.66^{b}	0.85^{b}	0.79	0.079
Firmicutes							
Butyrivibrio 2	0.48^{ab}	0.20^{b}	0.45^{ab}	0.75^{a}	1.13^{c}	0.21	0.001
Christensenellaceae R-7 group	3.33	1.74	3.28	3.50	4.44	1.19	0.198
Lachnospiraceae uncultured	0.74^{ab}	0.49^{b}	0.30^{b}	1.16^{a}	1.40^{a}	0.32	0.003
Lachnospiraceae NK3A20 group	1.10	0.38	0.44	0.52	1.36	0.46	0.114
Lachnospiraceae NK4A136 group	0.52	0.54	1.48	0.84	1.68	1.12	0.737
Oscillospira	0.05^{a}	0.18^{a}	0.16^{a}	1.79^{b}	1.88^{b}	0.53	0.001
Pseudobutyrivibrio	0.78^{a}	0.50^{a}	0.41^{a}	3.48^{b}	4.36^{b}	1.06	0.000
Roseburia	0.25	0.57	0.95	0.98	0.87	0.48	0.630

Table 3.2 (Continued)

	Wk00	Wk04		Wk16			
Bacteria genus	**Baseline**	**Ctrl**	**EW**	**Ctrl**	**EW**	**SED**	**P-val**
Ruminococcaceae uncultured	1.03	0.37	0.39	0.66	0.84	0.26	0.100
Ruminococcaceae NK4A214 group	0.00	5.45	5.21	1.36	15.96	7.57	0.356
Ruminococcaceae UCG-002	0.63^{a}	0.47^{a}	1.37^{a}	2.16^{ab}	3.68^{b}	0.99	0.013
Ruminococcaceae UCG-010	0.66^{abc}	0.29^{c}	0.55^{bc}	1.01^{ab}	1.27^{a}	0.30	0.011
Ruminococcaceae uncultured	0.36	0.55	4.29	0.30	0.30	3.53	0.638
Ruminococcus 1	2.55^{ab}	1.95^{b}	1.14^{b}	5.19^{a}	6.71^{c}	1.17	0.000
Selenomonas	3.90^{ab}	1.15^{b}	1.44^{b}	10.04^{a}	3.26^{b}	2.84	0.012
Selenomonas 1	0.20^{ab}	0.28^{b}	0.22^{b}	2.33^{a}	3.08^{c}	0.94	0.004
Subdoligranulum	0.59	0.45	0.31	0.71	0.57	0.39	0.830
Syntrophococcus	0.10	4.48	0.85	0.03	0.07	2.59	0.304
Proteobacteria							
Brenneria	0.04	1.89	5.11	0.01	0.01	4.11	0.580
Brevundimonas	0.98	0.48	0.64	0.54	0.23	0.44	0.655
Spirochaetae							
Treponema 2	0.40	0.26	0.66	1.18	0.46	0.48	0.300

[1] The bacteria community corresponded to 31 abundant genera (abundance ≥ 0.5% in at least one of the groups of lambs sampled).

[2] Group of lambs correspond to baseline (at arrival; wk 0), early weaning (EW; weaning at wk 4) and control (Ctrl; weaning at wk 6).

[3] Rearing periods corresponded to lambs separated from their dams at 0 wk (Wk00), reared in pens and with access to concentrates and hay at 4 wk (Wk04) and grazed in paddocks in a sward of mixed forages at 16 wk (Wk16) of rearing.

Figure 3.4 Taxonomic composition of the abundant archaea species in lambs during three different rearing periods. Rearing periods in lambs were defined as follow: separated from their dams and euthanized at wk 0 (base; Wk00); reared indoors, with free access to concentrate and fiber, and euthanized at wk 4 (Wk04); and reared outdoors, with free access to ryegrass/clover, and euthanized at wk 16 (Wk16). Lambs artificially-reared were divided in early weaning (EW) and control (Ctrl) groups. Abundant archaea corresponded to species with ≥1.0%. Columns in the charts correspond to the relative abundance (%) of the archaea species within each group of lambs and their corresponding rearing periods.

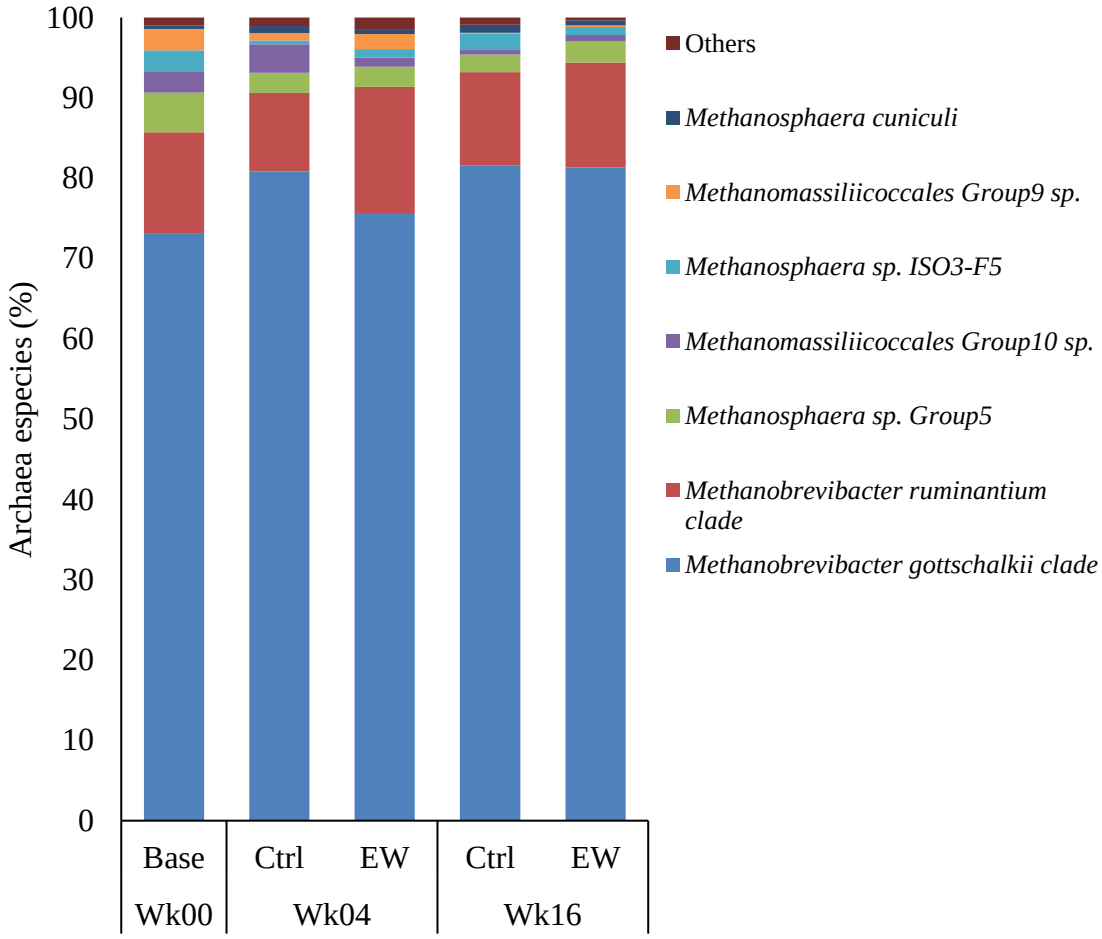


Table 3.3 Analysis of variance of the abundant archaea species[1] in different groups of lambs[2] at three rearing periods[3]. Results are natural log back transformed means and standard error of the differences (SED), P-value (P-val). Values in row with different letter indicate significant difference ($P < 0.05$) between groups of lambs.

	Wk00	Wk04		Wk16			
Species	**Baseline**	**Ctrl**	**EW**	**Ctrl**	**EW**	**SED**	**P-val**
Methanobrevibacter gottschalkii clade	73.14	80.86	75.53	81.60	81.32	8.944	0.85
Methanobrevibacter ruminantium clade	12.58	9.74	15.85	11.61	13.08	7.758	0.93
Methanosphaera sp. Group5	4.95	2.51	2.48	2.17	2.64	0.951	0.18
Methanomassiliicoccales Group10 sp.	2.64	3.54	1.12	0.55	0.76	1.992	0.44
Methanosphaera sp. ISO3-F5	2.60^{a}	0.50^{c}	1.07^{c}	2.11^{ab}	1.01^{bc}	0.614	0.02
Methanomassiliicoccales Group9 sp.	2.68^{a}	0.91^{ab}	1.88^{ab}	0.06^{b}	0.20^{b}	0.826	0.03
Methanosphaera cuniculi	0.45	0.88	0.54	1.02	0.63	0.592	0.86

[1] The archaea community was composed of 7 abundant species (abundance ≥ 1.0% in at least one of groups of lambs sampled).

[2] Group of lambs correspond to baseline (at arrival; wk 0), early weaning (EW; weaning at wk 4) and control (Ctrl; weaning at wk 6).

[3] Rearing periods corresponded to lambs separated from their dams at wk 0 (Wk00), reared in pens and with access to concentrates and hay at wk 4 (Wk04) and grazed in paddocks in a mixed sward of forages at wk 16 (Wk16) of rearing.

3.4.4 Fermentation

Total concentrations and individual proportions of SCFA did not vary between EW and Ctrl lambs at Wk04 (**Table 3.4**). At Wk16 proportions of caproate were 1.7 times greater in EW compared to Ctrl lambs, but the total concentration of SCFA and the individual proportions of the other SCFA did not differ between groups.

Table 3.4 Effect of weaning age[1] and rearing management[2] on ruminal short chain fatty acid (SCFA) total concentrations and individual proportions in the rumen of lambs. Data presented are predicted means, standard error of the difference (SED) and P-values (P).

Parameter	Wk04 [3]				Wk16			
	Ctrl	EW	SED	P	Ctrl	EW	SED	P
SCFA (mM)	87.6	114.5	23.43	0.27	85.2	101.6	13.21	0.24
Acetate (%)	57.2	56.5	4.13	0.75	60.4	60.9	2.02	0.81
Propionate (%)	28.0	29.7	5.72	0.70	23.0	20.8	1.82	0.25
Butyrate (%)	11.1	9.7	3.15	0.71	11.7	12.6	1.03	0.42
Valerate (%)	1.4	1.3	0.61	0.92	1.3	1.4	0.09	0.09
Caproate (%)	0.4	0.4	0.32	0.99	0.2	0.4	0.06	0.02
Isobutyrate (%)	1.1	1.1	0.55	0.88	1.5	1.8	0.23	0.29
Isovalerate (%)	1.0	1.3	0.58	0.57	1.8	2.1	0.28	0.33
Ace:Pro ratio	2.5	2.1	0.58	0.47	2.7	3.0	0.35	0.36

[1] Weaning age corresponded to wk 4 (early weaning; EW) and 6 (control; Ctrl).

[2] Rearing management were only analyzed at wk four (Wk04) and sixteen (Wk16).

[3] EW lambs were consuming 25% of their milk allowance and *ad libitum* concentrate, while the controls were consuming 75% of their milk allowance and *ad libitum* concentrate.

3.4.5 Canonical correlation analysis

The canonical correlation analysis (CCA) of the abundant bacterial genera and abundant archaea species with the fermentation profiles of both age groups of lambs (Wk04 and Wk16) is shown in **Figure 3.5**. The abundance of the bacterial genera *Prevotella* 1 and *Brenneria*, and archaea species *Mbb. gottschalkii*, *Mmc.* Group 9 sp. and *Mmc.* Group 10 sp. was positively correlated with total SCFA concentrations and propionate proportion. In addition, the abundance of those archaea species correlated with valerate. The abundance of the bacterial genera *Ruminococcaceae* NK4A214 group, *Selenomonas* 1, *Butyrivibrio* 2, *Pseudobutyrivibrio*, *Ruminococcus* 1 and *Christensenellaceae* R-7 group, and the archaea species: *Mbb. ruminantium* and *Mph.* sp. Group 5 correlated positively with proportions of isovalerate, isobutyrate, acetate and butyrate.

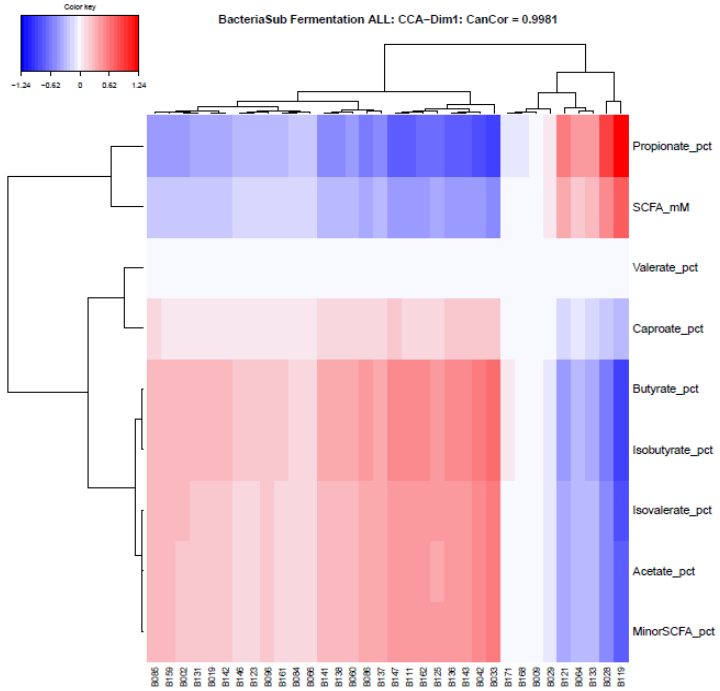
Further associations were done with regards to the ruminal microbiota and rumen morphology and blood metabolites. However, these analysis and results were removed from this Chapter into **Appendix C**, due to the potential confounding association effects and lack of biological explanation between changes in the rumen microbiota and rumen development and animal metabolism.

3.5 Discussion

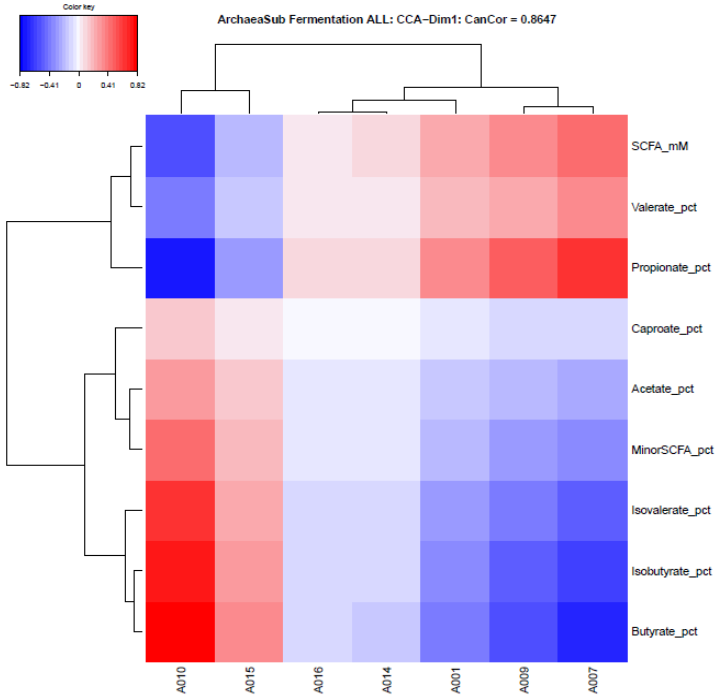
Rumen microbial colonization and establishment in the neonates is a complex process between the host and the rumen microbes, and is influenced by a variety of external factors. Early studies in the establishment of the rumen microbial community in lambs, using culture-dependent approaches, showed that, irrespective of the animal's age, changes in the appearance and concentration of different microbial groups were influenced by rearing practices, solid feed intake, and interaction with other animals (Fonty et al., 1984). In the present study, the acquisition of cellulolytic rumen microbiota in Wk00 lambs was similar to the observations of Fonty et al. (1987) and De Barbieri et al. (2015), who indicated that the bacterial communities in the rumen of newborn lambs could be altered by maternal inoculation during the first wks of life. One surprising finding in the present study was the high diversity of the microbial community of three to four day-old lambs (Wk00), which is different from findings in calves separated from their mothers at birth and artificially reared in pens (Rey et al., 2014), where the microbial diversity increased as the animals aged. However, our data might be influenced by the three to four days the lambs spent with their dams before they were brought to the facility. The observations that diversity decreased within the first two wks of life is consistent with previous studies by Fonty et al. (1987), where the isolation of lambs from their dams reduced the diversity in the rumen microbiota during the first days of life. The increases in the microbial diversity observed between Wk04 and Wk16 is likely to correspond to the acquisition of microbes from the environment when lambs graze together. However, the increased diversity may also be a direct effect of feeding forages as reported in calves by Kim et al. (2016). In summary, these results showed that the diversity of the microbial community in the rumen of growing lambs is affected by the rearing management and diet, independent of the animal's age.

Figure 3.5 Canonical correlation analysis clustering image maps of the associations between abundant rumen microbes and fermentation profiles of lambs reared at wk 4 and 16 of rearing. Rumen microbes and fermentation profiles were sampled at wk 4 (Wk04) and 16 (Wk16) of rearing. Abundant rumen microbes corresponded to 31 bacteria genera and 7 archaea species with a relative abundance of ≥1.00% and ≥0.50% in average across treatment groups and sampling times. **a)** Canonical correlation analysis (CCA) between abundant bacteria genus and fermentation profiles. **b)** CCA between abundant archaea species and fermentation profiles. The dark red and blue indicate positive and negative correlation coefficient values, respectively, whereas white color indicate zero correlation coefficient values. Microbial groups are identified by ID numbers in Table B.6 (Appendix B).

a)



b)



The proportions of *Fibrobacteres* in the rumen of Wk00 lambs were comparable to those found in buccal swabs of adult ruminants (relative abundances of ~2.2%) (Kittelmann et al., 2015). Increases of *Absconditabacteria* has been positively associated with increases of *Fibrobacteres* in adult ruminants consuming forages (AlZahal et al., 2017). The presence of *Saccharibacteria* phylum, which includes cellulose utilizing species (Opdahl et al., 2018), in the rumen of lambs still nursing from ewes may reflect maternal transfer. In contrast, *Spirochaetes*, a phylum that interacts and is associated with increases of cellulolytic bacteria (Stanton and Canale-Parola, 1980) was found at increased abundances in Wk16 lambs compared to Wk00 and Wk04, which could be attributed to the increased consumption and plant cell wall degrading taxa in these lambs. Therefore, the high abundance of cellulolytic and oral taxa, but lower relative abundance of *Spirochaetes* in Wk00 lambs, compared to Wk04 and Wk16, may reflect the potential microbial community inoculation of the offspring during the first days of life from the saliva of ewes grazing on the paddock. In contrast, the observed relative abundance of *Fibrobacteres* and orally-associated bacterial taxa were not age or diet dependent, since the rumen of Wk00 lambs had no functional activity (Baldwin et al., 2004). This was confirmed in the ruminal samples collected from Wk00 lambs with a white color and fermented milk odor, which might correspond to saliva, mucus, desquamated epithelial cells and milk spilled into the rumen (Jayne-Williams, 1979).

In growing ruminants, the rumen microbiota is affected by the animal's age and diet (Li et al., 2012b; Jami et al., 2013; Rey et al., 2014). Additionally, maternal transfer plays a key role during the early microbial establishment in the rumen of offspring (Abecia et al., 2014a; Abecia et al., 2014b; De Barbieri et al., 2015). In the current study, adult-like proportions of *Fibrobacter* were found in lambs ingesting only maternal milk at Wk00, which declined with age independently of the ingested diet. The adult like proportions may correspond to the bacteria inoculated by the ewe to the offspring (De Barbieri et al., 2015). These results differed from those reported in growing calves and goat kids, in which *Fibrobater* proportions remained constant with age independently of the age and diet (Jami et al., 2013; Rey et al., 2014). The intake of rich starch diets by Wk04 lambs decreased the abundance of cellulolytic microorganisms, whilst there was an increased abundance of saccharolytic bacteria taxa, i.e. *Alloprevotella* (Downes et al., 2013). Cellulolytic and hemicellulolytic genera from the phyla *Bacteroidetes* and *Firmicutes* increased again after

transferring the lambs to pasture at Wk16. Additionally, increases of *Pedobacter* and *Mucilaginibacter* in Wk16 lambs may be associated with the complex enzymatic systems that these two genera have for the degradation of cellulose and hemicellulose rich diets (López-Mondéjar et al., 2016). The ingestion of fresh pastures also increased the proportions of *Oscillospira* spp., as observed in a broad number of ruminant species, with the highest counts of this genus associated with the ingestion of fresh forage diets (Mackie et al., 2003). Recent studies have indicated that this butyrate producer bacteria is probably a slow grower (Gophna et al., 2017), therefore, its high abundance may be associated the ruminal transit times observed in ruminant consuming pasture diets (Van Soest, 1994). While, *Selenomonas* spp., which does not digest fiber in monoculture, but can utilize lactate and malate, has been associated with greater fiber digestion and propionate production when co-cultured with *Fibrobacter succinogenes* (Sawanon et al., 2011). Finally, increased proportions of *Butyrivibrio* spp., which is a butyrate-forming rumen bacterium, plays a key role in plant polysaccharide and protein degradation (Kelly et al., 2016).

The presence of methanogenic species in the rumen of newborns has been confirmed immediately post-partum, e.g. first 0 to 20 minutes of life (Guzman et al., 2015). In the present study, Wk00 lambs (~4 days of age) showed a complex archaea community structure, similar to that observed in grazing adult-like sheep (Seedorf et al., 2015). This data is in agreement with observations reported by Morvan et al. (1994), who showed that these microorganisms can be established in the rumen prior to the ingestion of solid feed. An interesting finding in the present study was the stability of the archaea community structure observed in lambs at different times despite the contrasting diets fed. Few changes were observed in archaea species with low relative abundance, such as *Methanosphaera* sp. ISO3-F5 in Wk04 lamb and *Methanomassiliicoccales* Group 9 in Wk16 lambs, similar to the reported by Seedorf et al. (2015) in sheep consuming different types or quality diets. Methanogens reduce specific substrates to produce CH_4 using H_2 as an electron donor, e.g. *Methanobrevibacter* spp. uses CO_2 (Miller et al., 1986; Seshadri et al., 2018), while *Methanosphaera* spp. utilizes methanol (Lang et al., 2015; Poehlein et al., 2018), and *Methanomassiliicoccales* spp. uses methylamines and methanol (Lang et al., 2015; Li et al., 2016). In milk fed ruminants, methanogens may use other H_2 sources rather than those from the fermentation of solid food (Guzman et al., 2015). In the present study, it is uncertain the mechanism through which the methanogen community in Wk00 lambs may obtain metabolizable substrates from milk reaching the rumen to maintain their relative

abundance in these lambs prior to ingestion of solid feed. The fact that these organisms are present also indicates that within the early rumen there are anaerobic niches in which methanogens can survive on H_2 produced by other microorganisms. Although, it might be assumed that the archaea community composition presented in these lambs may not be active and is just a result of ongoing inoculation form the dam. Further research is required to understand the archaea community composition, metabolism and association with potential microbial H_2 donors prior to establishment of solid feed intake in young ruminants.

The present study explored the relationship between abundant bacteria and short chain fatty acids (SCFA) profiles in Wk04 and Wk16 lambs. The abundance of *Prevotella* 1 was associated with greater total SCFA concentrations and propionate proportions. *Prevotella* is one of the most abundant and genetically divergent genera in the rumen (Bekele et al., 2010; Henderson et al., 2015), and is also known for utilizing a wide range of substrates and having the metabolic capacity to produce propionate (Seshadri et al., 2018). These indicate that *Prevotella* may play a key role in the fermentation of non-structural carbohydrates and proteins in the diet of young ruminants, producing high amount of propionate as a metabolic end-product. Interestingly, *Prevotella* was not associated with increased isovalerate and isobutyrate contractions from protein metabolism. This may be explained by what is observed in the present study in which the decrease in *Prevotella* proportions, during the intake of pastures, coincided with the increase of isoacids. The ruminal genera *Ruminococcaceae* NK4A214 group, *Ruminococcus* 1, *Christensenellaceae* R-7 group, *Butyrivibrio* 2, *Pseudobutyrivibrio* and *Selenomonas* 1 were associated with greater proportions of acetate and butyrate. These associations agree with a report of 6-week-old lambs fed a concentrate diet (Wang et al., 2016). The genera *Ruminococcaceae* NK4A214 group and *Ruminococcus* 1 are cellulolytic bacteria with acetate as main end-product (Koike and Kobayashi, 2001; Gagen et al., 2015). *Christensenellaceae* R-7 group, an acetate and butyrate producer (Morotomi et al., 2012) is highly abundant in diets rich in high fiber (Lima et al., 2015). *Butyrivibrio* 2 and *Pseudobutyrivibrio* produce butyrate from hemicellulose and other plant polysaccharides (Van Gylswyk et al., 1996; Kopečný et al., 2003; Kelly et al., 2010). While, *Selenomonas* 1 produces acetate and propionate from the degradation of different plant polysaccharides, and its enzymatic activity may enhanced the entry of the fibrolytic bacteria into plant cells (Sawanon et al., 2011). Therefore, the observed association in the present study indicates

that the abundance of these cellulolytic and hemicellulolytic bacteria favors the fermentation of diets rich in structural carbohydrates increasing the production of acetate and butyrate in the rumen.

On the other hand, abundant archaea and SCFA profile associations indicated that *Mbb. gottschalkii* and *Methanomassiliicocales* species, i.e. Group 9 sp. and Group 10 sp., were positively correlated with total SCFA concentrations and the proportions of propionate and valerate. These associations may not be biologically correct, since the production of propionate results in less H_2 formation per mole of feed monomer fermented in the rumen (Moss et al., 2000), which in turn affect the abundance of hydrogenotrophic archaea, e.g. Methanobrevibacter spp. (Miller, 2015). Additionally, *Methanomassiliicocales* spp. are methylotrophic methanogens (Li et al., 2016), and the production of methylamine, dimethylamine and trimethylamine in fiber-rich diets were associated with high ratios of acetate to propionate (Deusch et al., 2017). Whilst the abundance of *Mbb. ruminantium* and the increases of acetate and butyrate proportions may result from greater H_2 release during the production of these SCFAs (Moss et al., 2000). *Methanosphaera* spp. utilizes methanol during methanogenesis (Fricke et al., 2006). Methanol is derived from the hydrolysis of pectin and other methylated plant polysaccharides, which are abundant in clover and other non-grass pasture species which usually contain higher proportion of pectins than grasses (Dehority, 1969). Therefore, results of the association of *Mbb. gottschalkii* and *Methanomassiliicocales* species, i.e. Group 9 sp. and Group 10 sp., and total SCFA concentrations and the proportions of propionate and valerate, together with the associations of *Methanosphaera* sp. ISO3-F5 and proportions of acetate and butyrate may be a confounded effect of the diet fed and age of the lambs. Therefore, further correlation analysis should include other fermentation by-products such as methanol, methylamine, dimethylamine and trimethylamine, as well as gases such as CH_4 and H_2 to properly correlate the rumen fermentation and archaea communities under different dietary managements.

3.6 Conclusions

Inoculation from the dam plays a major role in the establishment of the rumen microbiota in lambs. Weaning strategy had very little effect on the microbial composition although some effects on diversity were observed. The biggest effect on rumen microbiota, however, was due to the different diets before and after weaning. These changes in the

bacterial community were similar to other studies in response to concentrate and forage feeding. Correlations of microbial community composition and fermentation by-products in the rumen corresponded to different fermentation metabolites; however, further analyses need to take place to understand and quantify the dynamic metabolism of the rumen microbiota, i.e. use of metagenomics and metatranscriptomics technologies, when animals are provided different diets.

Chapter 4: Effect of divergent feeding regimes during early life on the rumen microbiota in calves

General overview of the chapter:

A complex rumen microbiota was observed in four-day-old neonates, which was influenced by animal age and transition onto different solid diets in Chapter 3. However, it was not possible to elucidate if it was an age, diet or a combination of age and diet effect on the rumen microbiota. This chapter aimed to determine whether contrasting feeding regimes pre- and post-weaning produce a permanent change in the rumen microbiota with associated changes in rumen fermentation.

4.1 Abstract

The objective of this study was to determine whether divergent feeding regimens in calves in the first 7 months of life are associated with long-term changes in rumen fermentation and microbial community composition. Twenty-four Hereford-Friesian cross female calves were selected from a cohort of 200 animals. Calves were arranged in a 2 x 2 factorial design with two divergent treatments across three time periods. In period 1 (P01, 0-14 wks), calves were offered either a low-milk volume and concentrate solid feed diet with early weaning (CO) *vs.* high-milk volume and pasture solid feed diet and later weaning (FO). In period 2 (P02, 15-30 wks) half of the calves from each group were offered either a high-quality (HQ) *vs.* low-quality pastures (LQ). In period 3 (P03, 31-41 wks) a common pasture-only diet was offered to all calves. Treatment effects were evaluated within each of the three periods at wks 9, 19 and 41, respectively. Gas emissions (methane and hydrogen) and dry matter intake (DMI; kg/d) were measured over a two-day period in respiration chambers, followed by samplings for rumen short chain fatty acids (SCFA) and microbial communities. In P01, the FO group had lower DMI, but greater CH_4 yields (yCH_4) compared to the CO group. Acetate proportions and proportions of cellulolytic bacteria were greater in the FO than the CO group. The archaeal community was dominated by *Methanobrevibacter gottschalkii* in the FO group, while *Mbb. boviskoreani* was dominant in the CO group. In P02, DMI was lower in the LQ than HQ group, but CH_4 yield was similar between the groups. The proportion of acetate in the LQ group was higher as was the proportion of cellulolytic bacteria compared to the HQ group. Decreased proportions of *Methanosphaera* and increased proportions of *Methanomassiliicoccales* were observed in the LQ compared to the HQ group. *Methanobrevibacter* species were similar between treatment groups. No effects from P01 or interactions were observed for any of the parameters. In P3, all groups had similar DMI, gas emissions, SCFA proportions and microbial composition and no interactions with previous treatments were observed. These results indicate that the rumen microbial composition and the associated fermentation end-products are driven by the diet consumed at the time of sampling, and that previous dietary interventions do not lead to a detectable long-term microbial imprint or changes in rumen function.

4.2 Introduction

The rumen is a fermentation chamber occupied by a diverse, interactive and dynamic microbiota comprised of many species of bacteria, archaea, protozoa and fungi (Hobson and Stewart, 1997). These microorganisms convert the ingested feed into short chain fatty acids (SCFA) and microbial biomass, which are the main sources of amino acids and energy for ruminants, respectively (Bergman, 1990). Other fermentation end-products, including hydrogen (H_2) and carbon dioxide (CO_2), formic acid or methyl groups, are utilized by methanogens to produce methane (CH_4) (Moss et al., 2000; Liu and Whitman, 2008; Janssen, 2010). Methane production is considered a dietary gross energy loss to the ruminant (Bergman, 1990) and a greenhouse gas (GHG) (Johnson and Johnson, 1995). Manipulations of the ruminal ecosystem have been attempted to improve the efficiency of feed conversion and decrease the overall environmental impact of livestock production with little success or short-term effects after treatment cessation (Weimer, 1998).

In adult ruminants, the rumen microbial community is characterized by a high degree of redundancy and resilience, providing stability to the rumen environment and maintaining the digestive function of the host across a range of feeding and management conditions (Weimer, 2015). These properties represent a barrier to manipulate the rumen fermentation by selectively targeting groups of microorganisms. However, some studies suggest that the rumen microbial community may be more plastic, and therefore, easy to manipulate in early life (Yáñez-Ruiz et al., 2010; Abecia et al., 2014a; De Barbieri et al., 2015).

While sterile *in utero*, the rumen of newborn animals undergoes a rapid microbial colonization during and after birth by maternal (Ducluzeau, 1983b) and environmental sources (Dehority and Orpin, 1997; Curtis and Sloan, 2004). After the initial colonization, the rumen microbiota rapidly shifts towards obligate anaerobic microbes as young ruminants start to transition from milk to solid diets (Walters et al., 2011; Rey et al., 2014). Post-weaning, the exclusive consumption of a solid diet is associated with a progressive shift in the ruminal microbial composition towards a more diverse microbiota (Rey et al., 2014; Dill-McFarland et al., 2017). Therefore, the microbial community composition of the young ruminant is very responsive to the dietary interventions (Yáñez-Ruiz et al., 2010; Abecia et al., 2014a; Wang et al., 2017a). However, there is limited information about the effect of diet in early life on the microbial community composition when animals transition

between different diets through different stages of growth. The aim of this study was to determine whether contrasting feeding regimes pre- and post-weaning will imprint the rumen microbial community with associated changes in rumen fermentation.

4.3 Materials & Methods

Animal procedures were reviewed and approved by the Grasslands Animal Ethics Committee (AE 13297) and complied with the institutional Codes of Ethical Conduct for the Use of Animals in Research, Testing and Teaching, as prescribed in the New Zealand Animal Welfare Act of 1999 and its amendments.

4.3.1 Experimental design

In the present study, 24 calves (~1 week of age) were randomly chosen and balanced a cross dietary treatments from a larger production study using 200 Hereford-Friesian cross female calves. The study was carried out in a 2x2 factorial design with different dietary treatments across three time periods. In period 1 (P01, 0-14 wks), calves were grouped in a concentrate (CO) or pasture (FO) diet. In period 2 (P02, 14-30 wks) half of the calves from each group were reallocated to either a high-quality (HQ) or low-quality pasture (LQ) diet. In period 3 (P03, 30-41 wks) calves (same groups from P2) were grazed in paddocks with a common pasture. The pasture was composed of a ryegrass/white clover mixed sward.

4.3.2 Animal management

In P01, the FO group was penned during the first wk and moved to paddocks from wks two to twelve. These animals were offered ~8 L/calf/d of reconstituted whole milk powder (WMP; **Table 4.1**; NZ Agbiz, Auckland, NZ) for 12 wks and had free access to paddocks of ryegrass/white clover from wk 2. Milk was offered as follows: twice a day from one to seven wks, once a day from wks eight to ten, and step down weaned from wks 11 (20% of WMP allowance was removed every two days over a period of ten days) until the end of wk 12. The CO group was penned from arrival and transferred to paddocks from wk 7. These animals were offered ~4 L/calf/d of WMP for 8 wks and *ad libitum* starter concentrate during penning (**Table 4.1**; Denver Stock Feeds, Palmerston North, NZ). Milk was offered as follows: twice a day from 1 to 4 wks, once a day from 5 to 6 wks, and step down weaned from wk 7 until weaning by reducing 20% of milk allowance every two days. From wk 7, the CO group was transferred to paddocks with pastures of ryegrass/white clover and the concentrate capped at 2 kg/hd/d until wk 12. In P02, calves in the HQ and

LQ groups were grazed in paddocks of high-quality and low-quality pastures, respectively, from wks 14-30. Finally, in P03 both groups of calves were managed in two grazing mobs equally representing each treatment group in similar sward of mixed ryegrass and /white clover from wks 30-41.

4.3.3 Gas emissions and dry matter intake measurements

Methane (CH_4) and hydrogen (H_2) emissions and dry matter intake (DMI) were measured during wks 9 (P01), 19 (P02) and 41 (P03). Prior to gas emission measurements, calves were adapted to confinement conditions in covered yards from 5 to 7 days. Gas emission measurements were carried out in open circuit respiratory chambers (Pinares-Patiño et al., 2012b) over a 48 hour period. The air flow through the chambers was set at 700, 1000 and 1200 L/min during the three measurement periods, respectively, to account for the increasing gas emissions as the solid feed intake of the calves increased. Calves entered the chambers in the morning (0900h), when fresh solid feed (starter concentrate and/or cut and carry pastures) was offered. During the confinement condition adaptation and gas emission measurement periods, calves were feed accordingly to the assigned feeding group. Pasture for each respective feeding group of calves was cut daily, transported to the animal facility and offered *ad libitum*. Samples of concentrate and WMP were analyzed for chemical composition by wet chemistry methods (Hill Laboratories Ltd, Hamilton, New Zealand). Pasture samples of ryegrass/white clover mixed sward were analyzed by near infrared reflectance spectroscopy (NIRS; FeedTECH, Palmerston North, New Zealand). **Table 4.1** shows the chemical composition of the offered diets during the different rearing periods. Water was available *ad libitum*. Dry matter intakes (DMI) were determined during the gas measurement periods, as the difference between the allowance and the residual feed. Methane (CH_4) and hydrogen (H_2) production (pCH_4 and pH_2) was determined as the total amount of CH_4 or H_2 produced per day (g/d), while CH_4 and H_2 yield (yCH_4 and yH_2) was the total amount of CH_4 or H_2 per kilogram of DMI (g/kg of DMI).

Table 4.1 Chemical composition (% of dry matter) of the whole milk powder (WMP), concentrate and pastures[1] fed to calves in period 1 (P01), pastures of high[2] (HQ) and low-quality[3] (LQ) in period 2 (P02) and pastures fed to all calves in period 3 (P03).

	P01			P02		P03
Feed	**WMP**	**Pasture**	**Concentrate**	**HQ Pasture**	**LQ Pasture**	**Pasture [a]**
Dry matter (%)	95.2	18.7	93.8	21.5	37.7	14.9
Crude protein	24.1	14.8	19.8	19.8	7.3	19.2
NDF[4]	--	49.5	16.2	47.9	64.2	53.0
ADF[5]	--	25.4	5.9	25.2	35.7	27.0
Lignin	--	1.8	--	3.8	4.2	2.9
Lipids	28.4	1.1	2.3	2.3	1.1	2.8
Ash	5.5	6.4	6.2	8.8	5.5	10.3
Soluble sugars	41.5	19.3	8.0	9.3	9.0	11.4
Starch	--	--	36.8	--	--	--

[1] Pasture was composed of ryegrass/white clover mixed sward.

[2] Calves were grazed in irrigated pastures.

[3] Calves were grazed in unirrigated pastures.

[4] Neutral detergent fiber.

[5] Acid detergent fiber.

4.3.4 Sampling and fermentation analysis of rumen fluid

Rumen fluid samples from P01, P02 and P03 were collected at 9, 19 and 41 wks of age, respectively, in the mornings via stomach tubing after gas emission measurements and prior to feeding milk and/or solid feed. Each sample was subsampled for SCFA analysis and DNA extraction. Rumen samples were snap frozen and stored at -20°C until analysis. For SCFA analysis, rumen samples were prepared as described by Guyader et al. (2016). Gas chromatography was used to analyze SCFA composition (Attwood et al., 1998) using a Hewlett-Packard 6890 equipped with an auto-sampler fitted with a Zebron ZB-FFAP 30.0m x 0.53mm I.D. x 1µm film column and a flame ionization detector (Tavendale et al., 2005).

4.3.5 Extraction, amplification and purification of nucleic acids from rumen fluid

Nucleic acids (DNA) were extracted from 200 µl of the rumen fluid samples using the phenol-chloroform, bead beating with filtration kit for purification II (PCQI) (Henderson et al., 2013). Primers (**Table A.1, Appendix A**) and amplification reactions (30 and 35 cycles, respectively) used to PCR the V3-V4 region of bacterial and archaeal

16S rRNA genes were prepared in triplicate as described by Kittelmann et al. (2013). PCR products were pooled, and the correct sizes and the absence of signal from negative controls were verified by agarose gel electrophoresis and quantified by fluorescence using the Quant-iT dsDNA BR assay kit (Invitrogen, Carlsbad, CA, USA). Each amplicon (150 ng) from the same target gene and region (i.e., all bacteria and archaea amplicons) were pooled. Pooled samples were concentrated, and the final PCR product concentration was determined using Quant-iT dsDNA HS assay kit (Invitrogen, Carlsbad, CA, USA). Pools were purified using the NucleoMag NGS kit (Macherey-Nagel, Dueren, Germany). The final purification of amplicons was done using the QIAquick PCR Purification kit (QiaGen, Valencia, CA, USA) and the DNA concentration quantified using Quant-iT dsDNA HS assay kit (Invitrogen Carlsbad, CA, USA). Both pools were diluted to $6.0x10^9$ copies per µl and combined at a bacteria to archaea ratio of 5:1 (Kittelmann et al., 2013). Quality control (QC) were run in the pooled libraries using PerinElmer GX Touch HT Instrument using Bioanalyzer DNA High Sensitivity Assay. Amplicons were sequenced using Illumina MiSeq system (Massey University, Palmerston North, NZ). The pooled library was run on one Illumina MiSeq 2 x 250 base PE run version 2 chemistry (Reagent Kit v2, 500 cycles; Invitrogen, Carlsbad, CA, USA). Each Illumina MiSeq 2 x 250 base PE. A control library for the run, Illumina prepared PhiX, was loaded onto the Illumina MiSeq run at 20% volume. Sequence reads were provided in fastq format.

4.3.6 Phylogenetic analysis of sequencing data

Sequencing reads were quality-filtered using the DynamicTrim function of SolexaQA (Cox et al., 2010). Reads were then processed and analyzed using the QIIME software package 1.8 (Caporaso et al., 2010). Sequencing reads were grouped into operational taxonomic units (OTUs) sharing over 97 and 99% UCLUST similarity for bacteria and archaea, respectively (Edgar, 2010). Sequences were assigned to phylogenetic groups using BLAST (Altschul, 1990). Bacterial 16S rRNA genes were assigned using SILVA 123 (Henderson et al., 2019) and archaeal 16S rRNA genes using RIM-DB (Seedorf et al., 2014). QIIME generated OTU-tables were used for downstream statistical analysis.

4.3.7 Statistical analyses

Univariate analyses were performed using a linear mixed effect (LME) model via the restricted maximum likelihood (REML) framework as implemented in the NLME

package in R (Pinheiro et al., 2015; R CoreTeam, 2016). The resulting LME models were analyzed using an analysis of variance (ANOVA). Predicted means from the model, together with estimates of the standard error of the mean and pairwise comparisons (Tukey's test and Benjamini-Hochberg' test) were obtained, and back transformed (where applicable) using the PREDICTMEANS package of R (Luo et al., 2014b).

Dietary treatment effects and their interactions were analyzed for dry matter intake, rumen fermentation and gas emissions data. In P01, these data were analyzed using an LME model with dietary treatment from P01 (FO and CO) as fixed effect and animal as random effect. In P02 and P03, the data were analyzed using an LME model with dietary treatments from P01 (FO and CO) and P02 (HQ and LQ) as fixed effect, and animal as random effect. The resulted LME models were analyzed using a one-way ANOVA for P01 and a 2x2 factorial ANOVA for P02 and P03. Treatment effects were assessed and predicted means from the model, together with estimates of the standard errors of the means, were obtained and compared using Tukey's test. Statistical significance was declared at a P-value $\leq$ 0.05.The alpha diversity of the microbial community of calves under contrasting dietary management conditions was analyzed using the Chao1 and Shannon indexes in the vegan package of R (Oksanen et al., 2017). The dietary treatment effects within and between periods were compared for the Chao1 and Shannon indexes using an LME model with dietary treatment and periods as fixed effects, and animals as random effect. The differences of the LME models for Chao1 and Shannon indexes were assessed using ANOVA and post hoc analysis using Tukey's test. Statistical significance was declared at a P-value $\leq$ 0.05.

The beta diversity of the whole microbial community of each group of calves was analyzed using a partial least squares discriminant analysis (PLSDA; as justified in Chapter 3) using the mixOmics package of R (Lê Cao et al., 2016). Groups of calves were assigned combining period and treatments as follows: period one (P01) corresponded to groups from FO and CO; period two (P02) and three (P03) were the groups formed by the combination of dietary treatments from P01 and P02, resulting in FOHQ, FOLQ, COHQ and COLQ. The aim was to identify the effect of diet on rumen microbial communities and the groups of microorganisms associated with these effects(variable importance in projection or VIP microorganisms). Additionally, a PLSDA was conducted for abundant microbes. The abundant microbes were selected as follows: bacteria (minBacteria) and archaea

(minArchaea) with a relative abundance ≥ 0.50% and ≥ 1.00%, respectively. The aim was to identify if the abundant microbiota showed a similar cluster separation pattern to the observed in the whole microbiota. Association scores for bacteria and archaea were visualized via clustered image maps (CIM) representing the first two dimensions (Henderson et al., 2016).

Univariate analyses were used to determine the effect of different dietary treatments on the abundant microbial community. The abundant microbial community was assessed as follows: OTU taxonomic data table with an average relative abundance across periods ≥ 0.5% and ≥ 0.5% at bacteria phylum and genus level, respectively, and ≥ 1.0% at species level for archaea. After checking for normality, bacteria (phyla and genera) and archaea (species) community data were transformed using natural logarithm. All the analyses were performed using a LME model. The effect of dietary treatment in P01 for the abundant microbial community was compared using treatments in P01 as fixed effects and animal as random effect. The effects of dietary treatments for the abundant microbial community in P02 and P03 were assessed using dietary treatments in P01 and dietary treatments in P02 as fixed effects, and animals as random effects. The LME models were analyzed in P01 using a one-way ANOVA, and in P02 and P03 a 2 x 2 factorial ANOVA. Predicted means from the models, together with estimates of the standard error of the mean (SEM) were obtained and back transformed, and pairwise comparisons were done using Benjamini-Hochberg' test Statistical significance was declared at a P-value ≤ 0.05.

4.4 Results

4.4.1 Rumen function

In wk 9 (P01), calves from the FO group consumed 58% less (P<0.01) solid feed DM and had 47% higher (P<0.01) yCH_4 (g/kg DMI) than the CO group. No differences (P=0.41) in yH_2 were observed between groups. Total SCFA concentrations in the rumen were 31% lower (P<0.01) in the FO than CO group (**Table 4.2)**. The CO group had a decreased (P<0.01) proportion of acetate, and an increased (P<0.01) proportion of propionate, while the proportion of butyrate was similar (P=0.37) between the two groups. For the minor SCFA, the CO group had increased (P<0.01) proportions of valerate compared to the FO group and lower (P<0.01) proportions of isobutyrate and isovalerate. No difference (P=0.11) in the proportions of caproate was detected between groups. In wk 19 (P02) the DMI, pCH_4 and pH_2 were 29%, 25% and 57% lower (P<0.05), respectively,

in the LQ compared to the HQ group, while yCH_4 and yH_2 were similar (P>0.05) between the two groups. Total SCFA concentrations in the rumen were 26% lower (P<0.01) in the LQ than HQ group. Calves in the LQ group had greater (P<0.01) proportions of acetate, a lower (P<0.01) proportion of propionate, while proportions of butyrate had a decreasing trend (P=0.07) compared to the HQ group. Within the minor SCFA, the LQ group had low (P<0.01) proportions of valerate, isobutyrate and isovalerate compared to the HQ group. No interactions (P>0.05) between dietary treatments in P01 and P02 were observed for any of the parameters. In wk 41 (P03), when all calves were offered pasture with similar composition, the DMI, and CH_4 and H_2 emissions (production and yield), total concentrations and proportions of SCFA were similar (P>0.05) between groups, and no interactions (P>0.05) between P01 and P02 treatments were detected.

4.4.2 Microbial richness and diversity

A total of 8,087,270 sequences were obtained from the 72 samples, using Illumina MiSeq sequencing, with 97,286 bacteria and 15,037 archaea sequences per sample. The number of organizational taxonomic units (OTUs) were 1,509 and 41 for bacteria and archaea respectively. A total of 364 bacteria and 17 archaea taxa were analyzed among all the rumen samples after using a cut off of 70 reads per sample as minimum.

The bacterial Chao1 index in P01 showed greater (P<0.05) richness[3] in microbial communities from the FO group than the CO group (**Figure 4.1**). In P02, the bacteria richness was lower (P<0.05) in the FOHQ compared to the COHQ groups, no other differences between groups were observed. In P03, similar (P>0.05) bacteria richness was observed among all groups of calves (**Figure 4.1a**). The archaea Chao1 index showed a low (P<0.05) richness in the CO group compared to all other groups in the P01, P02 and P03 (**Figure 4.1b**).

[3] Species richness is simply a count of species, and it does not take into account the abundances of the species or their relative abundance distributions (Delang and Li, 2013).

Table 4.2 Effect of dietary treatments[1] on dry matter intake (DMI)[2], gas emissions[3] and fermentation profiles[4] in calves during three measurement periods. Results are the means and standard error of the differences (SED), P-value for treatment effect for FO *vs.* CO (P01), treatment effect for HQ *vs.* LQ (P02) and their interactions (P-int)[5].

	P01				P02								P03							
	FO	**CO**	**SED**	**P-1**	**FO**	**CO**	**HQ**	**LQ**	**SED**	**P-1**	**P-2**	**P-int**	**FO**	**CO**	**HQ**	**LQ**	**SED**	**P-1**	**P-2**	**P-int**
DMI (kg/d)	0.89	2.10	0.055	<0.01	3.50	3.31	3.98	2.83	0.286	0.52	<0.01	0.81	4.11	3.91	4.20	3.81	0.237	0.40	0.12	0.87
pCH4 (g/d)	14.59	18.20	1.789	0.06	67.80	62.90	74.80	56.00	3.280	0.15	<0.01	0.41	121.60	117.80	124.60	114.70	6.260	0.55	0.13	0.46
yCH4 (g/kg)	16.21	8.66	0.976	<0.01	20.60	19.42	18.87	21.16	1.998	0.56	0.27	0.91	29.81	30.41	29.91	30.32	1.275	0.65	0.75	0.68
pH2 (g/d)	0.123	0.211	0.0890	0.33	0.101	0.062	0.114	0.049	0.0281	0.18	0.03	0.76	0.033	0.036	0.034	0.035	0.0310	0.93	0.97	0.67
yH2 (g/kg)	0.143	0.101	0.0496	0.41	0.027	0.019	0.029	0.017	0.0071	0.26	0.11	0.15	0.008	0.009	0.007	0.009	0.0072	0.91	0.82	0.44
SCFA (mM)	70.10	101.60	8.700	<0.01	63.50	64.70	73.80	54.40	5.420	0.82	<0.01	0.28	74.50	71.00	76.20	69.40	8.330	0.68	0.43	0.18
Acetate (%)	62.48	45.05	1.527	<0.01	70.31	70.60	68.55	72.36	0.545	0.60	<0.01	0.80	67.96	67.89	68.02	67.84	0.775	0.93	0.82	0.26
Propionate (%)	21.83	39.14	1.501	<0.01	17.51	17.19	17.98	16.72	0.417	0.45	<0.01	0.75	16.53	16.63	16.41	16.75	0.603	0.87	0.58	0.92
Butyrate (%)	12.04	10.99	1.132	0.37	9.19	8.95	9.41	8.73	0.361	0.52	0.07	0.71	11.71	11.64	11.77	11.58	0.390	0.87	0.65	0.14
Valerate (%)	1.35	3.53	0.253	<0.01	0.94	1.00	1.20	0.74	0.056	0.32	<0.01	0.32	0.99	1.02	0.99	1.02	0.054	0.49	0.67	0.43
Caproate (%)	0.45	0.68	0.137	0.11	0.29	0.34	0.28	0.35	0.039	0.17	0.07	0.13	0.12	0.13	0.13	0.11	0.012	0.43	0.17	0.72
Isobutyrate (%)	0.92	0.28	0.059	<0.01	0.89	0.92	1.22	0.58	0.033	0.45	<0.01	0.86	1.30	1.27	1.28	1.93	0.076	0.74	0.84	0.25
Isovalerate (%)	0.94	0.33	0.115	<0.01	0.87	1.00	1.35	0.53	0.055	0.03	<0.01	0.88	1.40	1.42	1.41	1.41	0.092	0.88	0.96	0.28

[1] Dietary treatments corresponded to: Period 1 (P01) concentrate (CO) *vs.* pasture (FO) diets, and Period 2 (P02) high quality (HQ) *vs.* low quality (LQ) pastures, with measurements in P01 (9 wks), P02 (19 wks) and Period 3 (P03; 41 wks) when all calves were offered a common pasture diet.

[2] DMI (kg/d) was measured in two consecutive days during gas emission measurements.

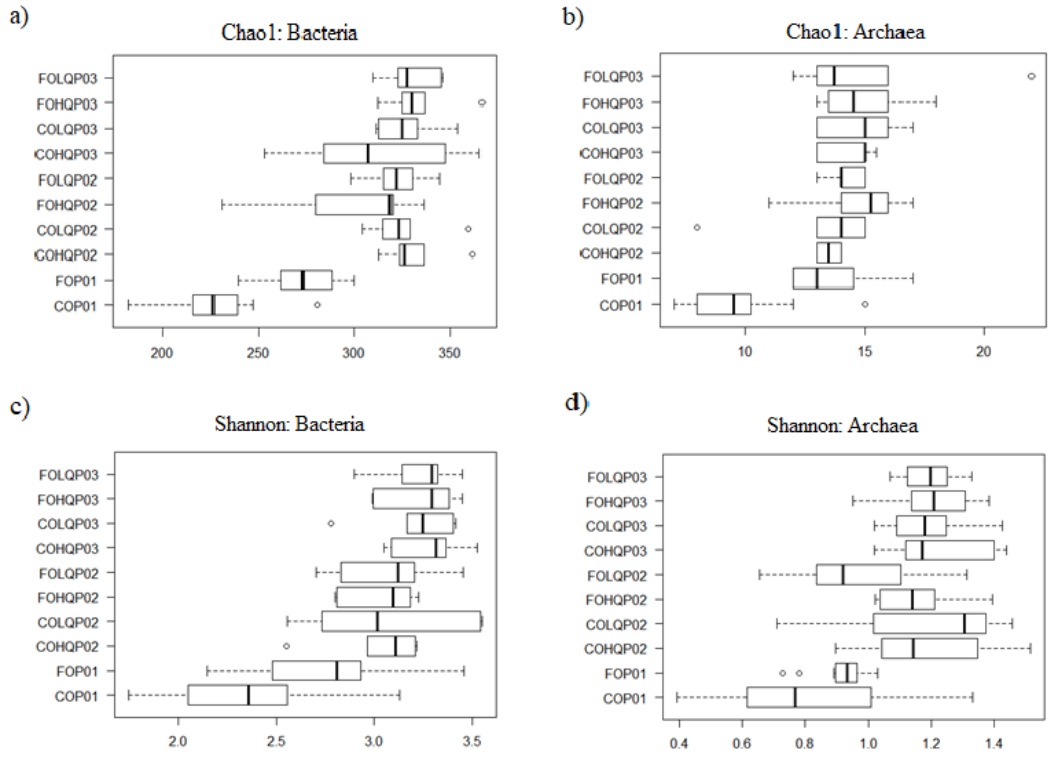
[3] Methane (CH_4) and hydrogen (H_2) production in two consecutive days (g/d) and yield per kilogram of DMI (y; g/kg DMI) measured.

[4] Total concentrations (mM) and individual proportions (%) of short chain fatty acids (SCFA).

[5] Dietary treatments in each period were evaluated as follow: a one-way ANOVA in P01 (9 wk) to analyze FO *vs.* CO diets, and a 2 x 2 factorial ANOVA in P02 and P03 to evaluate FO *vs.* CO and HQ *vs.* LQ dietary treatment effects and their interactions.

The bacterial Shannon index showed that the CO group had the lowest (P<0.05) bacteria diversity[4] compared to the FO group in P01. The FO group in P01 also had a lower (P<0.05) diversity compared to the communities in all other groups in P02 and P03, but no difference (P>0.05) in bacterial diversity was observed between all treatment groups in P02 and P03. (**Figure 4.1c**). The archaea Shannon index showed that calves from the CO and FO groups in P01 and calves in FOLQ from P02 had less (P<0.05) diverse communities than all the other groups of calves in P02 and P03 (**Figure 4.1d**).

Figure 4.1 Effects of dietary treatments in the alpha diversity indexes of the microbial communities in the rumen of calves during the three measurement periods. The alpha diversity of dietary treatment (FO and CO) during period one (P01) and the combination of dietary treatments from P01 and P02 HQ and LQ), resulting in FOHQ, FOLQ, COHQ and COLQ, evaluated during periods two (P02) and three (P03) are shown for: **a)** bacteria Chao1 index; **b)** archaea chao1 index; **c)** bacteria Shannon index; and **d)** archaea Shannon index. Boxplots represent the 25th and 75th percentiles, the whiskers extend to the most extreme data points, lines within boxes are the medians, and dots represent outliers.



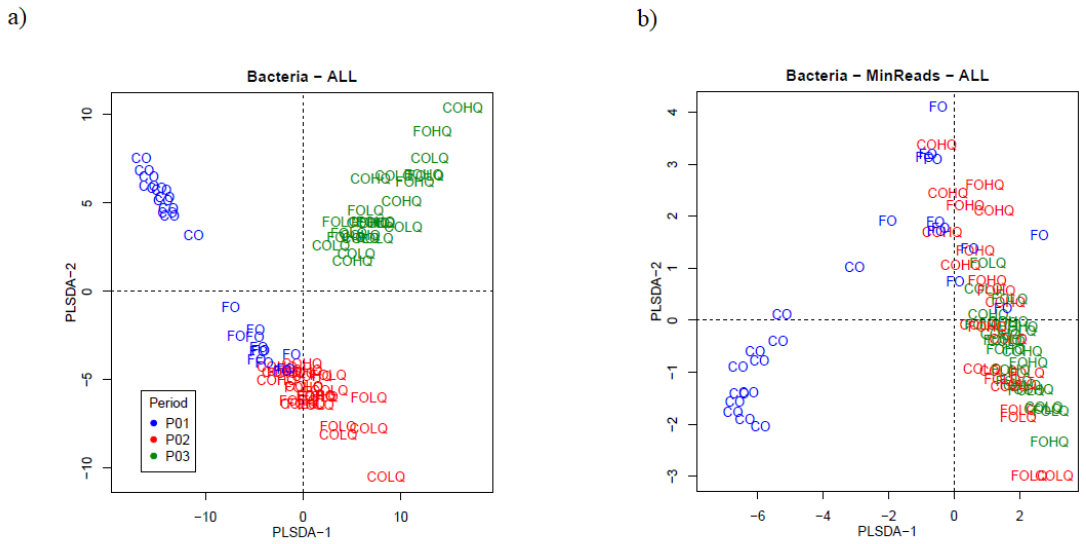
4.4.3 Bacterial beta diversity

The discriminant analysis of the 364 bacteria or main community (Bacteria - ALL) and 25 abundant (Bacteria - MinReads) bacteria communities at the genus level between periods

[4] Species diversity takes into account both species richness and species evenness (Delang and Li, 2013).

is shown in **Figure 4.2**. The PLSDA in the first dimension (PLSDA-1) showed that the CO group in P01 clustered separately from the other groups in P01-P03 (**Figure 4.2a** and **4.2b**). However, the second dimension of the PLSDA (PLSDA-2) showed that the Bacteria - ALL clustered the CO group in P01 and all the groups in P03 separately from the FO group in P01 and all the groups in P02 (**Figure 4.2a**), while no cluster separation of the PLSDA-2 was observed for the Bacteria - MinReads (**Figure 4.2b**).

Figure 4.2 Partial least square discriminant analysis (PLSDA) of the bacteria community at the genus level from calves fed different treatments and treatment-combinations in three sampling periods. Dietary treatments corresponded to: Period 1 (P01), concentrate (CO) *vs.* pasture (FO); and Period 2 (P02) high quality (HQ) *vs.* low quality (LQ) pastures. The treatment groups analyzed by period were: period one (P01) corresponded to groups from FO and CO; period two (P02) and three (P03) were the groups formed by the combination of dietary treatments from P01 and P02, resulting in FOHQ, FOLQ, COHQ and COLQ. **a)** PLSDA of the main bacteria community (Bacteria - ALL) – 364 bacterial genera; and **b)** PLSDA of the abundant bacteria (Bacteria - MinReads) – 25 abundant genera.



4.4.4 Phylum structure of the bacteria community

The bacterial taxa comprised 24 phyla (**Table D.1, Appendix D**), of which seven had a relative abundance of > 0.5% and corresponded to 98.2±0.97% of the bacteria sequences across samples (**Figure 4.3**). The analysis of the highly abundant phyla is shown in **Table 4.3** and include: *Bacteriodetes*, *Firmicutes*, *Tenericutes*, *Proteobacteria*, *Actinobacteria*, *Fibrobacteres* and *Cyanobacteria*. In P01, the FO group had 40.0- and 3.1-times greater (P<0.01) proportions of *Fibrobacteres* and *Tenericutes*, respectively, than the CO group. In P02, calves in the HQ group had 1.2 times greater (P=0.01) proportions of

Firmicutes, while the abundance of *Fibrobacteres* and *Cyanobacteria* were 6.2 and 3.7 times lower (P<0.01), respectively, than in calves from the LQ group. No effects (P>0.05) from dietary treatments or their interactions were observed in P02 for the most abundant bacteria phyla. In P03, no effect (P>0.05) from diets treatments or their interactions (P>0.05) were observed for the bacteria phylum.

Figure 4.3 Taxonomic composition of the main bacteria phyla in calves for the dietary treatments during the three sampling periods. Dietary treatments corresponded to: Period 1 (P01), concentrate (CO) *vs.* forage (FO); and Period 2 (P02) high quality (HQ) *vs.* low quality (LQ) forages. The treatment groups analyzed by period were: period one (P01) corresponded to groups from FO and CO; period two (P02) and three (P03) were the groups formed by the combination of dietary treatments from P01 and P02, resulting in FOHQ, FOLQ, COHQ and COLQ. Columns in the charts correspond to the relative abundance (%) of the most abundant bacterial phyla within each treatment and their corresponding periods.

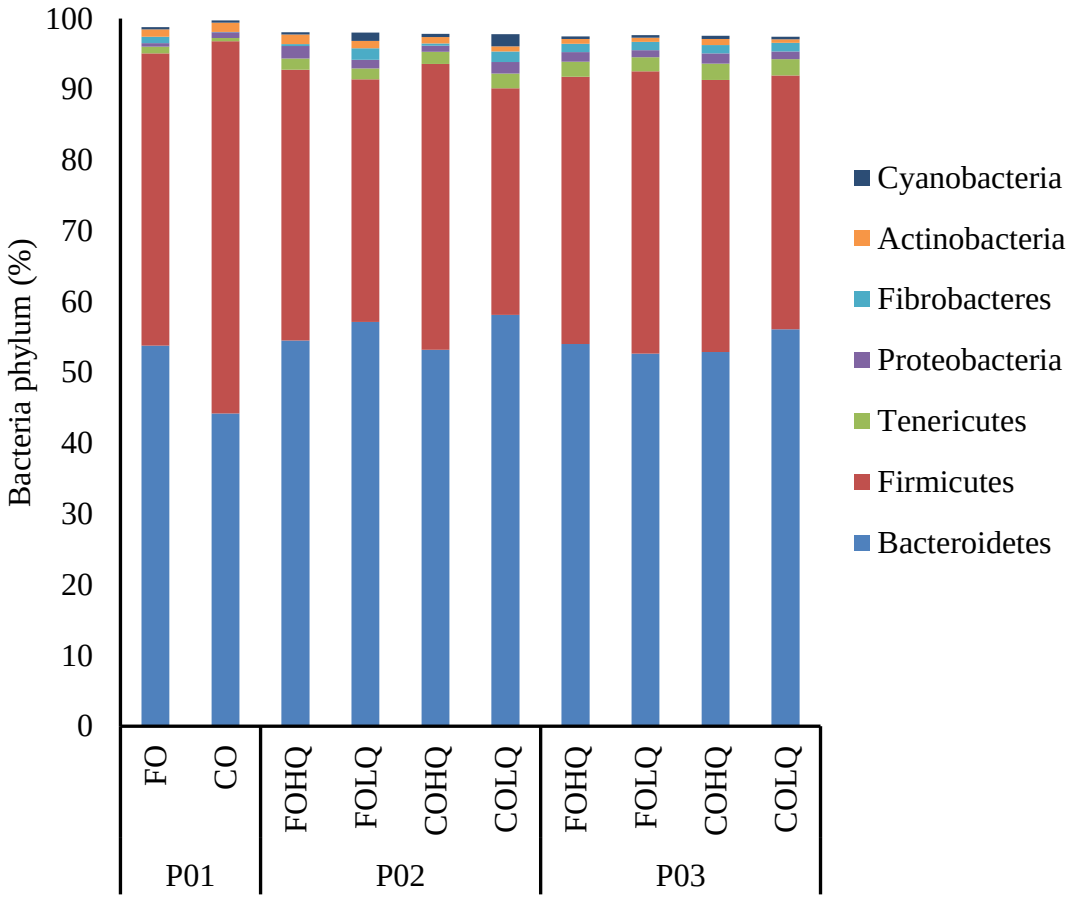


Table 4.3 Effect of dietary treatments[1] on the highly abundant bacteria phylum[2] during the three measurement periods. Results are natural log back transformed means and standard error of the differences (SED), P-value for treatment effect for FO *vs.* CO (P-1), treatment effect for HQ *vs.* LQ (P-2) and their interactions (P-int)[3].

	P01				P02								P03							
	FO	CO	SED	P-1	FO	CO	HQ	LQ	SED	P-1	P-2	P-Int	FO	CO	HQ	LQ	SED	P-1	P-2	P-Int
Actinobacteria	0.67	1.01	0.285	0.226	1.03	0.73	1.04	0.73	0.178	0.102	0.094	0.82	0.62	0.60	0.71	0.52	0.109	0.858	0.082	0.338
Bacteroidetes	52.93	40.45	3.877	0.074	55.46	55.23	53.59	57.15	2.804	0.935	0.218	0.627	53.10	54.27	53.19	54.17	2.120	0.586	0.646	0.289
Cyanobacteria	0.31	0.13	0.308	0.064	0.57	0.76	0.34	1.27	0.158	0.188	<.001	0.576	0.32	0.36	0.35	0.33	0.073	0.571	0.727	0.571
Fibrobacteres	0.40	0.01	0.496	<.001	0.53	0.57	0.22	1.38	0.188	0.798	<.001	0.512	1.10	1.01	1.04	1.07	0.233	0.712	0.873	0.686
Firmicutes	40.45	48.73	2.927	0.199	35.80	35.67	39.02	32.73	2.313	0.955	0.013	0.437	38.57	36.88	37.80	37.63	1.939	0.394	0.932	0.243
Proteobacteria	0.43	0.49	0.239	0.676	1.24	1.09	1.02	1.33	0.252	0.572	0.226	0.072	1.12	1.19	1.31	1.02	0.158	0.631	0.072	0.914
Tenericutes	0.89	0.29	0.348	0.005	1.49	1.79	1.56	1.72	0.233	0.212	0.508	0.728	2.02	2.27	2.19	2.10	0.204	0.230	0.663	0.882

[1] Dietary treatments corresponded to: Period 1 (P01) concentrate (CO) *vs.* pasture (FO) diets, and Period 2 (P02) high quality (HQ) *vs.* low quality (LQ) pastures, with measurements in P01 (9 wks), P02 (19 wks) and Period 3 (P03; 41 wks) when all calves were offered a common pasture diet.

[2] Measured effect corresponded to the 7 most abundant ruminal bacterial phyla.

[3] Dietary treatments in each period were evaluated as follow: a one-way ANOVA in P01 to analyze FO *vs.* CO diets, and a 2 x 2 factorial ANOVA in P02 and P03 to evaluate FO *vs.* CO and HQ *vs.* LQ dietary treatment effects and their interactions.

4.4.5 Bacterial composition – highly abundant genera

The bacteria taxa comprised 364 genera (**Table D.2, Appendix D**), from which 25 genera had a relative abundance of > 0.50%, corresponding to 78.9 ± 5.21% of the bacteria sequences across all samples. Dietary treatment effects on the abundant bacteria community across the three measurement periods is shown in **Table 4.4**. In P01, the FO group had greater proportions ($P < 0.05$) cellulolytic and proteolytic bacteria genera from the phyla *Bacteroidetes*, *Fibrobacteres*, *Firmicutes* and *Tenericutes*, while the CO group had greater abundance ($P < 0.05$) of amylolytic bacteria genera from the phyla *Bacteroidetes* and *Firmicutes*. In P02, calves in the HQ group had greater ($P < 0.05$) proportions hemicellulolytic and soluble carbohydrate degrader bacterial genera belonging to *Firmicutes*, while the LQ group had greater ($P < 0.05$) relative abundance of cellulolytic and hemicellulolytic degrader bacteria genera from the phyla *Bacteroidetes*, *Fibrobacter* and *Firmicutes*. In P02, no effects from dietary treatments or their interactions were observed in the abundant bacteria community. In P03, the high abundant bacteria community was similar ($P > 0.05$) among groups of calves with no dietary treatment nor interactions.

Table 4.4 Effect of dietary treatments[1] on the highly abundant bacteria genus[2] during the three measurement periods. Results[3] are natural log back transformed means and standard error of the differences (SED), P-value for treatment effect for FO *vs*. CO (P-1), treatment effect for HQ *vs*. LQ (P-2) and their interactions (P-int).

	P01				P02								P03							
BACTERIA TAXA	**FO**	**CO**	**SED**	**P-1**	**FO**	**CO**	**HQ**	**LQ**	**SED**	**P-1**	**P-2**	**P-Int**	**FO**	**CO**	**HQ**	**LQ**	**SED**	**P-1**	**P-2**	**P-Int**
Bacteroidetes																				
Bacteroidales																				
Bacteroidales BS11 gut group [4]	1.51	0.02	0.290	<.0001	3.28	2.59	1.72	4.95	0.712	0.309	<.001	0.692	3.77	3.66	3.71	3.71	0.428	0.795	0.993	0.198
Bacteroidales RF16 group [4]	0.86	0.00	0.117	<.001	0.90	0.88	0.63	1.27	0.332	0.95	0.063	0.981	0.73	0.89	0.82	0.79	0.176	0.385	0.894	0.026
Bacteroidales S24-7 group [4]	1.48	0.25	0.267	<.001	1.33	1.07	0.87	1.64	0.338	0.429	0.030	0.651	3.40	3.70	3.58	3.51	0.478	0.538	0.888	0.173
Prevotellaceae																				
Prevotella 1	39.31	0.78	12.873	<.001	35.13	35.27	37.82	32.76	3.87	0.972	0.205	0.620	32.74	33.59	32.68	33.65	2.718	0.758	0.725	0.839
Prevotella 7	0.35	18.38	6.386	<.001	0.06	0.06	0.11	0.03	0.023	0.706	0.001	0.990	0.08	0.06	0.08	0.06	0.020	0.383	0.526	0.823
Prevotellaceae UCG-001 [4]	0.86	0.87	0.410	0.985	2.16	2.85	2.92	2.11	0.610	0.262	0.192	0.224	2.56	2.60	2.45	2.71	0.333	0.907	0.447	0.154
Prevotellaceae UCG-003 [4]	1.17	0.00	0.177	<.001	2.60	2.85	1.46	3.25	0.505	0.116	0.002	0.305	2.42	2.64	2.63	2.43	0.358	0.539	0.599	0.598
Rikenellaceae																				
Rikenellaceae RC9 gut group [4]	1.66	0.23	0.220	<.001	4.62	4.27	3.62	5.46	0.671	0.610	0.012	0.254	3.69	3.71	3.64	3.76	0.324	0.937	0.706	0.754
Fibrobacteres																				
Fibrobacteraceae																				
Fibrobacter	0.40	0.01	0.147	<.001	0.53	0.57	0.22	1.38	0.188	0.798	<.001	0.512	1.10	1.01	1.04	1.07	0.233	0.712	0.873	0.686
Firmicutes																				
Christensenellaceae																				
Christensenellaceae R-7 group [4]	2.94	0.09	0.493	<.001	7.67	6.55	5.74	8.75	0.759	0.152	0.001	0.090	9.17	7.26	8.74	7.62	0.742	0.018	0.147	0.404
Lachnospiraceae																				
Butyrivibrio 2	0.41	0.01	0.055	<.001	0.93	0.92	1.41	0.61	0.284	0.955	0.005	0.636	0.62	0.61	0.67	0.56	0.112	0.960	0.370	0.645
Lachnospiraceae NK3A20 group [4]	1.96	5.95	1.716	0.015	2.17	1.86	2.26	1.79	0.342	0.361	0.180	0.341	0.86	0.69	0.73	0.81	0.100	0.109	0.472	0.607

Table 4.4 (Continued)

	P01				P02								P03							
BACTERIA TAXA	**FO**	**CO**	**SED**	**P-1**	**FO**	**CO**	**HQ**	**LQ**	**SED**	**P-1**	**P-2**	**P-Int**	**FO**	**CO**	**HQ**	**LQ**	**SED**	**P-1**	**P-2**	**P-Int**
Pseudobutyrivibrio	0.73	0.19	0.156	<.001	1.05	1.13	1.61	0.74	0.247	0.734	0.001	0.454	0.92	1.02	1.06	0.89	0.150	0.450	0.247	0.034
Roseburia	0.67	6.59	1.385	<.001	0.27	0.72	0.72	0.13	0.100	0.431	<.001	0.561	0.27	0.34	0.29	0.32	0.040	0.078	0.385	0.059
Ruminococcaceae																				
[Eubacterium] coprostanoligenes group [4]	0.94	0.30	0.132	<.001	1.16	1.07	1.00	1.24	0.137	0.511	0.093	0.036	1.23	1.23	1.15	1.32	0.165	0.972	0.31	0.386
Ruminiclostridium 9	2.71	0.00	0.525	<.001	0.62	0.59	1.34	0.27	0.342	0.881	<.001	0.672	0.13	0.12	0.13	0.11	0.047	0.840	0.608	0.133
Ruminococcaceae NK4A214 group [4]	1.89	0.07	0.306	<.001	2.09	1.93	2.41	1.67	0.292	0.577	0.019	0.946	2.98	2.87	3.07	2.79	0.274	0.705	0.325	0.81
Ruminococcaceae UCG-014 [4]	0.97	1.27	0.350	0.393	1.15	1.35	1.21	1.28	0.212	0.364	0.715	0.925	0.61	0.67	0.62	0.65	0.100	0.530	0.738	0.901
Ruminococcus 1	2.10	0.55	0.541	0.003	0.99	1.14	0.90	1.25	0.136	0.29	0.020	0.448	1.75	1.97	1.64	2.11	0.330	0.515	0.166	0.053
Saccharofermentans	0.74	0.00	0.155	<.001	0.68	0.78	0.59	0.90	0.104	0.309	0.007	0.136	1.00	1.02	0.97	1.05	0.094	0.851	0.419	0.117
Erysipelotrichaceae																				
Erysipelotrichaceae UCG-002 [4]	0.09	3.78	2.953	0.002	0.01	0.01	0.02	0.01	0.006	0.565	0.006	0.377	0.00	0.00	0.01	0.00	0.001	0.061	0.794	0.336
Kandleria	1.82	0.00	0.560	<.001	0.04	0.09	0.42	0.01	0.079	0.127	<.001	0.079	0.10	0.12	0.11	0.11	0.084	0.757	0.986	0.122
Acidaminococcaceae																				
Succiniclasticum	0.95	2.21	0.331	0.001	1.54	1.27	1.09	1.79	0.272	0.331	0.017	0.165	2.18	2.66	2.20	2.64	0.351	0.181	0.219	0.053
Veillonellaceae																				
Selenomonas 1	0.89	0.01	0.158	<.001	0.01	0.00	0.01	0.00	0.001	0.269	<.001	0.228	0.71	0.85	0.71	0.84	0.241	0.543	0.590	0.133
Tenericutes																				
Mollicutes RF9	0.80	0.28	0.213	0.011	0.01	0.01	0.01	0.01	0.002	0.213	0.607	0.782	1.33	1.46	1.36	1.42	0.197	0.517	0.776	0.870

[1] Dietary treatments corresponded to: Period 1 (P01) concentrate (CO) *vs*. pasture (FO) diets, and Period 2 (P02) high quality (HQ) *vs*. low quality (LQ) pastures, with measurements in P01 (9 wks), P02 (19 wks) and Period 3 (P03; 41 wks) when all calves were offered a common pasture diet.

[2] Measured effect corresponded to the 25-abundant ruminal bacterial genera.

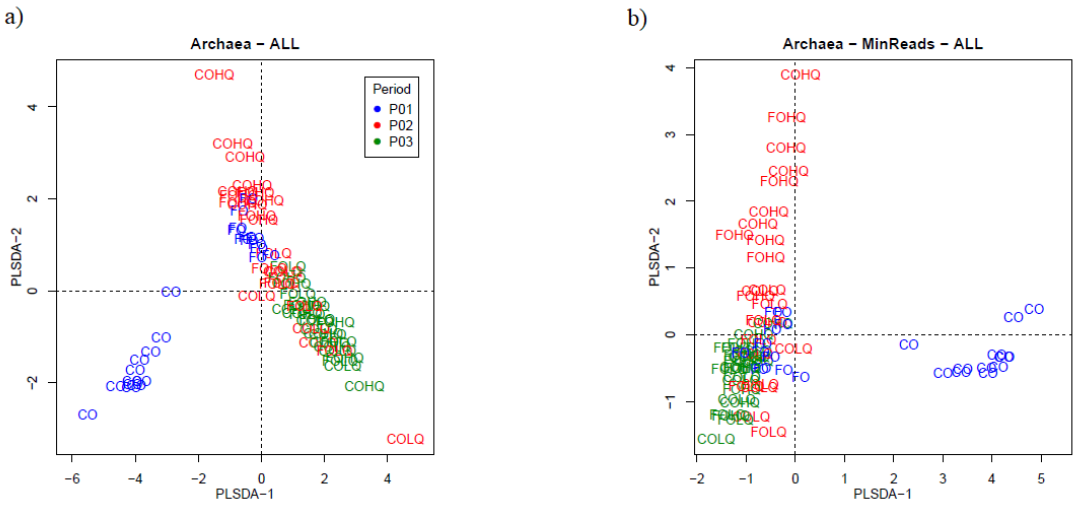
[3] Dietary treatments in each period were evaluated as follow: a one-way ANOVA in P01 to analyze FO *vs*. CO diets, and a 2x2 factorial ANOVA in P02 and P03 to evaluate FO *vs*. CO and HQ *vs*. LQ dietary treatment effects and their interactions.

[4] Indicates the group contains sequences not classified down to the genus level.

4.4.6 Archaea beta diversity

The analysis of the whole archaea species (Archaea – ALL; **Figure 4.4a**) showed a similar clustering to that observed in the abundant archaea species (MinReads – All; **Figure 4.4b**). In the first dimension (PLSDA-1), the archaea communities in the CO group in P01 clustered separately from the rest of the groups in P01-P03. In the second dimension (PLSDA-2), the HQ groups (FOHQ and COHQ) in P02 clustered separately from the other groups in P01-P03.

Figure 4.4 Partial least square discriminant analysis (PLSDA) of the archaea community at the species level from calves fed different treatments and treatment-combinations in three sampling periods. Dietary treatments corresponded to: Period 1 (P01), concentrate (CO) *vs.* pasture (FO); and Period 2 (P02) high quality (HQ) *vs*. low quality (LQ) pastures. The treatment groups analyzed by period were: period one (P01) corresponded to groups from FO and CO; period two (P02) and three (P03) were the groups formed by the combination of dietary treatments from P01 and P02, resulting in FOHQ, FOLQ, COHQ and COLQ. **a)** PLSDA of the main archaea community (Archaea - ALL) – 364 bacterial genera; and **b)** PLSDA of the abundant archaea (Archaea - MinReads) – 25 abundant genera.

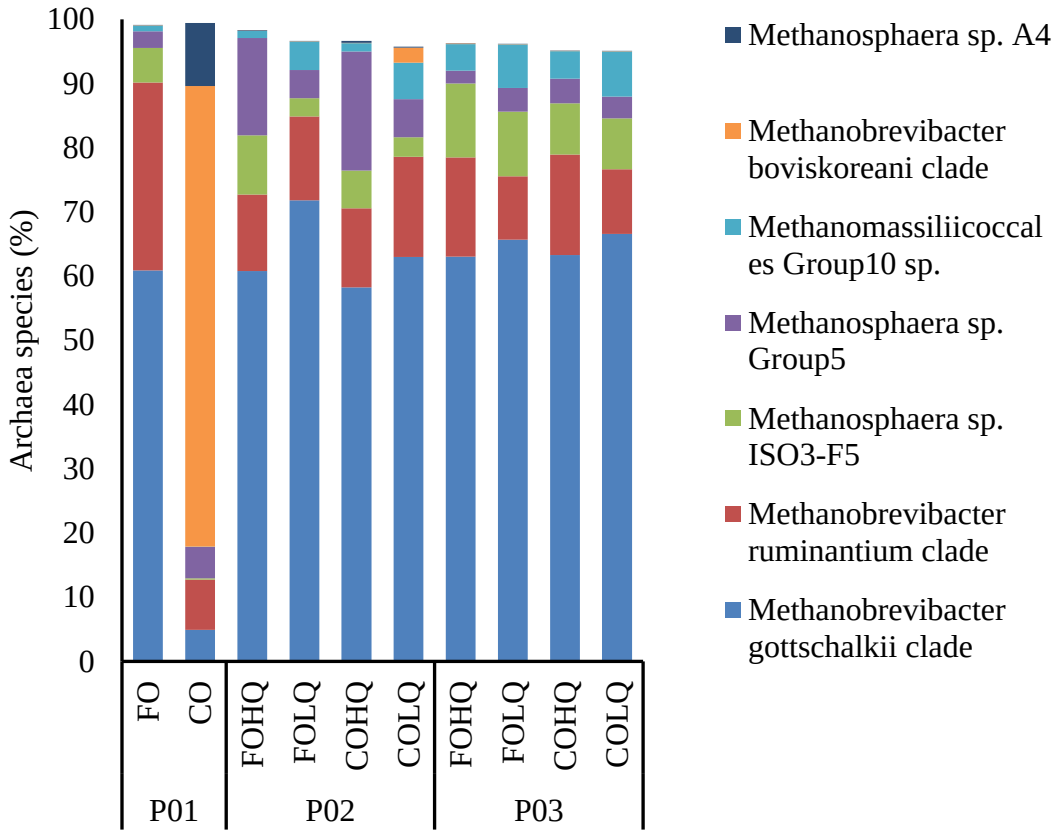


4.4.7 Archaea highly abundant species

The archaea taxa in this study included 17 species (**Table D.3, Appendix D**), of which seven had a relative abundance of > 1.0%, comprising 97.3±2.35% of the archaea sequences across samples (**Table D.4, Appendix D**). In P01, the FO group had greater abundance ($P < 0.001$) of *Methanobrevibacter gottschalkii* clade, *Methanobrevibacter ruminantium* clade, and *Methanosphaera* ISO3-F5, and lower abundance ($P < 0.01$) of the *Methanobrevibacter boviskoreani* clade, *Methanosphaera* A4 and *Methanosphaera*

Group5 compared to the CO group (**Figure 4.5**). In P02, the HQ group had higher relative proportions ($P < 0.05$) of the *Methanosphaera* Group 5, ISO3-F5 and sp. A4, and lower proportions ($P < 0.001$) of the *Methanomassiliicoccales* Group10 sp. compared to calves from the LQ group (**Figure 4.5**). In P02, no effects from dietary treatments ($P > 0.05$) or their interactions ($P > 0.05$) were observed for abundant archaea species. In P03, residual effects from dietary treatment in P02 were observed in calves from the HQ group with greater proportions ($P < 0.01$) of *Methanobrevibacter ruminantium* than from the LQ groups. Interactions were observed for *Methanobrevibacter boviskoreani clade* ($P = 0.016$) with the FOLQ showing 3.2- and 1.8-times lower proportions ($P < 0.05$) than the FOHQ and COLQ, respectively, with no differences ($P > 0.05$) for COHQ. No dietary treatment effects ($P > 0.05$) from P01 were observed in P03 for the highly abundant archaea.

Figure 4.5 Taxonomic composition of the main archaea species in calves for the dietary treatments during the three sampling periods. Dietary treatments corresponded to: Period 1 (P01), concentrate (CO) *vs.* pasture (FO); and Period 2 (P02) high quality (HQ) *vs.* low quality (LQ) pastures. The treatment groups analyzed by period were: period one (P01) corresponded to groups from FO and CO; period two (P02) and three (P03) were the groups formed by the combination of dietary treatments from P01 and P02, resulting in FOHQ, FOLQ, COHQ and COLQ. Columns in the charts correspond to the relative abundance (%) of the most abundant archaeal species within each treatment and their corresponding periods.



4.5 Discussion

Ruminal microorganisms are required for the degradation of plant cell walls (Hungate, 1966; Hobson and Stewart, 1997). The establishment of these microbes in the rumen has been shown to be a dynamic progression from birth to adulthood (Jami et al., 2013; Rey et al., 2014). Recent studies have suggested that early interventions in life might imprint the microbial community so that it persists during the animal's adult life (Yáñez-Ruiz et al., 2015). The key findings from this study are that the nature of the diet can influence DMI, rumen fermentation and rumen microbiota composition, but divergent dietary regimes from 1 to 14 and 14 to 30 wks of life were unable to elicit a permanent microbial or metabolic imprint.

4.5.1 P01: Pastures *vs.* concentrate

Dry matter intake, and rumen gas emissions and fermentation profiles

Calves from the FO group had a lower solid feed DMI compared to the CO group, which can be attributed to the high intake of milk in the FO group (Khan et al., 2011) and DMI from here on refers to the solid feed intake, since the milk DMI largely bypasses the rumen. As DMI is the main driver of CH_4 production (Jonker et al., 2016b), the lower CH_4 production in FO compared to CO calves was expected. The amount of CH_4 produced in the rumen is negatively correlated with propionate formation (Pinares-Patino et al., 2003; Goopy et al., 2006). During propionate formation, H_2 is consumed (Baldwin et al., 1963), while during acetate and butyrate formation H_2 is produced (Baldwin and Allison, 1983). Concentrate intake is associated with greater propionate production that lowers the release of ruminal H_2 for hydrogenotrophic methanogens (Janssen, 2010), and is also associated with lower rumen pH, which can decrease CH_4 yield (Van Kessel and Russell, 1996). Therefore, the observed decrease in CH_4 yield in the CO compared to the FO group in this study can be explained by these biological processes.

Microbial diversity

Compared to the FO group, the CO group had a lower richness and diversity of rumen microbiota. Our results agree with those reported by Kim et al. (2016) in calves transitioning from milk to concentrate diets that had lower bacterial richness and diversity than those transitioning fibrous diets. Similar effects have been observed in cows and goats, where the intake of fibrous diets also resulted in a more diverse microbial community than

in those fed high starch diets (Belanche et al., 2012; Mao et al., 2015; AlZahal et al., 2017). It has been reported that the richness and diversity of the microbial community is negatively correlated to ruminal pH (Mao et al., 2013; Liu et al., 2015; Kim et al., 2016). Therefore, feeding calves with concentrate diets may result in a lower microbial richness and diversity due to a potential drop in the ruminal pH compared to calves consuming pastures.

Bacterial phyla

At a phylum level, the microbiota composition observed in the present study is comparable to that reported elsewhere. In the newborn, the phyla composition in the rumen is represented by interchangeable abundance of *Bacteroidetes*[5], *Firmicutes*[6] and *Proteobacteria*[7], whose abundance changes as the animal grows and consumes solid diets (Li et al., 2012b; Jami et al., 2013; Rey et al., 2014). The proportion of *Firmicutes* in the CO calves was higher than the 10-15% observed in 6 to 9-week-old calves fed milk and concentrate (Jami et al., 2013; Rey et al., 2014), but similar to the 49-58% observed in 9 to 11-week-old calves weaned onto a high concentrate diet (Kim et al., 2016). Whilst, pasture-fed FO calves had similar *Firmicute* proportions to the 45-53% reported in 9 to 11-week-old weaned calves consuming a low concentrate diet (Kim et al., 2016). The pre-weaning proportions of *Bacteroidetes* in the FO group were similar to the 50 to 56% reported in 6 to 9-week-old pre-weaned calves with access to starter concentrates (Jami et al., 2013; Rey et al., 2014). Whilst the proportion of this phylum in CO calves was lower than that observed in FO calves and previous studies consuming concentrates, but higher than the 15-20% reported in 9 to 11-week-old calves consuming a high concentrate diet (Kim et al., 2016). These results together indicate that pre-weaned calves consuming high-quality pastures or concentrate/hay, favors the growth of *Bacteroidetes*, whilst in post-weaned calves increases in the ratio of starter concentrates in the diet favors the abundance of *Firmicutes*. In the current study, *Proteobacteria* proportions in both groups of calves were < 1.0%, while other studies in calves indicated relative abundances of 4.0-35.0% in calves

[5] This phylum is phenotypically a diverse group of Gram-stain-negative rods that do not form endospores. These bacteria are anaerobic or aerobic. The phylum is widely distributed in the environment, as well as in the gastrointestinal tract and on the skin of animals. This phylum is saccharolytic, although proteins and other substrates may be utilized, and its growth is accelerated in pH near to 7.0 and at 37°C (Krieg et al., 2010).

[6] A phylum of bacteria, most of which have Gram-positive cell wall structure. Phenotypically diverse. They are aerobes, facultative or strict anaerobes. Most of them are chemo-organotrophs, and grow at neutral pH, while some are acidophiles or alkaliphiles (Schleifer, 2009).

[7] This phylum comprises Gram-negative bacteria. Most members are facultatively or obligately anaerobic and have a wide variety in the types of metabolism.

receiving a concentrate diet (Li et al., 2012b; Jami et al., 2013; Rey et al., 2014). In these studies, ruminal pH was not measured so it was not possible to infer if changes in the abundance of *Proteobacteria* abundance was related to ruminal acidosis. However, in a recent study in calves, the abundance of *Proteobacteria* was between 5.5 and 9.5% when the ruminal pH was maintained at < 5.8, whilst the inclusion of hay into the starter concentrate mitigated ruminal acidosis and decreased *Proteobacteria* from 7.5 to 4.0% (Kim et al., 2016). The proportions of *Proteobacteria* have been inversely correlated with the acetate:propionate ratio (a proxy for low ruminal pH) (Petri et al., 2013b). The similarities in *Proteobacteria* proportions in the FO and CO group can be attributed to the fact that both groups had access to pastures, which may have buffer the ruminal pH in both groups and avoided ruminal acidosis (Auffret et al., 2017; Garcia et al., 2017). Pasture in the diet increased the relative abundance of *Fibrobacteres* in the FO group compared to CO calves, with similar proportions (0.3 ± 0.3%) to those observed in calves consuming concentrate with 10% of hay as solid feed (Rey et al., 2014). This is expected since *Fibrobacter* is one of the most active and specialized fiber-degrading organism in the rumen (Suen et al., 2011; Ransom-Jones et al., 2012). The abundance of bacteria phyla such as *Bacteroidetes*, *Firmicutes* and *Proteobacteria* is affected by variations in diet composition, i.e. proportion of starch to digestible fiber that affect the ruminal fermentation and pH. Therefore, further studies are needed to determine the effect that different starch:fiber ratios have on the bacteria phyla composition and its association with ruminal pH in neonatal ruminants.

Bacterial genera

The genus *Prevotella* is one of the most abundant ruminal microorganisms (Henderson et al., 2015). This genus plays a key role in the degradation and utilization of a large variety of carbohydrates (Cotta, 1992; Solden et al., 2016), and in ruminal protein degradation (Purushe et al., 2010). In the present study, calves in the FO had greater proportions of *Prevotella* 1 than the CO group, which includes the species *P. ruminicola*, *P. brevis*, and *P. bryantii* (Henderson et al., 2019), which produce mainly acetate and succinate (Avguštin et al., 1997), and very little propionate (Seshadri et al., 2018). In contrast, *Prevotella* 7, includes species such as *P. albensis* (Henderson et al., in press) that mostly ferment glucose, xylose and salicin to acetate and succinate (Avguštin et al., 1997), and propionate (Seshadri et al., 2018), were more abundant in the CO than the FO group.

The observed fermentation profiles for the CO and FO group are in agreement with the aforementioned end product formation described for *Prevotella groups* 1 and 7.

Consumption of the FO diet favored the growth of unclassified genera from *Bacteroidales* (S24-7 and BS11), *Prevotellaceae* (UCG-003), *Rikenellaceae* (RC9), *Christensenellaceae* (R-7) and *Ruminococcaceae* (NK4A214, *Ruminococcus* 1 and *Saccharofermentans*). These genera are associated with hemicellulose and cellulose rich diets (Lima et al., 2015; Mao et al., 2015; Ravachol et al., 2016; Seshadri et al., 2018). The proportions of these genera were lower in CO calves, which agreed with observations done in heifers transitioned from a pasture to a concentrate diet (Petri et al., 2013b). *Fibrobacter*, which is one of the main cellulose degraders in the rumen (Ransom-Jones et al., 2012), showed greater proportions in the rumen of the FO than CO group, corresponding to the greater intakes of cellulose in the diet of these calves. Interestingly, *Kandleria*, which is also a D-galactose utilizer (Kumar et al., 2018), was only found in calves from the FO group. This genus has been previously isolated from the rumen of young calves fed on only milk (Salvetti et al., 2011). Therefore, the appearance of *Kandleria* in the FO group may be associated with milk leaking into the rumen in this group.

The consumption of concentrates in the CO group increased the abundance of bacteria from the genera *Roseburia*, *Lachnospiraceae NK3A20 group* and *Erysipelotrichaceae* UCG-002 compared to the FO group. These genera have a high affinity for utilizing highly degradable mono- and polysaccharides (Stanton et al., 2009; Huo et al., 2014). Increases of these genera have been observed in the faces of cows during subacute rumen acidosis (Mao et al., 2012). *Roseburia* and *Lachnospiraceae NK3A20 group* are butyrate producing microorganisms (Duncan et al., 2002). However, despite the fact that the principle fermentation product of these organisms is butyrate, no effect on the proportion of this SCFA between the two groups was observed. This might be due to the fact that they made up only 6.6 and 6.0% of the community. The family *Erysipelotrichaceae* ferment a wide range of sugars to produce mainly lactic acid (Deusch et al., 2017). Studies in sheep with abundant members of the *Erysipelotrichaceae* family have been associated with low-methane emitting animals with an increased lactic acid production in which less hydrogen and thus less methane is formed (Kittelmann et al., 2014; Kamke et al., 2016). In the present study, the abundance of *Erysipelotrichaceae* UCG-002 in the CO group might have favored the production of propionate by lactate utilizing

bacteria (Kamke et al., 2016). *Succiniclasticum* is a succinate fermenter that produces propionate (Deusch et al., 2017), and was found in high abundances in the CO compared to the FO group. Increases of this genus has been negatively correlated with the abundance of methanogenic archaea and a decrease in CH_4 emissions (Wallace et al., 2015). This was attributed to the H_2 utilization for propionate formation, which competes with the most common hydrogenotrophic methanogens (Liu and Whitman, 2008; McCabe et al., 2015).

Archaeal species

Diet can be expected to have an effect on methanogens due to changes in pH, which can affect methanogen activity (Lana et al., 1998). Under normal physiological conditions, the rumen has a pH that ranges between 5.6-6.7 (Kolver and de Veth, 2002); with rumen methanogens being inhibited at lower pH. Calves from the CO group, receiving concentrates in their diets, showed an archaeal community dominated by *Mbb. boviskoreani* clade. This organism was isolated from and found in cattle fed high concentrate diets (Lee et al., 2013). This suggests that *Mbb. boviskoreani* could be more tolerant of lower pH as compared to the other *Mbb*. spp. which are known to be sensitive to low pH (Janssen, 2010). In the FO group, *Mbb ruminantium* and *gottschalkii* both dominated the fiber fed rumen contents as described in Seedorf et al. (2015) and Henderson et al. (2015). *Methanosphaera* spp. is a methanogen that generates methane by reducing methanol with H_2 and is dependent on acetate as a carbon source (Fricke et al., 2006). In the present study, the proportions of *Mph.* A4 and Group 5 were abundant in CO calves consuming concentrates. Previous reports of *Mph.* spp. indicated that these methanogens are negatively correlated with bacteria from the phyla *Bacteroidetes* and the family *Ruminococcaceae* (Dias et al., 2017). However, no such correlations were observed in the present experiment.

4.5.2 P02: high quality *vs.* low quality pastures

Dry matter intake, rumen gas emissions and fermentation profiles

In the LQ group, the DMI and the CH4 production (g/d) was lower compared to the HQ group. However, CH_4 yield (g/kg of DM) was not affected by pasture quality, results which agree with similar observations in adult cattle that were fed fresh cut pasture of different quality and produced similar CH_4 yields (Jonker et al., 2016b). The intake of highly digestible feeds increases the total concentration of SCFA and the propionate to acetate ratio (Owens and Basalan, 2016). In our study, increases in the total SCFA

concentrations and propionate:acetate ratio was observed in the HQ group consuming grasses with higher organic matter digestibility compared to the more fibrous material consumed by the LQ group. Calves in the LQ group also had a lower proportion of isobutyrate and isovalerate, which reflects the lower crude protein content in the LQ pasture, since these acids are breakdown products of amino acids (Brinkhaus et al., 2017). However, the low concentrations of these acids could also be a result of a higher uptake by cellulolytic bacteria in the low-quality pasture diet (Hoover, 1986).

Bacterial phylum

The composition of the rumen microbial community is shaped by the composition of the diet (Henderson et al., 2015). During the P02, HQ pasture increased the proportions of *Firmicutes*. Increases of the phylum *Firmicutes* have been observed in diets that are rich in non-structural carbohydrates (Deusch et al., 2017). High proportions of plant cell walls are associated with increases in *Fibrobacteres* (Abdul Rahman et al., 2016), which was observed in the FO group consuming a fiber rich diet.

Bacterial genera

In the rumen, *Ruminococcus* and *Fibrobacter* are major cell wall degraders (Koike and Kobayashi, 2001; Ransom-Jones et al., 2012; Abdul Rahman et al., 2016) and abundant when diets rich in plant cell walls are fed (Pitta et al., 2014a). Calves fed mature or low-quality pastures had an increased abundance of *Fibrobacter* and *Ruminococcus* relative to calves consuming high-quality pastures. Calves in the LQ group had greater proportions of the genera unclassified *Rikenellaceae* RC9, *Bacteroidales* and *Prevotellaceae* UCG-003, which belong to the phyla *Bacteroidetes*, and *Christensenellaceae* R-7 which belongs to the phyla *Firmicutes*. The genus *Rikenellaceae* RC9 has been identified as one of the dominant microbes in the rumen microbiota (Henderson et al., 2015; Li et al., 2017; De Mulder et al., 2018) and highly abundant in ruminants consuming fibrous diets (Petri et al., 2013b; Schären et al., 2017). Members of the *Bacteroidales* (BS11 gut group and S24-7) degrade hemicellulose and monomeric sugars (xylose, fucose, mannose and rhamnose), and produce acetate, butyrate, propionate and succinate (Ormerod et al., 2016; Solden et al., 2017). In the rumen of moose, it was observed that seasonal changes in diet, i.e. increases in structural carbohydrates and decreases in protein from spring to winter, was associated with a 800 fold increase in the abundance of *Bacteroidales* BS11 (Solden et al., 2017). The *Bacteroidales* S24-7 has been also found in high proportions in pre-partum

cows fed diets with high structural carbohydrates contents (Lima et al., 2015). Similarly, *Christensenellaceae* R-7 has been reported to be highly abundant (3.3% ± 0.16%) when dairy cows were fed a high fiber diet (Lima et al., 2015). *Christensenellaceae* isolated from human feces produces acetate and small amounts of butyrate as fermentation end products (Morotomi et al., 2012). In the current study, the high abundance of *Rikenellaceae* RC9, *Bacteroidales* BS11, *Bacteroidales* S24-7 and *Christensenellaceae* R-7 in the LQ group is consistent with previous reports.

Compared to the LQ the bacterial community, the HQ group had increased proportions of *Ruminococcaceae* NK4A214, *Butyrivibrio* 2, *Ruminiclostridium* 9 and *Pseudobutyrivibrio*. The increased DMI and ingestion of less structural carbohydrates, which are the preferred substrate of these genera (Rainey, 1996; Ravachol et al., 2016), may have favored the growth of these microbes in calves from the HQ group as compared to the LQ group. In the HQ group, the pastures with greater proportions of soluble carbohydrates increased the relative abundance of a saccharolitic genus like *Roseburia*, which produces butyrate (Stanton et al., 2009). The HQ diets also increased *Selenomonas*, which degrades a broad range of substrates (Seshadri et al., 2018). Studies of co-cultivation of *Selenomonas* and *Butyrivibrio* have resulted in improved growth of *Selenomonas* (Cotta, 1992; Cotta and Zeltwanger, 1995. Selenomonas and Butyrivibrio have both been reported in relatively high abundances in corn silage- and hay-diets {Deusch, 2017 #205). In the current study, calves in the HQ group ingesting pastures with less cellulose favored the growth of Roseburia, *Selenomonas* and *Butyrivibrio*.

Archaeal species

The archaeal community structure analyzed in this study is in accordance with observations from Seedorf et al. (2015), who found that the dominate archaea microbiota in rumen samples were *Mbb. ruminantium* and *Mbb. gottschalkii*. *Mph.* spp. and *Mmc.* spp. In P02, when calves were on different pastures, the most abundant methanogen was *Mbb. gottschalkii*, which has been mainly found in grazing ruminants (Jeyanathan et al., 2011; Henderson et al., 2015; Seedorf et al., 2015). *Mbb. boviskoreani* dominated the archaea in the concentrate fed group in P01, nearly vanished in P02 when these calves were maintained on pastures. *Methanosphaera* spp. a methanol utilizer methanogen (Fricke et al., 2006) were increased in the HQ group of calves consuming a fresh sward of ryegrass and white clover which may have produced more methanol than LQ pastures. Methanol is

derived from the hydrolysis of pectin and other methylated plant polysaccharides (Dehority, 1969). Methanol production and pectin degradation are influenced by pasture maturity, (Dehority et al., 1962). Clover and other non-grass pasture species usually contain higher proportion of pectins than grasses. *Methanomassiliicoccales* spp. are methylotrophic methanogens and utilize compounds like methylamines, dimethylamine, and trimethylamine. Plant-derived glycine, betaine and choline are rapidly metabolized by ruminal bacteria to trimethylamines (Neill et al., 1978; Mitchell et al., 1979). In our study, the fiber content in the diets and ratio of acetate to propionate was higher in the LQ group, which might indicate greater production of methylamines. Deusch et al. (2017) indicated that fiber-rich diets that had a high ratio of acetate to propionate produced higher concentrations of methylamine, dimethylamine and trimethylamine than corn silage-based diets. Therefore, we assume that the LQ group consuming a fiber-rich diet might potentially have a higher activity of methylotrophic methanogenesis than the HQ group. Our results indicate that the apparent methanogen structure community, specifically the low abundant archaea, are affected by changes in the chemical composition of the pasture consumed.

4.5.3 P3: similar pasture quality

Dry matter intake, rumen gas emissions and fermentation profiles

In P03, the DMI, CH_4 emissions and SCFA profiles did not differ between groups of calves grazed in the same grass swards. The observed similarities in rumen function (gas emissions and fermentation profiles) between groups of calves in our study agree with observations in lambs by Yáñez-Ruiz et al. (2010), where variations in SCFA profiles and CH_4 emissions were no longer apparent 16-20 wks after dietary treatments ceased and lambs were fed similar diets. These results indicate that the diet composition drives the rumen fermentation and methane production independently of the previous feeding regiment.

Bacterial composition

When calves were fed the same diet, no difference in the microbiota richness and diversity, and abundance of the major phyla *Bacteroidetes*, *Firmicutes*, *Tenericutes* and *Fibrobacteres* were observed. The proportion of the major bacteria phyla in our study were similar to those reported in 12 month old bulls fed a hay-based diet (Li et al., 2012b). The main bacteria genera were similar to that reported in grass fed animals by Henderson et al. (2015) in the global rumen census. Consistently, the most abundant genera in our study

during P03 was *Prevotella* 1, similar to the communities in pasture fed calves in P01 and P02. *Prevotella* has been identified as one of the most abundant genus in the rumen of growing (consuming solid diets) and adult ruminants (Li et al., 2012b; Rey et al., 2014; Henderson et al., 2015), due to its important role in the utilization of polysaccharides of plant origin, including xylans, pectins, and starch, and in the metabolism of peptides and proteins (Avguštin et al., 1997). *Christensenellaceae* R-7 was the second most abundant bacteria genus in the rumen during P03. Considering the high abundance that *Christensenellaceae* had during this period, it is likely that these bacteria play an important role in rumen dynamics of grazing.

Archaeal composition

Rumen archaea are much less diverse than rumen bacteria, which likely reflects the narrow range of substrates they use (Janssen, 2010; Seedorf et al., 2015). In P3 the proportions of *Mbb. gottschalkii* and *Mbb. ruminantium* found in the groups of calves agreed with those reported in global rumen census (Henderson et al., 2016) and in pasture-fed ruminants (Seedorf et al., 2015). On the other hand, hydrogen dependent methylotrophs, i.e. *Methanosphaera* spp. and *Methanomasilliicoccales* spp., showed a low relative abundance.

4.6 Conclusions

In conclusion, our results showed that the rumen microbial community in the growing calf is diet dependent with early life differences having only negligible effects on the microbiota of the growing ruminant. Different dietary regimes, pre- and post-weaning, were unable to leave a microbial imprint in the rumen of calves when the animals were fed a common diet. These findings showed that interventions after feeding colostrum to calves did not leave a permanent effect in the early microbial colonization and function in the rumen. Further studies should target earlier microbial interventions, specifically during microbial colonization of the rumen milieu, in an attempt to imprint the ruminal microbiota.

Chapter 5: Effect of methane inhibitors on microbial establishment during early life and its relationship with methane emissions, ruminal fermentation profiles and growth in calves

General overview of the chapter:

Contrasting dietary regimes pre- and post-weaning in Chapter 4 revealed that the rumen microbiota in the growing calf is diet dependent. Different dietary management was unable to permanently modify the ruminal microbiota of the growing ruminant. The present Chapter is focused on investigating whether feeding microbial inhibitors during early life may lead to a permanent change in the rumen microbiota and fermentation pathways, with associated gas emissions and growth alterations in cattle.

5.1 Abstract

In newborn ruminants, bacteria and archaea quickly colonize the gastrointestinal tract as solid feed intake increases. It is unknown whether during this time of rapid microbial and gut physiology change whether the rumen microbial community is susceptible to modifications that may be conveyed into adult life. The aim of this study was to determine whether inhibition of methanogens during early life leads to a permanent rumen change (imprint) in the rumen microbiota with potential long-term lower methane emissions. Twenty-four dairy calves (Friesian x Jersey cross) were randomly assigned to either a control or a treatment diet. The treatment consisted of a mixture of chloroform (CF) and 9,10 anthraquinone (AQ), two potent methane inhibitors, which were added to the diet for the first 14 wks of rearing. Subsequently, calves were managed as a single group on pasture. At wks 2, 4, 6, 8, 10, 14, 24 and 49, methane emissions were measured and samples for rumen metabolite and microbial community composition were collected. During rearing, dry matter intake was determined and the animals were weighed regularly over the duration of the experiment to monitor growth performance. When the inhibitor was fed to the calves during the first 14 wks methane emissions were lower compared to control calves. Methane inhibition did not affect DMI or growth. The acetate to propionate ratio decreased in treated compared to control calves during the first 14 wks but was similar at wks 24 and 41 after withdrawal of the inhibitor treatment. Methane inhibition decreased the proportion of *Methanobrevibacter* and *Methanosphaera*. Whilst some *Methanomasilliicoccales sp.* decreased during treatment, no differences in archaea proportions were observed at 24 and 49 wks. Although rumen metabolites were highly affected by methane inhibition, the bacterial community at the phylum level was similar. Even within the abundant genera (relative abundance ≥ 0.05%), differences in the community were negligible. Inhibition of methane emissions increased hydrogen emissions, altered the methanogen community and changed the rumen metabolite profile without an effect on the composition of the bacterial microbiota. This indicates that the major response of the bacterial community is not a change in composition but a change in metabolic pathways. However, when methane inhibition was stopped, methanogen composition, rumen metabolites and hydrogen emissions were similar between the two groups. This suggests that it is not possible to imprint a low methane rumen microbiota into the rumen.

5.2 Introduction

The rumen harbors a symbiotic community of microorganisms that degrade ingested plant components (Hobson and Stewart, 1997). Complex carbohydrates are hydrolyzed and fermented into short chain fatty acids (SCFA), mostly absorbed across the rumen wall and utilized as energy sources for the host ruminant (Van Soest, 1994). A by-product of acid formation in the rumen is hydrogen that is converted to methane, a potent greenhouse gas (Ellis et al., 2007). For decades, manipulations of the rumen microbiota have been attempted with the aim to improve animal performance or reduce methane emissions (Nagaraja et al., 1997; Weimer, 1998). Results from microbial manipulation, e.g. dietary interventions, microbial inhibitors, swabs of rumen contents, among others, in adult ruminants have shown no short-term post-treatment effects because of the well-established rumen microbiota (Weimer et al., 2010; Weimer, 2015). In young ruminants, it has been observed that microbial establishment progresses as solid feed intake increases and the rumen develops (Fonty et al., 1987; Jami et al., 2013; Rey et al., 2014; Dill-McFarland et al., 2017). Therefore, manipulations of the rumen microbiota during early life could be a feasible mechanism to promote changes in the community structure that will persist in later life (Yáñez-Ruiz et al., 2010; Abecia et al., 2014a; Abecia et al., 2014b).

In small ruminants, dietary interventions during early life have shown to alter the microbial composition and influence CH_4 emissions and SCFA production during treatment and for up to 3 months after treatment cessation (Yáñez-Ruiz et al., 2010; Abecia et al., 2013; Abecia et al., 2014b). Restriction of the acquisition or inhibition of ruminal microorganisms, i.e. protozoa and archaea, during early life has also resulted in different rumen microbial composition and fermentation end products (Morgavi et al., 2015; Wang et al., 2017b). However, these studies do not report on whether these differences were sustained once the full rumen microbiota was altered. These findings suggest that alterations in the early establishment of rumen microbiota may influence the microbial succession process and the host phenotype. Additionally, little evidence exists of the impact of microbial manipulation during early life on microbial establishment, rumen function and performance in calves.

In a normal functioning rumen, H_2 released during rumen microbial fermentation is mainly metabolized by methanogens, which use electrons generated to reduce CO_2 to CH_4 (Ungerfeld and Kohn, 2006; Janssen, 2010). Methanogenic archaea make up only 3-4% of

the rumen microbial population (Yanagita et al., 2000; Ziemer et al., 2000), playing an important role in H_2 removal. This is key to the re-oxidation of nicotinamide adenine dinucleotide reduced (NADH) and favors the microbial fermentation of feed in the rumen (Wolin et al., 1997; Joblin, 1999). The use of methanogen inhibitors in adult ruminants has been shown to increase H_2 concentrations in the rumen (Bauchop, 1967; Kung et al., 2003), change the feed fermentation patterns towards production of more propionate and butyrate (Knight et al., 2011; Martinez-Fernandez et al., 2016) and affect animal performance by reducing feed intake and live weight (Kung et al., 2003; Hristov et al., 2015; Veneman et al., 2015). However, it is not clear if alterations of the methanogen community during the first weeks of life through methane inhibitor treatment affects subsequent succession following discontinuation of treatment.

Chloroform (CF) and 9,10-Anthraquione (AQ) are methane inhibitors that interfere with the methyl-coenzyme M of methanogens during CH_4 formation (Gunsalus and Wolfe, 1978; Garcia-Lopez et al., 1996; Graham and White, 2002; Kung et al., 2003). Studies *in vitro* and *in vivo* have shown that CF and AQ are potent methanogen inhibitors (Bauchop, 1967; Garcia-Lopez et al., 1996; Kung et al., 2003; Knight et al., 2011). The use of these methane inhibitors can have adverse effects on feed intake, digestion, and rumen fermentation when added at high concentrations (Kung et al., 2003; Martinez-Fernandez et al., 2016). However, such studies have been aimed at examining changes in methanogen populations, ruminal fermentation and CH_4/H_2 production in mature ruminants. Based on the current knowledge, there is no evidence of the effects of feeding methanogen inhibitors (CF and AQ) during early-life on rumen microbial establishment, rumen function and performance in dairy calves. The objective of the present study was to determine whether feeding such methanogen inhibitors during early life may lead to an imprint on the rumen microbial community changing fermentation pathways, with associated gas emissions and growth alterations in cattle.

5.3 Materials & Methods

Animal manipulations were reviewed and approved (AE13132) by the Grasslands Animal Ethics Committee and complied with the institutional Codes of Ethical Conduct for the Use of Animals in Research, Testing and Teaching, as prescribed in the New Zealand Animal Welfare Act of 1999 and its amendments.

5.3.1 Experimental design and animal management

Twenty-four female dairy calves (Friesian x Jersey cross), from three to four days of age, were sourced from a commercial farm. On arrival to the animal facility, calves were weighed and randomly assigned to one of the experimental groups: control and treatment. The treatment group had the methane inhibitors mixed into their starter concentrate and total mixed ration (TMR) diets. The controls had no additions. The treatment lasted for 14 wks followed by a 35-week period in which both groups were managed as one mob on pasture.

During the first 10 wks of rearing, calves were housed in individual pens (1.5 x 3 m) bedded with wood shavings. Treatment groups were allocated in two different temperature-controlled rooms to avoid cross contamination of ruminal microbes between treatments. At wk 10 after weaning from milk, calves were moved into a treatment or control group pen. By wk 14, the calves from both groups were transferred into one mob to pasture. Live weight (LW) was determined weekly during the first 12 wks of life, fortnightly until wk 24 of age, and monthly thereafter.

5.3.2 Feeding management

After feeding colostrum during the first five to six days of life, calves were fed 2L of milk replacer (MR; 125g/kg dry matter; Milligans, Oamaru, NZ) twice a day at 0800 and 1600 h. At wk 2, concentrate starter (Denver Stock Feeds, Palmerston North, NZ) was offered *ad libitum*. At wk 4, calves were gradually transitioned to once per day milk feeding, where 4L was offered only in the morning. In addition to the starter feed, the calves had free access to total mixed ration (TMR). At wk 10, calves were fully weaned from MR over a 10-day period. The calves were weaned from the starter concentrate feed from wks 12 to 14. After wk 14, calves were moved to a paddock with a mixed sward of ryegrass and clover, where the TMR was available until weaning from starter diets during wks 15 to 17. Fresh water was available *ad libitum*.

The ingredients used in the concentrate and TMR diets are given in **Table 5.1**. The chemical composition of the concentrate and TMR (**Table 5.2**) diets was determined by wet chemistry at the Nutrition Lab at Massey University (Palmerston North, NZ). Compositional analyses were carried out according to the methods of the Association of Official Analytical Chemists (AOAC, 1990, 2010, 2012). During the methane

measurement period, pasture samples were taken and scanned by infrared reflectance spectroscopy (NIRS; FeedTECH, AgResearch Ltd., Palmerston North, NZ) with calibration curves for ash, lipids, crude protein (CP), neutral detergent fiber (NDF) and WSC (Corson et al., 1999). Dry matter intake (DMI) of MR, starter concentrate diet and TMR were determined on a daily basis as long as the animals were kept in single pens.

Table 5.1 Ingredients of the starter concentrate and the total mixed ration (TMR) diet.

Starter concentrate	g/kg	TMR diet	g/kg
Maize	108	Hay	500
Barley	432	Barley	290
Peas	173	Soya	100
Soya	205	Molasses	100
Molasses	54	Di-calcium-phosphate	5.5
Sodium bicarbonate	20	Salt	3.0
Salt	5.0	Mineral/vitamin mix	1.5
Calf pre-mix	1.0		
Bovatec	0.6		
Rumasweet palatant	0.2		

Table 5.2 Chemical composition (g/kg) of the starter concentrate, total mixed ration (TMR) diet and forages.

Diet	Milk replacer[1]	Starter concentrate	TMR diet	Forage Wk24	Forage Wk49
Dry matter [2]	97.0	88.7	82.8	17.6	20.2
Crude protein [3]	22.7	20.8	13.3	21.8	18.9
Sugars [4]	49.5*	56.1	5.1	11.6	8.7
NDF [5]	na	15.5	43.8	43.3	49.3
Ether extract [6]	20.6	1.7	1.1	3.5	2.1
Ash [7]	6.2	5.9	5.6	9.5	9.2

[1] Manufacturers data
[2] Method 945.15; AOAC, 2010.
[3] Method 992.15; AOAC, 2010.
[4] Paul, A.A and Southgate, D.A. The Composition of Foods. 4th Edition, 1978.
[5] Neutral detergent fiber (NDF); Method 7.074; AOAC, 1990
[6] Method 954.02; AOAC, 1990
[7] Method 942.05; AOAC, 2012
* Lactose

5.3.3 Methane inhibitors

The inhibitors used in this study were 9,10-anthraquinone (AQ) and chloroform (CF). The CF was complexed with cyclodextrin to stop evaporation from the feed and render it odorless. Concentrations of AQ and CF were 500 and 50 mg/kg of feed, respectively. Both AQ and CF were pre-mixed into 1/5 of the amount of starter meal required using a food processor and then mixed into the total batch amount using a concrete mixer. The same process was used for the TMR diet, where the inhibitors were mixed into the soybean meal and then in a mixing wagon with the rest of the ingredients. The final mixes were done twice a wk and stored in a cold room at 4°C until used.

5.3.4 Methane and dry matter intake measurements

Methane (CH_4) and hydrogen (H_2) emissions were measured at 2, 4, 6, 8, 10, 14, 24 and 49 wks of age. Measurements were carried out in open circuit respiratory chambers (Pinares-Patiño et al., 2012a) for 24 h. The air flow through the chambers was adjusted to 600 L/min to account for the low CH_4 emissions of a young animal. For the last two measurements, the airflow was increased to 1000 and 1500 L/min, respectively. Calves entered the chambers in the morning (0800h), when solid feed (starter concentrate and TMR diet, or fresh grass) was offered. During the milk feeding period, MR was offered before entering to the chambers in the morning (0800h) and on wks 2 and 4 also before the afternoon feed allocation (1600h). For the last two measurements, pasture was cut daily (Aorangi Farm, AgResearch, New Zealand) and transported to the Animal Facility at Grasslands. Calves were adapted to eat fresh cut grass in confinement pens from five to seven days prior to entering to the chambers. Cut fresh forage was offered *ad libitum* and refusals were collected to determine DMI as the difference of feed allowance and refusals.

5.3.5 Rumen fluid sampling

Rumen samples were taken via stomach tubing after removing the calves from the respiration chambers. Each sample was subsampled for short chain fatty acid (SCFA) analysis (1.8 ml) and DNA extraction (0.9 ml). Samples for SCFA analysis were centrifuged (20,000 g, 10 min, 4 °C) and an aliquot of 0.9 ml of the supernatant was collected into 0.1 ml of internal standard (19.8 mM ethylbutyrate in 20% v/v phosphoric acid) and stored at -20 °C until analyzed. Rumen samples for subsequent DNA extraction and microbial community analysis were snap-frozen and stored at -20 °C.

5.3.6 Short chain fatty acid analysis

SCFA samples were thawed and centrifuged (20,000 g, 10 min, 4 °C) and 0.8 ml of the supernatant was collected into a crimp cap glass vial. Gas chromatography was used to analyze SCFA composition (Attwood et al., 1998) in a HP 6890 gas chromatograph equipped with and flame ionization detector using a Zebron ZB-FFAP 30.0m x 0.53mm I.D. x 1µm film column (Tavendale et al., 2005).

5.3.7 DNA extractions

Nucleic acids were extracted from 200 µl of the rumen fluid using the phenol-chloroform, bead beating, with filtration kit for purification II (PCQI) method (Henderson et al., 2013). Primers (**Table A.1, Appendix A**) and amplicon reactions (30 and 35 cycles, respectively), targeting the V3-V4 region of the 16S rRNA genes in the microbial bacteria and archaea, were prepared as described by Kittelmann et al. (2013). Triplicate PCR products were pooled, and the correct sizes of PCR amplicons and the absence of signal from negative controls were verified by agarose gel electrophoresis and quantified by fluorescence using the Quant-iT dsDNA BR assay kit (Invitrogen, Carlsbad, CA, USA). For each amplicon, 150ng from the same target gene and region (i.e., all bacteria and archaea amplicons) were pooled and concentrated. The pooled PCR product concentration was determined using Quant-iT dsDNA HS assay kit (Invitrogen, Carlsbad, CA). Each pool was then purified using the NucleoMag NGS kit (Macherey-Nagel, Dueren, Germany), with a final purification of the amplicons done with the QIAquick PCR Purification kit (QiaGen, Valencia, CA, USA). The resulting DNA concentration was quantified using Quant-iT dsDNA HS assay kit (Invitrogen, Carlsbad, CA, USA). Both pools were then diluted to $6.0x10^9$ copies per µl and combined at a bacteria to archaea ratio of 5:1 (Kittelmann et al., 2013). Before sequencing, pooled libraries were checked for quality control (QC) using PerinElmer GX Touch HT Instrument using Bioanalyzer DNA High Sensitivity Assay. Amplicons were sequenced at the Genome Service/New Zealand Genomics Limited using Illumina MiSeq system (Massey University, Palmerston North, NZ). The pooled library was run on one Illumina MiSeq 2 x 250 base PE run version 2 chemistry (Reagent Kit v2, 500 cycles; Invitrogen, Carlsbad, CA, USA). An Illumina prepared PhiX control library for the run was loaded onto the Illumina MiSeq run at 20% volume. Sequence reads were provided in fastq format.

5.3.8 Phylogenetic analysis

Sequencing reads were quality-filtered using the DynamicTrim function of SolexaQA (Cox et al., 2010). Reads were then processed and analyzed using the QIIME software package 1.8 (Caporaso et al., 2010). Sequencing reads were grouped into operational taxonomic units (OTUs) sharing similarities over 97% for bacteria and 99% for archaea (Edgar, 2010). Sequences were assigned to phylogenetic groups using BLAST (Altschul, 1990). Bacterial 16S rRNA genes were assigned using SILVA 123 (Henderson et al., 2019) and archaea 16S rRNA genes using RIM-DB (Seedorf et al., 2014). QIIME generated OTU-tables were used for downstream statistical analysis.

5.3.9 Statistical analysis

Univariate analyses were performed using a linear mixed effect (LME) model via the restricted maximum likelihood (REML) framework as implemented in the NLME package in R (Pinheiro et al., 2015; R CoreTeam, 2016). The LME models were analyzed using analysis of variance (ANOVA) on repeated measurements. Predicted means from the model, together with estimates of the standard error of the mean and pairwise comparisons (Tukey's test and Benjamini-Hochberg' test) were obtained, and back transformed (where applicable) using the PREDICTMEANS package of R (Luo et al., 2014b).

After checking for normality, body weight was the only transformed data using natural logarithm. The effects of methane inhibitors on dry matter intake (DMI), live weight (LW), CH_4 and H_2 emissions, SCFA total concentrations and individual proportions were fitted in a linear mixed-effect model with treatment and age as fixed effects, and animal as a random effect. Initial body weight was used as covariate for the analysis live weight. Treatment effects from the LME models were assessed using ANOVA on repeated measurements and predicted means, together with estimate standard error of the mean (SEM) were obtained, back-transformed (where applicable) and pairwise compared using Tukey's test. Values of $P \leq 0.01$ were considered highly significant, $P \leq 0.05$ considered significant, $P \leq 0.10$ considered a trend and $P > 0.10$ not significant.

The alpha diversity of the ruminal microbiota during and after methane inhibitors interventions was analyzed using the Chao1 and Shannon indexes in the VEGAN package of R (Oksanen et al., 2017). The pre- and post-treatment effect of using methane inhibitors on the ruminal microbiota were compared for the Chao1 and Shannon indexes using an

LME model with treatment and age as fixed effects, and animals as random effect. The obtained LME models were assessed using ANOVA on repeated measurements and post hoc analysis using Tukey's test. Statistical significance was declared at a P-value ≤ 0.05.

The beta diversity of the microbial community of calves was analyzed using a partial least squares discriminant analysis (PLSDA) using the mixOmics package of R (Lê Cao et al., 2016). This multivariate analysis was used to classify and discriminate (Gonzalez et al., 2012) the treatments at different ages on the ruminal microbial community. Classes were assigned by combining treatments and age. The aim was to identify the effect of methane inhibitors pre- and post-interventions on the ruminal microbiota, as well as, the groups of microbial organisms associated with these effects (variable importance in projection or VIP microorganisms). An additionally PLSDA was conducted for the abundant microbiota. The abundant microbiota was selected as follows: bacteria (minBacteria) and archaea (minArchaea) with a relative abundance $\geq 0.50\%$ and $\geq 1.00\%$, respectively. The aim was to identify if a reduce number of microorganisms, such as the abundant microbiota, showed a similar cluster separation pattern to the observed in the whole microbiota. Association scores for bacteria and archaea were visualized via clustered image maps (CIM) representing the first two dimensions (Henderson et al., 2016).

A univariate analysis was carried out to determine the effect of methane inhibitors on the abundant microbial community during pre- and post-treatment with methane inhibitors. Abundant bacteria phyla, bacteria genera and archaea species were defined as organisms with an average proportion $\geq 1.0\%$, 0.5% and 1.0%, respectively. After checking for normality, bacteria (phyla and genera) and archaea (species) community data were transformed using natural logarithm. All the analyses were performed using a LME model. The effect of treatment pre- and post-intervention on the bacteria and archaea communities was analyzed using treatment and age as fixed effect, and animal as random effect. Predicted means from the models, together with estimates of the standard error of the mean were obtained and back transformed, and pairwise comparisons were done using Benjamini-Hochberg' test. Statistical significance was declared at a P-value ≤ 0.05.

5.4 Results

5.4.1 Animal performance and rumen fermentation.

Dry matter intake was not affected by treatment (P=0.25), increasing over time in both groups (P<0.01; **Table 5.3**). Live weight differed (P<0.01) between groups but only after treatment cessation with 2.1 ± 1.5% decreased (P<0.05) during wk 24.

Yield of methane (CH_4) and hydrogen (H_2) decreased (P<0.01) and increased (P<0.01), respectively, in treated compared to control calves. Methane inhibition decreased (P<0.01) the proportion of acetate and increased (P<0.01) propionate, valerate, caproate and isovalerate, with no effect on (P>0.05) butyrate and isobutyrate. No treatment effects (P>0.05) were observed after treatment cessation at wk 24 and 49. Significant interaction (P>0.01)were observed for yield of CH_4 and H_2, and proportions of acetate, propionate, valerate, caproate and isovalerate.

5.4.2 Rumen microbiota diversity

A total of 8,064,593 sequences were obtained from 190 rumen samples, using the Illumina MiSeq platform, with an average of 42,445 bacteria and 7,699 archaea sequences per sample. The number of operational taxonomic units (OTUs) was 1,500 and 40 for bacteria and archaea, respectively. A total of 245 bacteria and 17 archaea taxa were analyzed after using a minimal sample cut off of 200 reads.

The Chao 1 (**Figure 5.1a**) and Shannon (**Figure 5.1b**) indexes for bacteria increased (P < 0.001) over time but were similar between the two treatment groups (P > 0.05) with no treatment x time interaction (P > 0.05). In archaea, the Chao richness (**Figure 5.1c**) was lower in the treated calves until wk 14 (P < 0.05) but was similar thereafter (P > 0.05). The Shannon index for archaea (**Figure 5.1d**) had a treatment x time interaction (P = 0.005), where compared with the controls, the treated calves had increased diversity at wk 2, but decreased diversity (P < 0.05) at wk 14.

The beta diversity of the bacteria dataset is shown in **Figure 5.2**. A PLSDA analysis of the whole dataset in **Figure 5.2a** shows no clustering for the treatments, but a continuum across the two dimensions according to animal age. **Figure 5.2b** shows the analysis for the abundant bacteria (> 0.5% of all bacteria) where no clustering treatments and a much weaker clustering for animal age is observed.

Table 5.3 Effect of methane inhibitors[1] on live weight (LW), dry matter intake (DMI), methane (CH_4) and hydrogen (H_2) production (g/d) and yield (g/kg DMI), and total concentrations (mM) and individual proportions (%) of short chain fatty acids (SCFA) in dairy calves. Results[2] are the means and standard error of the differences (SED), P-value for treatment (P-Tx), time (P-Tm) and interaction (P-Int). Significant differences (pairwise comparisons) between treatment are shown with different letters at each sampling time.

		Rearing period (weeks)											
	Treatment	**2**	**4**	**6**	**8**	**10**	**14**	**24**	**49**	**SEM**	**P-T**	**P-A**	**P-Int**
LW [3]	Ctrl	38.74	48.46	60.20	70.41	82.30	96.58	124.50^{a}	259.30	1.957	<0.01	<0.01	0.67
	Trt	39.03	47.77	58.57	68.56	80.01	95.09	119.20^{b}	253.24				
DMI	Ctrl	0.24	0.48	0.77	1.21	2.23	2.46	3.05	6.84	0.247	0.87	<0.01	0.66
	Trt	0.18	0.32	0.72	1.07	1.98	2.33	2.87	7.28				
pCH_4	Ctrl	1.53	7.44^{a}	19.04^{a}	25.80^{a}	44.04^{a}	50.81^{a}	63.80	145.40	3.037	<0.01	<0.01	<0.01
	Trt	0.69	1.04^{b}	1.50^{b}	3.81^{b}	2.70^{b}	12.28^{b}	58.76	143.13				
yCH_4	Ctrl	5.16	15.72^{a}	25.80^{a}	23.99^{a}	20.20^{a}	21.08^{a}	22.33	21.86	2.161	<0.01	<0.01	<0.01
	Trt	4.58	4.38^{b}	2.27^{b}	3.85^{b}	1.41^{b}	5.39^{b}	21.08	20.26				
pH_2	Ctrl	0.17	0.14	0.42^{a}	0.07^{a}	0.02^{a}	0.04^{a}	0.08	0.29	0.388	<0.01	<0.01	<0.01
	Trt	0.35	0.82	2.48^{b}	3.29^{b}	5.93^{b}	4.35^{b}	0.09	0.44				
yH_2	Ctrl	0.98	0.38^{a}	0.89^{a}	0.07^{a}	0.01^{a}	0.02^{a}	0.03	0.07	0.437	<0.01	<0.01	<0.01
	Trt	1.68	2.64^{b}	3.41^{b}	2.93^{b}	2.97^{b}	1.88^{b}	0.02	0.06				
SCFA	Ctrl	81.60	77.36	76.26	80.46	85.48	80.17	75.47	74.39	7.190	0.31	0.67	0.72
	Trt	72.57	84.39	78.00	73.13	79.50	71.69	74.47	72.57				
Acetate	Ctrl	53.67^{a}	54.50^{a}	62.92^{a}	61.86^{a}	62.66^{a}	67.21^{a}	67.56	69.61	2.131	<0.01	<0.01	<0.01
	Trt	48.71^{b}	44.82^{b}	47.77^{b}	47.71^{b}	49.11^{b}	52.46^{b}	67.73	69.22				

Table 5.3 (Continued)

		Rearing period (weeks)											
	Treatment	**2**	**4**	**6**	**8**	**10**	**14**	**24**	**49**	**SEM**	**P-T**	**P-A**	**P-Int**
Propionate	Ctrl	26.01[a]	26.53[a]	22.23[a]	23.22[a]	22.36[a]	17.73[a]	18.63	17.68	2.115	<0.01	<0.01	0.00
	Trt	32.90[b]	35.45[b]	27.78[b]	28.76[b]	33.73[b]	25.54[b]	18.47	18.15				
Butyrate	Ctrl	15.18	13.46	10.09	10.06[a]	10.67	10.55	9.80	8.42	2.198	0.15	0.01	0.25
	Trt	12.83	12.82	14.05	14.89[b]	11.12	13.82	9.77	8.48				
Caproate	Ctrl	1.17	0.64	0.68[a]	0.67[a]	0.78	0.58	0.38	0.25	0.267	0.03	<0.01	<0.01
	Trt	0.93	0.76	1.81[b]	1.94[b]	1.14	0.74	0.33	0.25				
Valerate	Ctrl	2.72	2.55[a]	1.89[a]	1.62[a]	1.69[a]	1.47[a]	1.13	1.06	0.366	<0.01	<0.01	<0.01
	Trt	3.05	3.87[b]	4.80[b]	3.94[b]	3.48[b]	2.79[b]	1.13	1.03				
Isobutyrate	Ctrl	0.64	1.06	0.97	1.11	0.75	1.07	1.09	1.36	0.120	0.91	<0.01	0.46
	Trt	0.71	0.92	1.13	1.20	0.61	0.95	1.13	1.33				
Isovalerate	Ctrl	0.62	1.27	1.23[a]	1.46	1.09	1.39[a]	1.41	1.61	0.432	0.07	<0.01	<0.01
	Trt	0.87	1.36	2.62[b]	1.56	0.81	3.69[b]	1.45	1.54				

[1] Methane inhibitors were dosed from arrival until wk 14 of the rearing period, after which both groups were on the same diet.

[2] Repeated measurements were used to analyze the long-term effects of the methane inhibitors.

[3] Live weight (LW) was adjusted to initial LW and results are natural logs (LN) back transformed.

Figure 5.1 Alpha diversity indexes of the rumen microbiota in control and treatment calves across different ages. **a)** Bacteria Chao1 index; **b)** bacteria Shannon index; **c)** archaea Chao1 index; and **d)** archaea Shannon index. Boxplots represent the 25th and 75th percentiles, lines within boxes are the medians, the whiskers extend to the most extreme data points and dots represent the outliers.

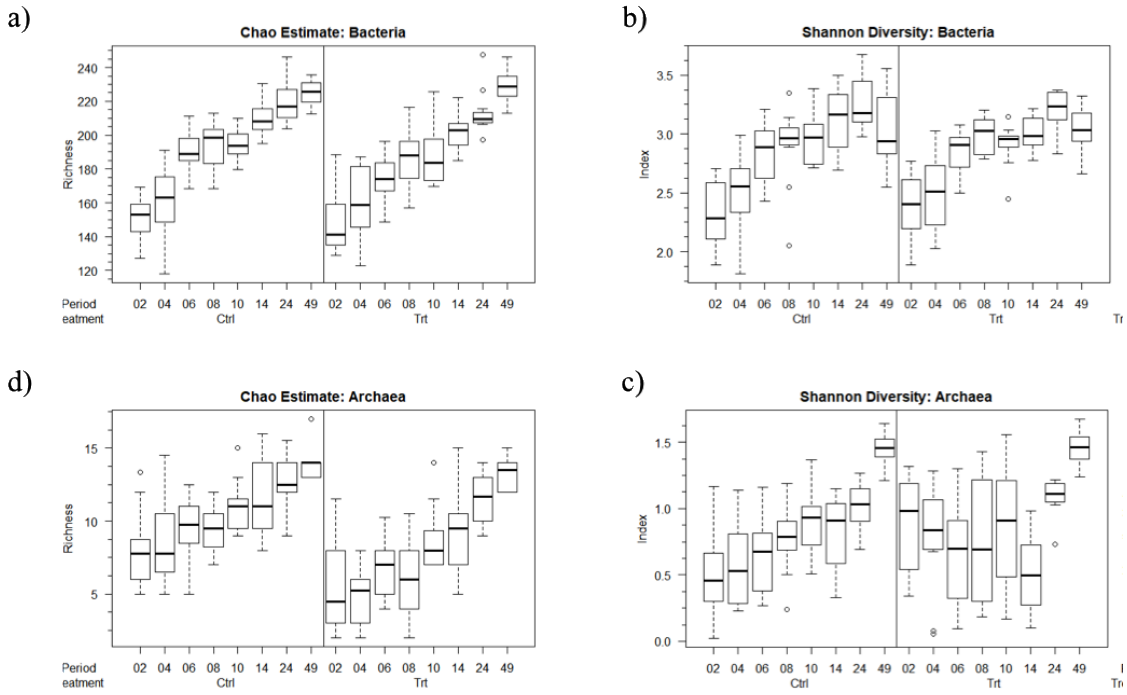
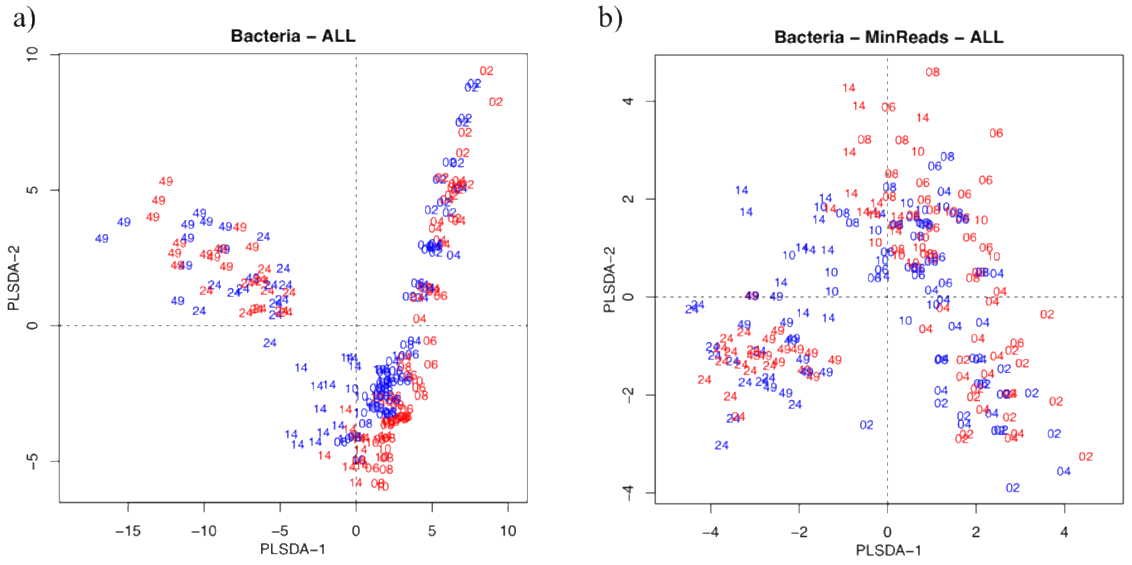
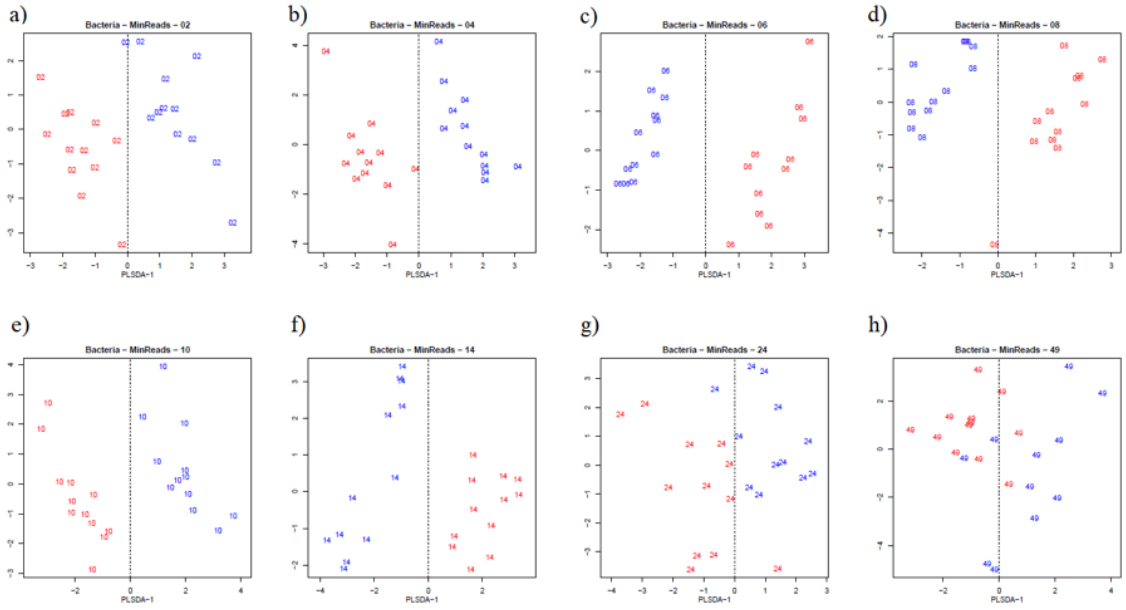


Figure 5.2 Partial least square discriminant analysis (PLSDA) of the bacteria community in treatment (chloroform/anthraquinone) and control dietary treatment across sampling periods. **a)** PLSDA of the whole bacteria community; and **b)** PLSDA of the abundant (MinReads) bacteria at the genus level. Calves from the treated and control groups are represented in red and blue, respectively. The numbers correspond to the sampling time (wks).



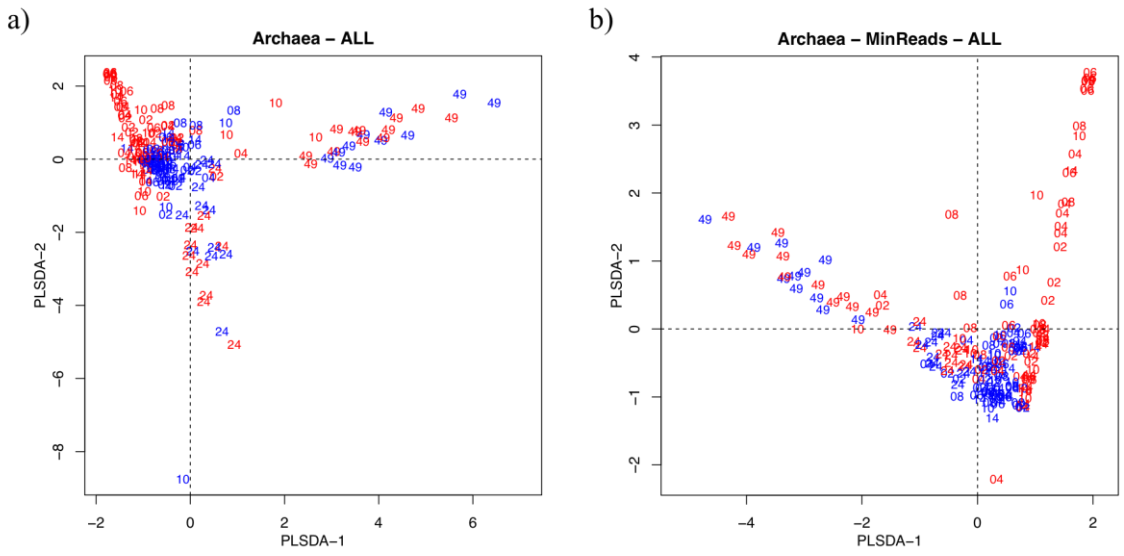
The analysis of the abundant bacteria for each sampling point showed a treatment separation (**Figure 5.3**). This separation is very clear for wks 2 to 14 (**Figures 5.3 a-f**), when methane inhibitors were fed. At wk 24 one could speculate about a separation, but this was completely gone at wk 49 (**Figures 5.2 g-h**).

Figure 5.3 Partial least square discriminant analysis (PLSDA) of the abundant bacteria community (MinReads) at the genus level within each sampling period. Calves from the treated and control groups are represented in red and blue, respectively. The number corresponds to the sampling time (wks). **a-b)** calves fed milk twice a day and *ad libitum* concentrates; **c-e)** calves fed milk once a day, and *ad libitum* concentrates and total mixed ration diet (TMR); **f)** calves step-down weaned of concentrates and fed *ad libitum* TMR diets; and **g-h)** calves grazing a mixed sward of ryegrass/clover as one mob.



The beta diversity of the archaeal community is shown in **Figure 5.4**. The whole and abundant archaea community of treated calves showed a cluster separation from control calves during the first 14 wks (**Figure 5.4a** and **5.4b**). While at wk 24 and 49, both groups clustered together within two defined clusters in the whole archaea community (**Figure 5.4a**), but in the abundant archaea only the community at wk 49 of both treatments clustered separately (**Figure 5.4b**).

Figure 5.4 Partial least square discriminant analysis (PLSDA) of the archaea community for treated and control calves across sampling times. **a)** PLSDA of the whole (ALL) archaea community; and **b)** PLSDA of the abundant (MinReads) archaea community at the species level. Calves from the treated and control groups are represented in red and blue, respectively. The number correspond to the sampling time (weeks).



5.4.3 Bacterial community composition

The bacterial community composition from the selected cut offs of OTUs showed 18 different phyla. These phyla corresponded to 32 classes, 46 orders, 81 families and 244 genera (**Tables E.1, E.2, E.3, E.4 and E.5, Appendix E**). In **Figure 5.5** shows only phyla with an abundant > 1% across samples. *Firmicutes* (43.1±6.89%) and *Bacteroidetes* (43.1±7.22%) were the most abundant phyla. *Proteobacteria*, *Spirochaetae*, *Tenericutes*, *Fibrobacteres*, *Actinobacteria* and *Cyanobacteria*[8] together represented only 12.8+4.71% of the bacteria phyla in the rumen. **Table 5.4** shows the effect of methane inhibitors on the abundant bacterial phyla at different sampling times. On average methane inhibition had no effect on the bacterial composition at the phylum level ($P > 0.05$). Significant time affects were observed in the abundant bacterial phyla composition ($P < 0.001$), except for the *Actinobacteria*. The proportions of *Bacteriodetes* and *Firmicutes* were constant until wk 49, when *Bacteriodetes* dominated the ruminal community. Other changes over time were the general decline in *Proteobacteria* and at the same time an increase in *Spirochaetes*

[8] This group of photosynthetic bacteria that live in a wide variety of moist soils and water either freely or in a symbiotic relationship with plants. This phylum of bacteria obtains their energy through photosynthesis and are the only photosynthetic prokaryotes able to produce oxygen. While in the rumen, these microbes might be opportunistically acquired from the environment.

until weaning (wk 10), which decreased thereafter. Significant treatment x time interactions were only observed in *Fibrobacteres* (P = 0.048) and *Cyanobacteria* (P = 0.003). Pairwise analysis indicated that in treated calves the intake of methane inhibitors decreased the proportions of *Fibrobacteres* at wk 6 (5.3-fold decreased). The relative abundance of *Cyanobacteria* decreased in treated calves at wks 6, 10 and 14 (2.9, 6.1 and 10.8-fold, respectively; P < 0.050) with respect to control calves.

Figure 5.5 Relative abundance of the abundant bacterial phyla in the rumen of control and treated calves. Data shown in columns are the percentage of total sequences identified per group at different times of rearing. Sampling times were from 2-49 wks. Calves were arranged in a control (Ctrl) and treatment (Trt) group. Chloroform (CF) and 9,10-anthraquinone (AQ) were applied to the concentrate and total mixed ration diet (TMR) until wk 14. Calves in both groups were fed as follows: milk twice day and *ad libitum* concentrate diets at wks 2 and 4; milk once a day and *ad libitum* concentrates and TMR diets at wks 6, 8 and 10; concentrates step-down weaned and TMR diets fed *ad libitum* until wk 14; and grazing a mixed sward of ryegrass/clover as one mob at wks 24 and 49.

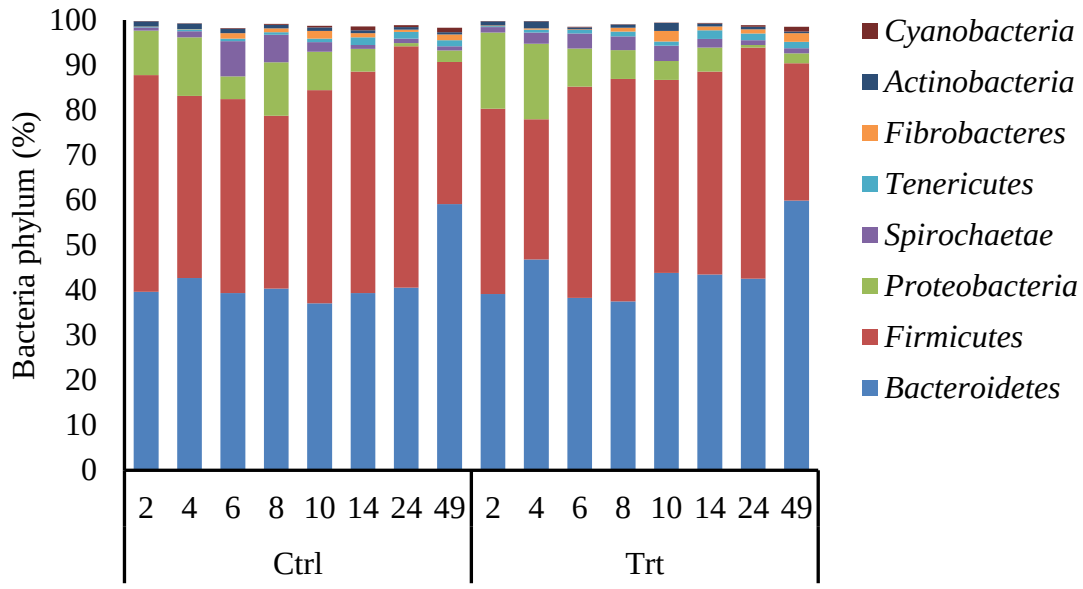


Table 5.4 Analysis of the effect of methane inhibitors[1] on the abundant bacterial phyla[2] at different sampling times[3]. Calves were arranged in a control (Ctrl) and treatment (Trt) group. Results[4] are natural log back transformed means and standard error of the differences (SED), P-value for treatment (Trt), time (Time) and interaction (Int).

		Time (wk)									P-value		
Taxa- Phylum	**Treatment**	**2**	**4**	**6**	**8**	**10**	**14**	**24**	**49**	**SED**	**Trt**	**Time**	**Int**
Firmicutes	Ctrl	44.03	37.35[a]	41.97	36.52[a]	46.37	48.82	53.30	30.99	5.066	0.301	0.000	0.100
	Trt	36.61	29.24[b]	45.48	47.67[b]	42.04	44.23	50.59	30.10				
Bacteroidetes	Ctrl	36.17	41.46	38.54	39.92	36.51	38.57	40.22	58.75	5.133	0.264	0.000	0.540
	Trt	37.00	46.13	37.59	35.73	43.25	42.96	41.73	59.61				
Proteobacteria	Ctrl	2.53[a]	4.71	1.10[a]	5.11	4.59	2.85	0.68	2.30	0.417	0.482	0.000	0.052
	Trt	8.09[b]	9.57	3.36[b]	2.27	2.59	2.31	0.56	2.09				
Spirochaetae	Ctrl	0.15	0.51	2.90	4.21	1.70	0.68	0.77	0.89	0.172	0.535	0.000	0.264
	Trt	0.35	0.77	1.80	2.14	2.63	1.28	0.91	0.93				
Tenericutes	Ctrl	0.12	0.24	0.34	0.37	0.62	1.44	1.42	1.34	0.096	0.625	0.000	0.771
	Trt	0.06	0.23	0.34	0.60	0.81	1.84	1.49	1.38				
Fibrobacteres	Ctrl	0.01	0.04	0.22[a]	0.27	0.76	0.65	0.39	1.03	0.053	0.751	0.000	0.048
	Trt	0.03	0.09	0.04[b]	0.17	0.86	0.21	0.58	1.63				
Actinobacteria	Ctrl	0.78	0.62	0.42	0.49	0.49	0.59	0.48	0.50	0.065	0.574	0.125	0.126
	Trt	0.33	0.76	0.24	0.60	0.90	0.44	0.55	0.36				
Cyanobacteria*	Ctrl	0.01	0.01	0.06[a]	0.11	0.28[a]	0.75[a]	0.36	0.91	0.030	0.001	0.000	0.003
	Trt	0.01	0.01	0.02[b]	0.04	0.05[b]	0.07[b]	0.27	0.95				

[1] Chloroform (CF) and 9,10-anthraquinone (AQ) were applied to the concentrate and total mixed ration diet (TMR) until wk 14.

[2] Abundant bacteria phyla were defined as organisms with an average proportion ≥ 1.0%.

[3] Sampling times were from 2-49 wks. Calves during the rearing time (wk) were fed as follows: milk twice a day and *ad libitum* control and treatment concentrates at wks 2 and 4; milk once a day and *ad libitum* control and treatment concentrates and TMR diet at wks 6, 8 and 10; concentrates step-down weaned and TMR diets fed *ad libitum* until wk 14; and grazing a mixed sward of ryegrass/clover as one mob at wks 24 and 49.

[4] A repeated measurement analysis was carried out to determine the effect of methane inhibitors on the bacteria community structure and their carry-over effects.

* This is an environmental bacteria phylum.

At the genus level, forty-one bacteria genera had a relative abundance of ≥ 0.50% across sampling times. These abundant bacteria accounted for 85.7±5.38% of the community across sampling times. *Prevotella* 1 was the most abundant genus (~24.7%), followed by *Christensenellaceae* R-7 group (~5.2%), *Rikenellaceae* RC9 gut group (~4.3%), *Ruminococcus* 2 (~4.1%) and *Sharpea* (~3.2%) (**Table E.5, Appendix E**). The abundant bacteria genera for treated and control calves during different sampling times is shown in **Table 5.5**. Treatment effects ($P < 0.05$) were observed in 7 of the abundant genera. Samples from inhibitor-treated calves indicated proportional increases of *Rikenellaceae* RC9 gut group, *Succiniclasticum* and *Ruminococcaceae* UCG 002, whilst decreases of *Ruminococcus* 1, *Ruminococcaceae* NK4A214 group, *Ruminoclostridium* 5 and *Ruminococcaceae* UCG 005 when compared to control calves. Most of the abundant bacterial genera were significantly ($P < 0.05$) affected by time, except for the *Eubacterium ventriosum* group ($P > 0.05$). Treatment x time interactions ($P < 0.05$) were observed in 15 of the abundant bacteria genera, which showed significant pairwise comparison differences only between wks 2 and 14 (intake of inhibitors).

5.4.4 Archaea community

The archaea community was represented by 16 species at a cut off of > 200 amplicons across sampling times. The archaea community of treated calves had a reduced number of amplicons during the administration of methane inhibitors compared to control calves (**Figure 5.6**). Community analysis of the abundant community of archaea is showed in **Table 5.6**. Treated calves had decreases of *Mbb. gottschalkii* clade proportions compared to control calves. During the treatment until wk 14 there was a ~24 ± 22.5-fold increase in the abundance of the *Mmc*. Group 12 ISO4-H5 in treated calves. An opposite effect was observed for *Msp.* ISO3-F5, whose proportion was ~16 ± 13.7-fold decreased in treated calves relative to control calves. However, after inhibitor, the archaeal groups did not differ between treatments, with *Mmc*. Group 12 ISO4-H5 was almost completely lost from the rumen of calves. Time effects ($P < 0.05$) were observed across all the abundant archaea species. While, *Mbb. ruminantium* clade, *Mmc.* Group12 sp. ISO4-H5 and Group4 sp. MpT1, and *Msp.* sp. ISO3-F5 showed a treatment by time interaction ($P < 0.05$).

Table 5.5 Effect of methane inhibitors[1] on the abundant bacterial genera[2] at different sampling times[3]. Results[4] are natural log back transformed means and standard error of the differences (SED), P-value for treatment (Trt), time (T) and interaction (Trt x T). Pairwise comparison for treatment within each sampling time are shown with different letters.

		Time (wk)									P-value		
Taxa- Genus	**Treatment**	**2**	**4**	**6**	**8**	**10**	**14**	**24**	**49**	**SED**	**Trt**	**T**	**Trt x T**
Prevotella 1	Ctrl	18.36	19.61	20.21[a]	19.24[a]	18.14	18.93	28.10	39.13	4.280	0.945	<.0001	0.007
	Trt	21.82	27.44	13.03[b]	11.71[b]	23.91	19.84	29.09	39.91				
Christensenellaceae R-7 group	Ctrl	0.34	1.00	4.44	4.63	5.38	4.56	3.79	5.43	0.780	0.608	<.0001	0.544
	Trt	0.33	0.57	3.85	8.38	6.37	8.41	4.46	5.26				
Rikenellaceae RC9 gut group	Ctrl	0.13	0.99	2.04	3.14	2.75	4.13	2.04	4.64	0.637	0.007	<.0001	0.515
	Trt	0.55	1.93	5.32	6.12	5.02	9.31	2.24	4.53				
Ruminococcus 2	Ctrl	2.62	1.19	1.91	2.85	4.22	3.87	0.43	0.27	0.354	0.074	<.0001	0.288
	Trt	0.52	0.41	4.25	1.99	3.24	2.03	0.44	0.25				
Sharpea	Ctrl	3.55	1.41	0.61	0.53	1.79	0.07	0.01	0.01	0.231	0.644	<.0001	0.855
	Trt	5.46	2.18	0.93	0.92	2.14	0.26	0.00	0.01				
Bacteroidales BS11 gut group	Ctrl	0.13	1.59	2.95	2.25	1.69	3.05	1.62	2.49	0.324	0.392	<.0001	0.846
	Trt	0.09	1.08	1.23	2.16	1.49	1.31	1.76	3.02				
Bacteroidales S24-7 group	Ctrl	0.09	0.62	2.80	4.44	2.08	1.05	1.96	1.81	0.333	0.876	<.0001	0.281
	Trt	0.11	0.29	2.05	1.90	2.52	2.43	2.11	2.39				
Ruminobacter	Ctrl	0.02	0.04	0.02[a]	1.18	2.43	0.42	0.02	0.03	0.079	0.363	<.0001	0.016
	Trt	0.03	0.13	0.85[b]	0.62	0.66	0.32	0.02	0.03				
Ruminiclostridium 9	Ctrl	0.03	0.05	0.17	0.20	0.35	2.42	5.86	0.32	0.204	0.508	<.0001	0.662
	Trt	0.04	0.06	0.20	0.45	1.03	1.59	4.54	0.28				
Lachnospiraceae UCG 005	Ctrl	0.39	0.37	0.02[a]	0.02	0.01	0.01	0.01	0.03	0.018	0.484	<.0001	0.017
	Trt	0.24	0.09	0.21[b]	0.07	0.01	0.01	0.01	0.02				
Treponema 2	Ctrl	0.02	0.09	0.88	2.00	0.96	0.43	0.65	0.72	0.153	0.284	<.0001	0.867
	Trt	0.06	0.21	1.42	1.59	1.76	0.81	0.76	0.77				
Roseburia	Ctrl	2.05	0.82[a]	0.23[a]	0.06	0.07	0.24	1.48	0.46	0.171	0.081	<.0001	0.001
	Trt	4.08	2.35[b]	1.25[b]	0.15	0.14	0.11	0.85	0.41				
p-2534-18B5 gut group	Ctrl	0.01	0.01	0.02[a]	0.19[a]	0.23	0.07[a]	0.10	0.02	0.100	0.000	<.0001	0.000
	Trt	0.01	0.04	5.13[b]	1.41[b]	0.53	0.74[b]	0.11	0.02				
Lachnospiraceae NK3A20 group	Ctrl	0.01	0.02[a]	0.08[a]	0.36[a]	1.23	1.70	1.22	1.14	0.186	0.001	<.0001	0.010
	Trt	0.02	0.18[b]	1.09[b]	1.76[b]	1.61	3.28	1.23	1.13				

Table 5.5 (Continued)

		Time (wk)									P-value		
Taxa- Genus	**Treatment**	**2**	**4**	**6**	**8**	**10**	**14**	**24**	**49**	**SED**	**Trt**	**T**	**Trt x T**
Succinivibrio	Ctrl	0.68	0.77	0.03	0.05	0.08	0.02	0.00	0.02	0.050	0.604	<.0001	0.526
	Trt	1.96	0.55	0.03	0.01	0.06	0.01	0.00	0.02				
Succiniclasticum	Ctrl	0.21	1.09	0.76	1.51	0.81	0.65	0.98	1.56	0.237	0.004	<.0001	0.151
	Trt	0.70	2.47	2.24	1.82	1.99	1.07	1.10	1.47				
Ruminococcus 1	Ctrl	0.45	0.60	1.18	1.44	1.74	1.85	3.17	1.65	0.247	0.009	<.0001	0.666
	Trt	0.18	0.37	0.70	1.01	1.36	1.42	2.44	1.71				
Ruminococcaceae UCG 014	Ctrl	0.35	0.43	0.85	1.07	1.53	1.77	1.23	0.97	0.171	0.167	<.0001	0.827
	Trt	0.14	0.29	0.80	1.30	0.97	0.95	1.11	0.93				
Succinivibrionaceae UCG 002	Ctrl	0.06	0.03[a]	0.03	0.10	0.06	0.09	0.05	0.07	0.018	0.615	0.261	0.011
	Trt	0.02	0.64[b]	0.11	0.06	0.09	0.04	0.02	0.07				
Ruminococcaceae NK4A214 group	Ctrl	0.36[a]	0.27	0.82	0.86	0.91	0.76	2.75	2.38	0.197	0.031	<.0001	0.044
	Trt	0.09[b]	0.31	0.58	0.63	0.57	0.63	2.52	2.48				
Ruminiclostridium 5	Ctrl	0.31[a]	0.93[a]	1.35[a]	0.77[a]	0.39	0.99[a]	0.28	0.24	0.076	<.0001	0.273	0.001
	Trt	0.08[b]	0.04[b]	0.08[b]	0.11[b]	0.18	0.22[b]	0.32	0.24				
Prevotellaceae UCG 001	Ctrl	0.06[a]	0.48	0.65	0.45	0.51	0.95	0.89	1.67	0.125	0.630	<.0001	0.034
	Trt	0.23[b]	0.51	0.24	0.28	0.39	0.33	1.05	2.12				
Prevotellaceae UCG 003	Ctrl	0.07	0.05	0.39	0.49	1.49	1.14	0.87	2.60	0.150	0.676	<.0001	0.105
	Trt	0.07	0.16	0.34	0.46	0.49	0.60	1.26	2.48				
Selenomonas 1	Ctrl	0.27	0.23	0.16	0.10	0.16	0.29	3.41	0.91	0.130	0.059	<.0001	0.294
	Trt	0.50	0.35	0.12	0.23	0.39	0.35	2.87	0.87				
Fibrobacter	Ctrl	0.01	0.04	0.22[a]	0.27	0.76	0.65	0.39	1.03	0.081	0.751	<.0001	0.048
	Trt	0.03	0.09	0.04[b]	0.17	0.86	0.21	0.58	1.63				
Pseudobutyrivibrio	Ctrl	0.13	0.16	0.15	0.19	0.16	0.75	1.93	1.42	0.115	0.559	<.0001	0.869
	Trt	0.19	0.14	0.15	0.29	0.27	0.53	2.07	1.41				
Mollicutes RF9	Ctrl	0.04	0.17	0.26	0.32	0.60	1.22	1.12	0.88	0.119	0.106	<.0001	0.985
	Trt	0.05	0.25	0.28	0.55	0.76	1.79	1.13	0.84				
Prevotella 7	Ctrl	0.68	0.10	0.01	0.01	0.01	0.01	0.44	0.03	0.027	0.771	<.0001	0.755
	Trt	0.54	0.21	0.03	0.01	0.01	0.01	0.23	0.03				
Kandleria	Ctrl	0.00	0.00	0.01	0.02	0.07	0.02	1.93	0.03	0.047	0.985	<.0001	0.759
	Trt	0.00	0.00	0.00	0.01	0.02	0.03	1.83	0.03				
Succinimonas	Ctrl	0.02	0.02	0.02	0.02	0.02	0.01	1.00	0.01	0.027	0.436	<.0001	0.824
	Trt	0.09	0.02	0.02	0.03	0.04	0.02	1.00	0.01				

Table 5.5 (Continued)

		Time (wk)									P-value		
Taxa- Genus	**Treatment**	**2**	**4**	**6**	**8**	**10**	**14**	**24**	**49**	**SED**	**Trt**	**T**	**Trt x T**
Ruminococcaceae UCG 005	Ctrl	0.15	0.19	0.57	0.70	0.43	0.62	0.53	0.58	0.075	0.015	<.0001	0.291
	Trt	0.06	0.09	0.16	0.54	0.52	0.35	0.57	0.45				
Eubacterium coprostanoligenes group	Ctrl	0.03	0.12	0.52	0.70	0.71	1.03	0.96	0.91	0.100	0.136	<.0001	0.417
	Trt	0.03	0.10	0.28	0.38	0.37	0.53	1.04	0.91				
Erysipelotrichaceae UCG 002	Ctrl	0.00	0.01	0.03	0.01	0.05	0.01	0.05	0.00	0.004	0.884	0.002	0.116
	Trt	0.00	0.00	0.01	0.01	0.01	0.09	0.02	0.00				
Atopobium	Ctrl	0.41[a]	0.19	0.28[a]	0.35	0.39	0.51	0.30	0.32	0.060	0.254	0.015	0.036
	Trt	0.13[b]	0.30	0.09[b]	0.40	0.67	0.29	0.31	0.21				
Lachnospiraceae uncultured	Ctrl	0.15	0.14	0.57	0.20	0.15	0.17	0.21	0.25	0.043	0.482	0.029	0.172
	Trt	0.18	0.15	0.23	0.33	0.22	0.30	0.21	0.26				
Prevotellaceae NK3B31 group	Ctrl	0.01[a]	0.05[a]	0.04	0.02	0.08	0.09	0.20	0.27	0.024	0.275	0.001	0.002
	Trt	0.17[b]	0.43[b]	0.19	0.05	0.04	0.02	0.19	0.23				
Sphaerochaeta	Ctrl	0.10	0.23	0.34	0.66	0.29	0.18	0.09	0.04	0.042	0.764	<.0001	0.572
	Trt	0.19	0.29	0.21	0.30	0.44	0.21	0.06	0.03				
Lachnospiraceae NK4A136 group	Ctrl	0.05	0.18	0.14	0.20	0.29	0.51	0.60	0.38	0.059	0.338	<.0001	0.584
	Trt	0.10	0.14	0.40	0.29	0.33	0.67	0.46	0.36				
Ruminococcaceae UCG 002	Ctrl	0.04[a]	0.19	0.36	0.27[a]	0.25	0.33	0.49	0.37	0.073	0.001	<.0001	0.033
	Trt	0.25[b]	0.37	0.68	0.66[b]	0.52	0.76	0.38	0.34				
Bacteroidales RF16 group	Ctrl	0.01	0.01	0.03[a]	0.25	0.47[a]	0.85[a]	0.57	1.28	0.070	0.073	<.0001	0.000
	Trt	0.01	0.02	0.13[b]	0.18	0.10[b]	0.11[b]	0.66	1.33				
Eubacterium ventriosum group	Ctrl	0.02	0.04	0.06	0.05	0.04	0.05	0.04	0.06	0.007	0.092	0.274	0.714
	Trt	0.02	0.01	0.01	0.02	0.02	0.04	0.04	0.05				

[1] Calves were arranged in a control (Ctrl) and treatment (Trt) group. Chloroform (CF) and 9,10-anthraquinone (AQ) were applied to treated calves in the concentrate and total mixed ration diet (TMR) until wk 14.

[2] Abundant bacteria genera were defined as organisms with an average proportion ≥ 0.5%.

[3] Sampling times were from 2-49 wks. Calves during the rearing time (wk) were fed as follows: milk twice a day and *ad libitum* control and treatment concentrates at wks 2 and 4; milk once a day and *ad libitum* control and treatment concentrates and TMR diet at wks 6, 8 and 10; concentrates step-down weaned and TMR diets fed *ad libitum* until wk 14; and grazing a mixed sward of ryegrass/clover as one mob at wks 24 and 49.

[4] A repeated measurement analysis was carried out to determine the effect of methane inhibitors on the bacteria community structure and their carry-over effects.

Figure 5.6 Color-coded bar plot showing the archaea amplicons as a function of interventions modulating the rumen methanogens in control (Ctrl) and treatment (Trt) calves during different sampling times. Data shown in columns correspond to the number of total Illumina read numbers for each OTU identified (Y axis) within each group of calves at different times of rearing (X axis). Calves are arranged in a control (Ctrl) and treatment (Trt) group. Chloroform (CF) and 9,10-anthraquinone (AQ) were applied to the concentrate and total mixed ration diet (TMR) until wk 14. Calves during the rearing time in both groups were fed as follows: milk twice a day and *ad libitum* concentrate diets at wks 2 and 4; milk once a day and *ad libitum* concentrates and TMR diets at wks 6, 8 and 10; concentrates step-down weaned and TMR diets fed *ad libitum* until wk 14; and grazing a mixed sward of ryegrass/clover as one mob at wks 24 and 49.

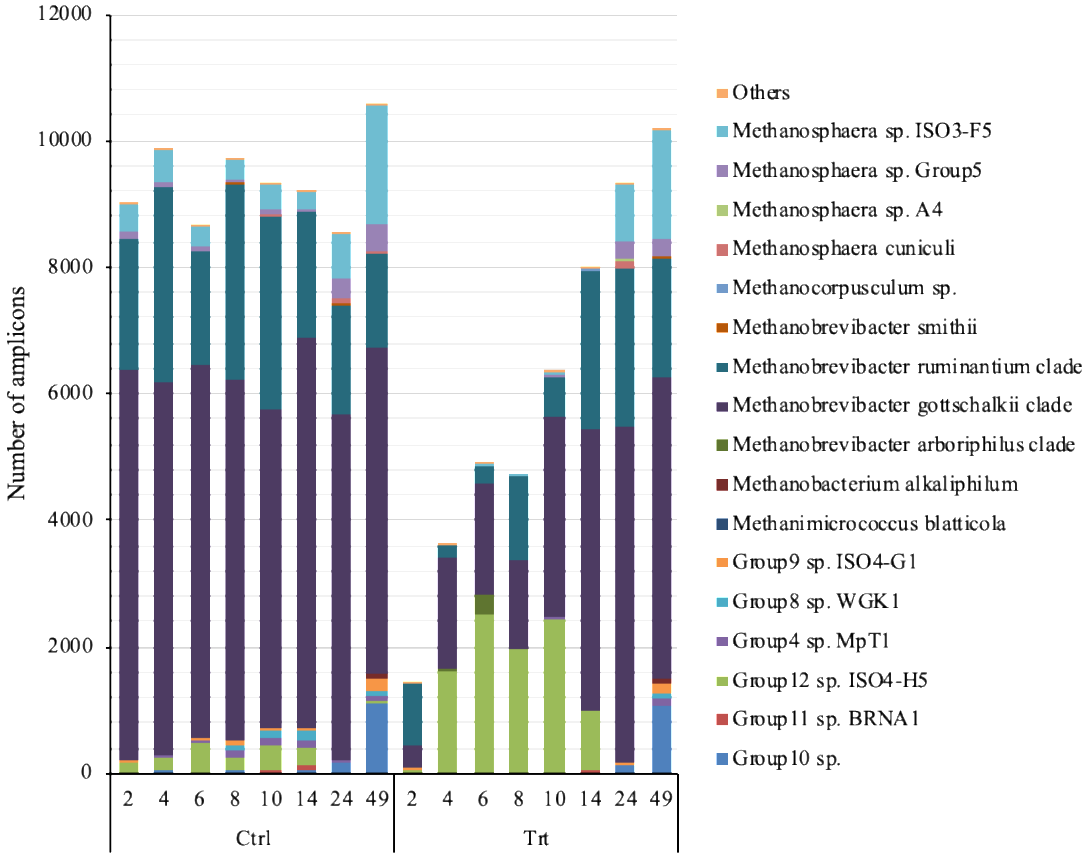


Table 5.6 Effect of methane inhibitors[1] on the abundant archaeal species[2] at different sampling times[3]. Results[4] are natural log back transformed means and standard error of the differences (SED), P-value for treatment (Trt), time (Time) and interaction (Int).

Relative Abundance		**Time (wk)**									**P-value**		
Taxa- Genus	**Treatment**	**2**	**4**	**6**	**8**	**10**	**14**	**24**	**49**	**SED**	**Trt**	**Time**	**Int**
Methanobrevibacter gottschalkii clade	Ctrl	46.52	49.71	52.03	45.77	46.54	59.39	59.89	48.63	15.849	0.028	0.061	0.183
	Trt	31.59	45.54	14.89	21.23	36.53	40.96	56.46	46.19				
Methanobrevibacter ruminantium clade	Ctrl	2.75 [a]	4.55	4.80	13.07	16.97	10.05	15.56	11.72	3.943	0.843	0.016	0.011
	Trt	26.58 [b]	7.74	2.74	7.06	7.37	3.81	23.07	16.78				
Methanomassiliicoccales Group12 sp. ISO4-H5	Ctrl	0.11 [a]	0.33 [a]	1.63 [a]	0.96 [a]	1.36 [a]	1.51	0.07	0.13	1.460	0.000	0.000	0.000
	Trt	7.48 [b]	7.52 [b]	15.37 [b]	15.02 [b]	8.22 [b]	4.72	0.06	0.17				
Methanosphaera sp. ISO3-F5	Ctrl	2.03	2.99	2.73 [a]	1.73	3.04 [a]	2.18 [a]	7.01	16.97	1.531	0.004	0.000	0.000
	Trt	1.03	0.98	0.22 [b]	0.68	0.67 [b]	0.07 [b]	8.91	16.51				
Methanomassiliicoccales Group10 sp.	Ctrl	0.05	0.10	0.02	0.07	0.12	0.15 [a]	1.48	9.08	0.470	0.397	0.000	0.137
	Trt	0.65	0.09	0.06	0.07	0.15	0.03 [b]	0.72	7.96				
Methanosphaera sp. Group5	Ctrl	0.13	0.21	0.11	0.06	0.11	0.07	2.24	2.46	0.281	0.202	0.000	0.569
	Trt	0.54	1.66	0.05	0.05	0.19	0.02	2.80	1.73				
Methanomassiliicoccales Group4 sp. MpT1	Ctrl	0.19 [a]	0.39	0.38 [a]	0.51 [a]	0.81	1.14 [a]	0.09	0.81	0.199	0.051	0.000	0.000
	Trt	1.53 [b]	0.53	0.01 [b]	0.06 [b]	1.24	0.02 [b]	0.12	0.96				

[1] Calves were arranged in a control (Ctrl) and treatment (Trt) group. Chloroform (CF) and 9,10-anthraquinone (AQ) were applied to treated calves in the concentrate and total mixed ration diet (TMR) until wk 14.

[2] Abundant archaea species were defined as organisms with an average proportion ≥ 1.0%.

[3] Sampling times were from 2-49 wks. Calves during the rearing time (wk) were fed as follows: milk twice a day and *ad libitum* control and treatment concentrates at wks 2 and 4; milk once a day and *ad libitum* control and treatment concentrates and TMR diet at wks 6, 8 and 10; concentrates step-down weaned and TMR diets fed *ad libitum* until wk 14; and grazing a mixed sward of ryegrass/clover as one mob at wks 24 and 49.

[4] A repeated measurement analysis was carried out to determine the effect of methane inhibitors on the bacteria community structure and their carry-over effects

5.5 Discussion

This study utilized known methane inhibitors that were incorporated into the solid diet of calves to inhibit methane production so as to investigate impacts on the rumen microbial composition, fermentation profiles, gas emissions and animal growth.

In adult ruminants, the administration of methane inhibitors have shown negative dose effects on DMI and LW (Henderson et al., 2018). In the present study, the inclusion of a CF/AQ mix in the starter and TMR diets did not affect DMI or the growth of treated compared to control calves during the intake of methane inhibitors. The performance in both groups of calves was in accordance with those observed in goat kids drenched with the methane inhibitor bromochloromethane, in which DMI was not affected, (Abecia et al., 2013). However, in goat kids receiving bromochloromethane, the LW of treated animals appeared to be greater, while no such effect was observed in this calf study. In contrast, the administration of AQ at a level of 66 ppm for 6 wks to sheep decreased their average daily gain, corresponding to decreased DMI (Kung et al., 2003). Feed aversion associated with the inclusion of the methane inhibitors in the diet it has not been documented, but studies in adult ruminants have shown that the introduction of novel feeds or feed components might produce neophobia or flavor aversion (Provenza, 1995). This condition may be decreased during early life when herbivores develop greater preferences to food with particular flavors or components in the diets (Provenza and Balph, 1988). In the present study therefore, corresponding DMI levels between groups may have been resulted from the early life conditioning of the treatment group to the flavor of methane inhibitors. These results indicate that the flavor aversion to methanogen inhibitors might influence the nutrient intake and consequently affect animal growth.

Feeding methane inhibitors in the solid feed to calves decreased their methane emissions and increased hydrogen emissions over a period of 14 wks. Similar results have been shown in dairy cows and steers (Knight et al., 2011; Martinez-Fernandez et al., 2016) using chloroform, and in sheep (Kung et al., 2003) using 9-10 anthraquinone. Hydrogen disposal, via molecular hydrogen, was an important part of the changes in fermentation pathways in treated calves. In total, molecular hydrogen accounted for 26% of the hydrogen that was not captured in methane. This proportion of molecular hydrogen is in good accordance to the 15 to 30% value observed by Martinez-Fernandez et al. (2016) in adult

cattle. In response to methane inhibition the proportion of propionate was increased in the current trial. The propionate fermentation pathway consumes hydrogen and is in direct competition with methanogenesis. (Iannotti et al., 1973; Wolin, 1976; Morvan et al., 1996). During propionate formation, pyruvate is reduced to propionate in one of two multi-step pathways (Baldwin et al., 1963), while the reduction of protons (H+) results in H_2 formation in the rumen (Hegarty, 1999; Hegarty and Gerdes, 1999; Wang et al., 2016b). However, because only rumen concentrations were measured in this experiment no quantitative estimate of the hydrogen captured in propionate could be made. Acetogenesis, is another potential pathway of hydrogen disposal in the rumen, but can be excluded here since chloroform inhibits the acetyl-CoA cleavage pathway of acetogens (Scholten et al., 2000).

The use of CH_4 inhibitors did not affect the diversity and richness of the bacterial community in calves. This was a new observation in young calves and was an unexpected result since the rumen metabolite profiles (gases and SCFA) were significantly altered by the two methane inhibitors. The results in the present study agree with observations reported in lambs also receiving methane inhibitor from birth until weaning, in which alterations of the fermentation pathways corresponded to similar bacterial diversity in the rumen (Abecia et al., 2018). Bacterial community richness and diversity was mainly influenced by temporal variation in the present study. Such increases have been shown for growing calves in previous studies (Jami et al., 2013; Dill-McFarland et al., 2017). In addition to animal age, the bacterial community diversity and richness can be affected by diet (Kim et al., 2016; Martinez-Fernandez et al., 2016). However, in our study, animal age and diet composition were confounded, so the effects of time on bacteria community diversity reported here are a combination of animal age and changing diet.

Methanogens normally present in the rumen have been found in neonatal ruminants, indicating that microbial colonization of the gastrointestinal tract (GIT) occurred before or during birth (Guzman et al., 2015). The inclusion of CH_4 inhibitors in the diet of calves from this study reduced the archaeal community richness and diversity as expected since different archaea have different susceptibilities to different inhibitors. However, this returned to control levels 10 wks after treatment ceased. Such observations have been seen previously by Abecia et al. (2013) in goat kids ingesting halogenated compounds that showed reductions in the archaeal diversity while ingesting inhibitors, but 12 wks after treatment ceased, all groups exhibited similar diversities. These studies indicate that in

addition to the inhibition of the archaeal community, the unoccupied H_2 sink is rapidly utilized up by these niche microorganisms after inhibitors are removed. Additionally, the inhibited archaeal community showed rapid adaptation to prevailing dietary and fermentation conditions in the rumen, as shown by the similar archaea diversity and richness when growing ruminants are transitioned onto different diets.

It has been suggested that the composition of ruminal methanogenic community changes as the ruminant matures (Guzman et al., 2015; Friedman et al., 2017). In the present study the beta diversity analysis showed changes in the archaea community as the animal ages, however, it was not possible to elucidate if such changes were a result of changes in age or diet. In adult ruminants, *Methanobrevibacter* spp. and *Methanosphaera* spp., which belong to the *Methanobacteriales*, may represent up to 90% of the rumen archaea, being the most important and dominant archaeal order. This has also been described (Henderson et al., 2015; Seedorf et al., 2015; Friedman et al., 2017) for cattle. In the present study, the addition of CF/AQ in the diet of treated calves decreased the relative proportions of *Mbb. gottschalkii* and *Methanosphaera* ISO3-F5, whereas *Methanomassiliicoccales* Group 12 sp. ISO-H5 was increased more than 5-fold during the CH_4 treatment. Similar results have been noted previously in adult cows, where the infusion of CF via cannula reduced the diversity of the *Mbb. gottschalkii* and *Methanosphaera* spp. (Knight et al., 2011). Additionally, Knight et al. (2011) found that less prevalent archaeal groups became more prevalent when the main groups of archaeal species were suppressed. The most abundant species of *Methanobrevibacter* reduce H_2 and CO_2 to CH_4 (Miller et al., 1986), while *Methanospheara* spp. is a methanogen that reduces methanol with H_2 and is dependent on acetate as a carbon source (Fricke et al., 2006). However, *Methanomassiliicoccales* spp. are obligately hydrogen-dependent methylotrophic methanogens and require compounds like methanol, methylamine, dimethylamine, and trimethylamine as a major energy and carbon source (Lang et al., 2015; Li et al., 2016). In the present study, it is not clear how the use of two different methanogen inhibitors affect the growth and abundance of the different archaea species. Beta diversity showed that in early life the methanogens differed between the treated and the control diets, however, the introduction of a common forage diet increased the methanogen community complexity and reduced this difference. In summary, on average the changes in the archaeal community were mainly driven by animal age or diet offered at the time except for *Mmc.* Group 12,

which proportionally increased during the intake of methane inhibitors, an order discovered only very recently (Borrel et al., 2014).

In the present study, despite the differences in intra-ruminal H_2 concentrations, the bacterial community at the phylum level was similar between groups. This result differed from those reported in fistulated steers that showed increases in abundance of *Bacteroidetes* and decreases in *Firmicutes* in response to a moderate decrease in CH_4 and increase in the intraruminal pressure of H_2 (Martinez-Fernandez et al., 2016). Changes in the abundant bacterial genera were small (Table 5.5) despite the large fermentation shifts observed in the rumen (Table 5.3). Major change (more than 5-fold) in the treated group were only seen in the *Lachnospiraceae* NK3A20 group and P2534-18B5 gut group. The relative abundance of both genera were increased during inhibitor treatment and resultant CH_4 inhibition and have been recently determined to be major groups in Holstein Friesian cattle (Paz et al., 2016). The increase of *Lachnospiraceae* NK3A20 observed in treated calves was in accordance with data from CH_4 inhibition studies in goat kids supplemented with rhubarb (which contains anthraquinone as the active agent) (Wang et al., 2017a). *Lachnospiraceae* spp. possess one of the largest and most diverse glycoside hydrolases (GH) and polysaccharide lyases (PL) repertoires (Seshadri et al., 2018). Additionally, this bacterial family has the capacity to ferment polysaccharides or fumarate to acetate, succinate, and CO_2, while no H_2 is formed (Janssen and Hugenholtz, 2003). On a lower level, CH_4 inhibition in the present study appeared to increase *Rikenellaceae* RC9 gut group, *Roseburia* and *Succiniclasticum* to different extents. This is similar to the observation where these groups were affected by the ingestion of concentrate diets (Henderson et al., 2015), in the sense that these diets are known to lead to lower methane emissions, because starch utilizing bacteria tend to produce less H_2 (Stewart et al., 1997; Janssen, 2010). Therefore, the metabolism of these bacteria is not likely to be affected by the partial pressure of H_2 in the rumen.

Cellulolytic microbes like *Fibrobacter* were not affected by CH_4 inhibition, which is in accordance with previous observations done by Martinez-Fernandez et al. (2016) in steers dosed with 1.6 and 2.6 g of CF/100 kg of LW. These results indicate that *Fibrobacter* are not affected by H_2 accumulation (Wolin et al., 1997) because its major end product is succinate (Abdul Rahman et al., 2016). In contrast, growth of cellulolytic *Ruminiclostridium* and *Ruminococcus* is reduced with increased H_2 concentrations. These

genera are known to possess cellulosomes and degrade cellulose (Bensoussan et al., 2017; Bule et al., 2018). Inhibition of these cellulolytic bacteria genera has been observed previously during *in vitro* (batch and continuous incubations) experiments, in which inhibition of CH_4 production with bromochloromethane reduced *Ruminococcus* spp., while *Fibrobacter* numbers increased (Goel et al., 2009). The class *Clostridia* predominated the hydrogen-producing cellulolytic bacteria, and the hydrogen accumulation significantly inhibited their hydrogen-producing activity (Lay, 2001). The partial pressure of hydrogen influences the metabolism of these fiber-degrading genera by inhibiting NADH oxidation, while H_2 is diverted to form other end products such as succinate and ethanol (Wolin et al., 1997). Therefore, the degradation of cellulose by these *Ruminiclostridium* and *Ruminococcus* (*Ruminococcaceae* family) may be impaired by the increased H_2 pressure in the rumen. However, the fact that the DMI was similar both groups of calves and the LW only decreased by 2.1% in the treated group at 24 wks indicates that such an effect is not major in the rumen, e.g. differences may be due to differential gut fill at the time of weighting.

The supply of CH_4 inhibitor in the starter diets of calves produced fluctuations in the relative abundance in some of the most abundant bacteria genus. Compared to controls, the proportions of *Prevotella* 1 were increased in some inhibitor-treated animals but decreased in others. Such fluctuations of *Prevotella* spp. have also been observed in goat kids fed rhubarb (Wang et al., 2017a) and may be a reflection of different species composition and susceptibility. *Prevotella* spp. have the capacity to degrade a broad spectrum of polysaccharides and peptides in the diet, possessing enzymes that also degrade endogenous glycans secreted in the saliva (Purushe et al., 2010; Seshadri et al., 2018), which may explain their abundance in the rumen microbiota (Henderson et al., 2015). There is not a clear explanation for the partial decrease of *Prevotella* 1 during wks 6 and 8 in treated calves, however, this observation could be associated with the inclusion of a TMR diet. After wk 10, the genera present at similar proportions in the treatment as well as the control group, showed a marked metabolic capacity change to adapt to increasing H_2 pressure in the rumen by likely producing more propionate and succinate, and releasing less hydrogen (Marounek and Dušková, 1999). Therefore, the administration of AQ/CF in the diet of calves produced minor changes in the composition of the abundant bacteria, while large shifts in ruminal fermentation were observed. The adaptive changes in rumen microbial ecology and metabolism due to a complete inhibition of methanogens is not well

understood, although the increase in propionate production has been observed in many studies. Further investigations are necessary to elucidate the mechanisms that the rumen bacterial population has to adapt to high intra-ruminal H_2 pressure, produced by the inhibition of methanogen microbes with CF/AQ in the diet, and develop new strategies for imprinting the rumen microbiota.

5.6 Conclusions

Collectively, this study showed that CF/AQ mix applied to the starter diets during early life did not affect the DMI and growth of dairy calves. This animal productivity observation is important considering the research efforts taking place to reduce methane emissions from livestock. Additionally, the proportions of the highly abundant archaeal species were considerably impacted, resulting in decreased CH_4 emissions, but few changes in the composition of the abundant bacterial community. The observed differences in the abundant bacterial community are likely due to the altered rumen fermentation profiles brought about due to reduced methanogenesis and increased hydrogen partial pressures. After discontinuation of the treatment, a short-term lasting effect was observed on the archaeal and bacterial community and related rumen function. This observed effect in calves during early life however did not have a long-lasting effect on ruminal metabolism and the rumen microbiota. The dynamic interaction within and between the complex bacteria-archaea microbiota during rumen development and the relevant factors and mechanisms require further investigations. This study provides information on the establishment of the rumen bacterial and archaeal community and the related structural and metabolic variations associated with dietary interventions. Further research is needed to elucidate the dynamic interactions of the structural composition and metabolism of the rumen bacteria and archaea, as well as the long-term impact of manipulation during early life on ruminant production.

Chapter 6: General Discussion & Conclusions

This chapter compiles and highlights significant research findings obtained from Chapters 2-5. This Chapter also provides a discussion of the results from all experimental work pertaining to the main objectives outlined in Chapter 1. Finally, study limitations and implications are discussed, and recommendations for future directions are presented.

6.1 Overview

In adult ruminants, the impact of microbial manipulation on productivity and greenhouse gas emissions is limited by the resilient nature of the microbial ecosystem of the rumen. Research efforts to date have only achieved short- or non-lasting effects after microbial interventions. Microbial colonization of newborn ruminants occurs soon after birth, providing a potential window of opportunity to manipulate the rumen microbiota in order to induce a change that lasts into adult life. The ability to manipulate the rumen microbiota could offer an opportunity to enhance animal performance and reduce greenhouse gas emissions especially in pastoral systems where dietary interventions are limited.

The rumen microbiota of young ruminants has been manipulated using dietary intervention or inhibiting certain groups of microbes. However, most previous studies have only involved short-term monitoring of the microbiota and have thus provided little information on the extent to which these interventions persist.

To investigate the impact of rumen manipulation, a series of separate studies, with distinct objectives, are described in this thesis: (i) to determine the effect of early weaning from milk on rumen development and function in artificially-reared lambs; (ii) to characterize the impacts of early weaning in lambs from (i) above on the composition of the rumen microbiota at wk 4 and 16 of rearing and to examine potential relationships between rumen microbiota composition and rumen fermentation profiles; (iii) to assess the effect of divergent rearing regimes (milk and solid feed diets) in the first 7 months of life on the short and long term stability of the microbial community structure, fermentation profiles and methane (CH_4) emissions in the rumen of dairy-beef calves; and (iv) to evaluate the effect of CH_4 inhibitors on rumen microbiota composition, rumen fermentation profiles, and rumen gas emissions during interventions and 35 wks after treatment cessation in dairy calves.

6.2 Findings and implications

This thesis presents several significant research findings, summarized below:

In Chapter 2, the effect of weaning artificially-reared lambs from milk replacer at 4 (EW) and 6 wks of age (Ctrl) on DMI, fermentation profiles, rumen metabolism and rumen

morphology were evaluated at wk 4 and 16. During wk 4, EW lambs had greater concentrations of plasma β-Hydroxybutyrate (BHBA) than Ctrl lambs, indicating greater ketogenesis in rumen epithelial cells and thus earlier metabolic development of the rumen. No weaning age effects were observed for DMI, fermentation profiles and rumen morphology at wk 4 and 16 of rearing. These results indicate that morphological and physiological development of the rumen can support weaning of artificially-reared lambs at 4 wks, using a step-down weaning system. Additionally, comparing the morphology of the different rumen sacs showed that papillae morphology and muscular thickness differed across rumen sites at wk 4 and wk 16 of rearing, indicating that future studies in lambs evaluating the impact of different diets should involve representative sampling across the rumen to more accurately study rumen development and ontogenic changes (Chapter 2).

In Chapter 3, the impact of weaning age in lambs (from Chapter 2) on the rumen microbiota within the first 16 wks of rearing was assessed. The diversity of the bacterial community in post-nursing lambs at 3-4 days of age was comparable to the bacterial community diversity observed in ~4-week-old artificially reared lambs and post weaning (~16 wks) lambs on pasture. However, transitioning lambs from starter diets onto forage-based feed in grazing conditions was associated with an increase in the diversity of the rumen bacterial community. The microbiota in 3-4-day-old lambs was characterized by a highly cellulolytic flora. Proportions of saccharolitic bacteria were greater in ~4-week-old lambs that received a high proportion of concentrates in their diet, while hemicellulolytic and cellulolytic bacteria genera were greater in ~16-week-old pasture fed lambs. The age at weaning did not affect archaea diversity of lambs across the different stages of rearing. The genus *Methanobrevibacter* (*Mbb.*) was not affected, however, the proportions of the less abundant *Methanomassiliicoccales* (*Mmc*.) and *Methanosphaera* (*Mph*.) were increased in ~4- and ~16-weeks-old lambs, respectively. These results indicate that weaning lambs at 4 or 6 wks of rearing has little effect on the microbiota diversity and composition between groups at ~4 and ~16 wks of age, when lambs were artificially reared from 3-4 days of age. The major changes in the diversity and composition of the microbiota were observed between measurements, which represent a combination of animal age and dietary changes. A correlation analysis indicated that in these lambs the proportions of *Bacteroidetes* (e.g. *Prevotella*), were positively associated with total concentrations of ruminal SCFA concentrations and proportion of propionate. Whereas proportions of ruminal genera from *Firmicutes* were positively correlated with the proportions of acetate,

butyrate, isobutyrate and isovalerate. These results indicated that increases in the proportions of abundant phyla *Bacteroidetes* and *Firmicutes* were associated with changes in fermentation profiles. The high abundance of these two phyla may influence fermentation of the diets entering the rumen, affecting the concentrations and proportions of SCFA, nutrient supply, for the rumen development and metabolism.

In Chapter 4, the effect of divergent rearing regimes (milk and solid feed diets) in the first 7 months of life was assessed on the short- and long-term stability of the microbial community structure, fermentation profiles and CH_4 emissions in the rumen of dairy-beef calves. Solid diets rich in starch favored the growth of amylolytic bacterial genera, whilst increased forage intake resulted in greater proportions of cellulolytic and hemicellulolytic bacterial genera. However, the largest effect of the dietary regimen was observed for archaea where the community in starch fed animals was dominated by *Mbb. boviskoreani*, while the methanogens in forage fed calves was dominated by *Mbb. gottschalkii*. Calves consuming concentrates had greater solid feed intake and total SCFA concentration, but lower acetate:propionate ratio (A:P) and CH_4 yields than calves receiving high milk volumes and forage diets. After weaning each group was split into a high- and a low-quality pasture group. The high-quality pasture group had greater and lower proportions of hemicellulolytic and cellulolytic bacteria, respectively, compared to calves grazing low-quality forages. The ingestion of low fiber high-quality pasture showed high proportions of *Mph.* spp. but low proportions of *Mmc.* spp. in the rumen of calves. The consumption of high-quality forages resulted in greater DMI and concentration of SCFA, and lower A:P, but no differences in CH_4 yield. During the third period, all calves grazing a similar forage quality showed no differences in DMI, fermentation profiles and microbial community composition. In none of the data sets was it possible to detect an effect that transferred form the earlier treatment into the next one. These results showed that the microbial composition and associated fermentation end-products were driven by the dietary regimen, and that dietary interventions from wk 1 to 30 of age (this should be the end of summer grazing) did not lead to microbial and rumen function change by 41 wks (end of the measurement period).

In Chapter 5, the effect of methanogen inhibitors added to the starter diets of calves was evaluated on rumen microbiota composition, rumen fermentation profiles, and rumen gas emissions over the pre- and post-weaning. Calves consuming methanogen inhibitors in

starter diets produced 90% less methane compared to the controls, but these animals released large amounts of molecular H_2. They had reduced proportions of H_2-producing and increased proportions of H_2-consuming bacteria compared to control calves. CH_4 inhibitor, decreased the relative abundance of *Mbb.* spp. and *Mph.* spp. but increased the proportion of *Mmc.* spp.. DMI and total SCFA concentrations were similar between groups but the A/P ratio was decreased during methane inhibition. Differences in microbial community composition, fermentation profiles and gas emissions were observed two wks after cessation of inhibitor treatment, however, no effects were detected 14 and 39 wks after weaning. These results indicated that inhibition of methanogenic archaea increased the intra-ruminal pressure of H_2, reducing the production of H_2 in the rumen by changing fermentation pathways, while have only minor effects on the composition of the bacterial microbiota. In contrast pronounced effects were observed on the archaeal community. However, when the treatment stopped, so did all the metabolic effects and the archaeal community rebounded.

This work showed that early weaning of lambs improved morphological and functional development of the rumen in a manner that supported weaning transition onto solid diets (Chapter 2). The transition from milk to starter is accelerated by reducing the age at weaning through the use of step-down weaning system, practices that could contribute to the optimization of artificial rearing management options for commercial farming systems focused on reducing the cost of rearing. Additionally, future studies evaluating the effect of diet on rumen morphology in young ruminants should include samples from the four ruminal sacs in order to get a representative description of rumen development. The present thesis has also given promising insights into the role of early dietary intervention (Chapter 3 and 4) and methanogen inhibitor utilization on rumen microbial manipulation of young ruminants (Chapter 5). After colostrum intake, dietary interventions produced short-term effects in the rumen microbiota due to the rapid adaptation of the microbes to dietary shifts. Inhibition of hydrogenotrophic archaea was associated with changes in the ruminal environment, but there were no significant changes in the more abundant bacterial genera. Further studies are needed to elucidate the metabolic capacities that the abundant bacteria in the rumen have allowing adaptation to a high partial pressure of H_2. The maternal role in the rumen microbial imprint is not clear. However, the observed microbial diversity and composition was more similar between samples from lambs after separation from their dams (grazing on farm) and lambs weaned onto pastures,

which may give some insight of microbial transfer and early imprinting. The study has also given some insights into the associations of abundant bacteria taxa belonging to the phyla *Bacteroidetes* and *Firmicutes* with ruminal fermentation, rumen development and animal performance (Chapter 3). The data from this study thus suggests that, bacteria from these two abundant phyla play a pivotal role in the transitioning of young ruminants from functionally monogastric into true ruminants.

6.3 Discussion

6.3.1 Rumen development

The development of a functional rumen facilitates the weaning transition of a young ruminant from milk into solid feeds (Baldwin et al., 2004). In Chapter 2 of the present work, it was observed that weaning off milk at either 4 or 6 wk of age does not affect rumen histomorphology at week 4, implying that the step-weaning method smoothed the transition into solid feed in both groups. Similarities in rumen epithelium development are explained by the equivalent time of exposure to comparable concentrations of SCFA observed in both groups at week 4 and 16. On the other hand, in this study the SCFA production was not measured, which could explain the increased rumen function in EW lambs when compared to Ctrl lambs at week 4. A sudden increase of SCFA produced in the rumen, as shown by Steele et al. (2012), may result in greater absorption of fermentation end products by the rumen epithelium, despite the ruminal surface area, resulting in greater ketogenic activity and concentrations of plasma BHBA. Therefore, the age and period of exposure to elevated concentrations of SCFA stimulates the ruminal papillae development in lambs; however, accelerated metabolic development of the rumen in EW compared to Ctrl lambs might result from an adaptation mechanism to enable absorption of increased production of SCFA.

Histomorphometry differences across ruminal sacs were evident when lambs consumed different diets, which is consistent with reports in calves (Lesmeister et al., 2004) and red deer calves (Thompson et al., 2008). In the current work, the observed differences may reflect an anatomical adaptation to the diet consumed, whereby 4 wk lambs consuming concentrates and small component of dietary fiber supported a more homogenous rumen morphology compared to 16 wk that grazed in a mixed sward, resulting in a greater diet stratification (Evans et al., 1973; Van Soest, 1994). The observed differences in papillae

development across ruminal sacs have been suggested as an anatomical adaptation to the stratification of the rumen contents (Clauss et al., 2009).

6.3.2 Rumen microbial diversity

In the current work, the dietary management imposed at different ages resulted in changes and adaptations in the rumen microbial communities. In these studies, from birth to two wks of age, young ruminants consumed mainly milk, after which the intake of solid starter diets (mostly concentrate grain-based diets) steadily increased until weaning off milk, when the animals were transferred onto forage diets. In Chapter 3, the microbial diversity in 3-4-day-old lambs was comparable to that recorded at 4 and 16 wks. The fact that at this age the community was highly cellulolytic with no solid food going in the rumen suggests that the community found was an inoculation from an adult animal, the dam, which serves as a founder community rather than being a functional part of the rumen at this stage. The results reported in Chapter 3 indicate that neonatal lambs reared by the mother acquire a complex microbiota, as seen in previous studies involving nursing offspring (Fonty et al., 1987; Abecia et al., 2017).

In adult ruminants, diet has the greatest effect on microbial community diversity and structure in the rumen, as supported by previous studies (Tajima et al., 2001; Fernando et al., 2010; Kong et al., 2010; Henderson et al., 2015; AlZahal et al., 2017). In the present work, the intake of concentrate diets, compared to forage diets, resulted in a lower microbial diversity in the rumen of lambs (Chapter 3) and calves (Chapter 4 and 5), which agrees with observations in calves and goat kids consuming diets rich in non-structural carbohydrates (Kim et al., 2016; Abecia et al., 2017). The low microbial diversity was likely a reflection of the intake of a reduced variety of substrates available in the concentrate-based diets (high starch) compared to the forage-based diets (high structural and non-structural carbohydrates) to the bacteria. Reduction in microbial diversity was mainly driven by a low richness (number of bacteria genera and archaea species) coupled by the increase in dominance of bacterial genera, i.e. *Prevotella*, and archaea species, i.e. *Methanobrevibacter* spp., similar to what was observed in goat kids (Wang et al., 2017a). In contrast to the dietary treatments, methanogens inhibitors did not affect the bacterial diversity and increased the diversity of the archaeal community, which were the target organisms for the inhibitors, so this effect was not unexpected. This indicates that a change

in diet, substrate structure changes the microbial community, while a change in molecular hydrogen/electrons only causes the existing microbes to change their metabolic pathway.

6.3.3 Bacterial community

Variation in the composition of the bacterial community at the phylum and genus level in young ruminants across trials are likely to be caused by differences in feeding practices (Chapter 3), diet composition (Chapter 4) and methanogen inhibitors (Chapter 5). These factors affecting the bacterial community in the present work were similar to those reported in different species of ruminants across the world, in which variations in microbial community composition were likely to be caused by differences in diet, climate, and farming practices (Henderson et al., 2015).

In the current work, the complexity at the phylum and genus level of the bacterial community in four to five day-old lambs (Chapter 3) was similar to that reported in dairy calves of a similar age (Jami et al., 2013; Rey et al., 2014; Guzman et al., 2015), in which all major types of rumen bacteria, including proteolytic and cellulolytic species, as well as some niche specialists, were present in the rumen microbiota. In neonatal lambs from the current work, the similar proportions of cellulolytic bacteria, i.e. *Fibrobacteres* and *Tenericutes*, to those in the oral cavity of adult ruminants (Kittelmann et al., 2015), coupled with the low abundance of *Spirochaetes*, which increases during cellulose degradation (Stanton and Canale-Parola, 1980), may indicate a vertical transfer of microbiota from the mother. The presence of this complex and specialized array of microorganisms transferred from the mother can allow the fermentation of substrates entering the rumen, §rumen development and the transition onto solid diets in the neonate ruminant.

In this work, the *Bacteroidetes*:*Firmicutes* (B:F) ratio was independently influenced by dietary management (Chapter 3, 4 and 5). The phylum *Bacteroidetes* is highly represented by *Prevotella* spp., which was the most abundant genus in the rumen across trials from the present work. This observations agreed with the proportions reported in a broad diversity of diets and methanogen inhibitors fed to both young (Rey et al., 2014; Kim et al., 2016; Wang et al., 2017a) and adult ruminants (Pitta et al., 2010; Henderson et al., 2015; Martinez-Fernandez et al., 2016; Liu et al., 2017b; O'Callaghan et al., 2018). *Prevotella* is characterized by its high degree of genetic divergence (Bekele et al., 2010; Rubino et al., 2017). Additionally, members of this genus are characterized for their fictional diversity to different substrates (Bekele et al., 2010; Seshadri et al., 2018) and

adaptation to high H_2 levels by shifting fermentation to succinate and propionate (Mitsumori et al., 2012; Denman et al., 2015). These characteristic allowed *Prevotella* spp. to become abundant under different diets, composition (Chapter 3 and 4) and high intra-ruminal H_2 pressure when methanogens were inhibited (Chapter 5). Whilst bacterial genera from the phylum *Firmicutes* also increase during the intake of concentrate diets. Elevated proportions of saccharolytic bacteria, i.e. *Roseburia*, *Selenomonas* 1, *Lachnospiraceae* NK3A20 group and *Erysipelotrichaceae* UCG-002, enhanced by the intake of high grain diets and CH_4 inhibitor (Chapters 3, 4 and 5). The results from the present work agreed with those observed in bovines consuming high concentrate diets (Mao et al., 2012; Henderson et al., 2015) and in goat kids receiving CH_4 inhibitors (Wang et al., 2017a). The increased abundance of these genera in young ruminants fed concentrate-based diets and CH_4 inhibitors was favored by their capacity to degrade non-structural carbohydrates and produce less or no H_2 (Stewart et al., 1997; Janssen, 2010). These finding indicate that the bacterial phyla composition is modified by the ingested ratio of forage to concentrate in the diet, e.g. in mixed diets, steady decreases in the forage:concentrate ratio result in a rapid increase of *Prevotella*, however, based on evidence from studied in sub-acute rumen acidosis (SARA), as ruminal pH reaches rumen acidosis, the more resistant *Firmicutes* genera may increase their abundance in the rumen.

The transition of calves and lambs from concentrate onto forage-based diets induced an increase of the abundance of cellulolytic and hemicellulolytic *Bacteroidetes* and *Firmicutes* bacteria, i.e. *Fibrobacter*, *Rikenellaceae* RC9 gut group, *Ruminococcaceae* (NK4A214 group and UCG 014), *Prevotellaceae* (UCG 003), *Bacteroidales* S24-7 group, *Christensenellaceae* R-7 group, *and Ruminococcus* 1 (Chapters 3, 4 and 5). These results are in agreement with reports from young ruminants consuming higher ratios of forage:concentrate in the diet (Kim et al., 2016). The intake of forage rich in hemicellulose and soluble carbohydrates also resulted in greater abundances of *Pseudobutyrivibrio*, *Selenomonas* 1 and *Lachnospiraceae* NK3A20 group (Chapters 3, 4 and 5), which degrade a broad range of substrates (Rainey, 1996; Sawanon et al., 2011; Grilli et al., 2013; Seshadri et al., 2018). The ingestion of diets with a greater diversity of structural carbohydrates favors the development of a more complex bacterial community.

The compilation of these results showed that the dominant bacterial phyla and genus identified in young ruminants in the different trials from this work corresponded to the

dominant bacteria taxa in adult ruminants (Henderson et al., 2015). This abundant bacteria group was present in the rumen prior to solid feed intake, changing the proportional community abundance of saccharolytic, hemicellulolytic and cellulolytic bacteria as the animals transitioned across diets of differing composition. The addition of methanogen inhibitors in the diet produced few changes in the relative abundance of the dominant group of bacteria, showing only increases in the population of hydrogenotrophic bacteria genera and decreases in the population of hydrogen-producing bacteria genera. Therefore, the acquisition of the microbes during early life from the mother may set a range of microbes able to degrade different food substrates and that may facilitate the transitioning from milk into solid diets.

6.3.4 Archaea community

In young ruminants from the present work, the composition of the archaea community was dominated by species belonging to the orders *Methanobacteriales* and *Methanomassiliicoccales*, similar to findings in adult ruminants (Seedorf et al., 2015). *Mbb. gottschalkii* and *Mbb. ruminantium* were the most abundant archaea species (Chapter 3, 4 and 5), as observed in adult ruminants of different species and consuming different pastures and pasture:concentrate mixed ratios (Henderson et al., 2015; Seedorf et al., 2015). These two archaea species are hydrogenotrophic, and their abundance is increased in diets with high proportions of acetate. Whilst, in Chapter 4, *Mbb. boviskoreani* predominated in calves fed high proportions of concentrates in the diet, with a ruminal environment characterized by high concentrations of total SCFA and proportions of propionate. The elevated proportions of *Mbb. boviskoreani* in calves from Chapter 4 agreed with reports in beef steers consuming high grain diets (Lee et al., 2013). However, this archaea species was not observed in lambs from Chapter 3 and calves from Chapter 5 consuming high proportions of concentrate in the diet when reared indoors, which differed from calves in Chapter 4 on high concentrate diets reared on paddocks. These results may indicate that this archaea species is able to tolerate low ruminal pH, but not below ruminal pH of 5.6 (Lee et al., 2013); however, further studies *in vivo* are required to support this statement when ruminants are fed diets with high proportions of concentrate.

In the low abundant archaea species, *Methanosphaera* spp., a methanol utilizer methanogen (Fricke et al., 2006), increased their abundance during the fermentation of highly digestible diets. Whilst *Methanomassiliicoccales* spp., a methylotrophic

methanogen, had greater abundance in low digestible diets. These findings indicate that changes in the proportions of low abundant methanogens may be produced by the intake of precursors of methanol and methylamines in the diet.

The intake of methanogen inhibitors in the starter diets showed greater inhibition effects in the proportion of species from the order *Methanobacteriales* than the order *Methanomassiliicoccales* (Chapter 5). These methanogen inhibitors interfere with methyl-coenzyme M reductase (Mcr), which is the enzyme catalyzing the last step in methanogenesis and is conserved in all methanogenic archaea. These compounds may also affect the transcripts of key enzymes such as methyl-H4MPT:HS-CoM methyltransferase (mtrA), involved in methanogenesis from H_2 and CO_2 by *Methanobrevibacter*; methanol-specific methyltransferase transcripts (mtaB), involved in methanogenesis from methanol by Methanosphaera and Methanomassiliicoccales; methylamine-specific methyltransferases (mtMA), involved in methanogenesis from methylamines by Methanomassiliicoccales (Söllinger et al., 2018). However, further work is needed to explore the effect of methanogen inhibitors on the transcripts of key enzymes in taxon-specific methanogenesis pathways.

This work has shown that *Methanobrevibacter* is the main genus across diets in young ruminants, and that the low abundant archaea genus *Methanosphaera* and order *Methanomassiliicoccales* are affected by the intake of high and low digestible diets respectively. Additionally, the intake of methanogen inhibitors had greater effects on the relative abundance of the order *Methanobacteriales* than the *Methanomassiliicoccales*.

6.3.5 Rumen microbiota and host interactions

The establishment of the rumen microbiota is a co-evolutionary process with a two-way interaction between the host and the microbes in the rumen (Van den Abbeele et al., 2011). Rumen microbiota -host interactions are affected by external factors such as maternal and environmental microbiota, diet, and microbial inhibitors during early life (Chapters 3, 4, and 5). The correlation analysis between rumen microbiota and SCFA in lambs fed either concentrates or forages showed that increases in the abundance of *Prevotella* were associated with high propionate proportions and total concentrations of SCFA. Although this is not proof for causation, similar observation have been made in 6-week-old lambs consuming concentrates (Wang et al., 2016c). The *Prevotella* genus is numerically predominant in both forage-fed and grain-fed ruminants, and has the capacity

to utilize a broad range of substrates as energy sources, producing succinate as the major fermentation end product which is rapidly converted to propionate (Purushe et al., 2010). Whilst, a greater abundance of genera from the phylum *Firmicutes*, i.e. plant cell call degrader *Butyrivibrio* 2 and *Pseudobutyrivibrio*, were associated with higher abundances of isoacids, acetate and butyrate. These results were similar to the associations reported by Wang et al. (2016c) in lambs fed starter concentrates at 6 wks of age. Cellulolytic bacteria utilize isoacids for protein synthesis during the degradation of structural plant polysaccharides, with acetate as their major end product (Russell and Sniffen, 1984; Nagaraja et al., 1997). Genera such as *Butyrivibrio* and *Pseudobutyrivibrio* are butyrate producers (Rainey, 1996Rainey, 1996; Kelly et al., 2010). These results showed that there are strong associations between abundant ruminal bacteria and the relative proportions of SCFA in the rumen. However, the association between rumen microbes and fermentation profiles may differ under different dietary management (Chapter 4 and 5) due to the different functions of species within a genus and the functional redundancy that the bacteria genera have in their metabolism and fermentation end-products (Weimer, 2015; Seshadri et al., 2018).

6.4 Potential limitations and future directions

There are still many gaps in our understanding of how the rumen microbiota becomes established and how this process is affected by feeding management of the newborn, rumen fermentation and host performance; areas that require further work to fill knowledge gaps.

Although rumen morphological development, fermentation profiles and microbial community composition were unaffected by weaning age in lambs, early weaning was associated with increased metabolic development of the rumen, as indicated by greater BHBA levels. In pre-ruminants, higher plasma concentrations of BHBA suggest increases in the metabolism of SCFA by rumen epithelial cells. Findings in lambs have shown that the increased production of SCFA in the rumen can affect their absorption by the rumen epithelium and ultimately the capacity of the epithelial cells to produce ketone bodies (Steele et al., 2012; Sun et al., 2018). However, to understand how early feeding strategies and microbial fermentation (amount of total SCFA produced) affect rumen development, future studies in intra ruminal concentrations and production of SCFA coupled with transcriptomic analysis of the rumen epithelium should be considered. The aim should be

to elucidate the molecular mechanism responsible for the up- and down-regulation of cellular development, and the absorption and metabolism of different intra ruminal concentrations of SCFA. This knowledge will help to better understand how the combined effects of exposure time with intraruminal production and concentration of SCFA may affect the development and metabolism of the rumen epithelium. Additionally, understanding how the production of fermentation metabolites affect the expression of genes in the ruminal epithelium will provide new insights into nutritional interventions during rumen development to facilitate the weaning process in the young ruminant.

The diversity and composition of the rumen microbiota of three to four-day-old lambs was associated with the potential role that the mother may play during the microbial imprinting in the newborn. Recent studies on microbial colonization of the rumen have indicated that the ruminant fetus has a sterile rumen during the last third of gestation (Malmuthuge and Griebel, 2018). It has not been elucidated when the microbial colonization of the rumen takes place, however, the presence of ruminal microbes can be detected a few minutes after birth (Guzman et al., 2015), which is further enriched by microbial transfer from the mother during nursing (Fonty et al., 1987; De Barbieri et al., 2015; Abecia et al., 2017). In Chapter 3, the rumen microbiota of the mother was not characterized, which limits the assumptions of a microbial transfer and potential imprint from the mother to the young lambs. Therefore, further studies on rumen microbial establishment in the newborn ruminant may require the analysis of the microbiota (e.g. fecal, oral, vaginal) of the mother, especially if the offspring was naturally born or nursed by its dam during the first days of life.

The use of qualitative techniques, i.e. sequencing of the 16S rRNA gene with the Illumina platform, showed considerable changes in the proportions of prokaryote microbes when young ruminants were offered contrasting diets (Chapter 3 and Chapter 4) and CH_4 inhibitors (Chapter 5). The consumption of diets rich in concentrates produced higher proportions of *Prevotella* 7 and *Mbb. boviskoreani* in calves from Chapter 4, which contrasts to the observed proportions of *Prevotella* 1 and *Mbb. gottschalkii* observed in lambs from Chapter 3 and calves from Chapter 5. Increases of fiber in the diet showed greater proportions of cellulolytic bacteria (Chapter 3, 4 and 5), whilst the intake of CH_4 inhibitors altered the relative proportions of archaea genera and H_2-producing and -utilizing bacteria (Chapter 4). However, it was not assessed the bacterial diversity within and

between samples, and the metabolic capabilities associated with the bacterial microbiota were inferred during treatment interventions (Chapter 3, 4 and 5). Assessing of the functional attributes of the rumen microbiota is essential for understanding their role on host metabolism. The metabolic capacity of the microbiota can be inferred or catalogued from 16S rRNA gene and shotgun metagenomics libraries, respectively (Jovel et al., 2016; Quince et al., 2017). The 16S rRNA gene sequencing, which profile selected organisms or single marker genes, rely on the correlation between phylogenetic trees and clusters of genes shared between taxa (Langille et al., 2013). Shotgun metagenomics, on the other hand, delivers a direct assessment of the functional attributes of the microbiome (Riesenfeld et al., 2004; Sharpton, 2014), with results depending on sequencing depth. Therefore, the integration of full 16S rRNA genes and Shotgun metagenomics sequencing (metagenomes) may help to profile taxonomic composition and functional potential of microbial communities during dietary interventions and CH_4 inhibitors.

The current work attempted to link functional metabolic aspects associated with the most abundant components of the rumen microbiota in young ruminants fed different diets (Chapter 3, 4 and 5) based on the assumptions from previous studies on rumen microbiology and rumen fermentation (e.g. Seedorf et al., 2015; Henderson et al. 2015; Wang et al., 2016; Seshadri et al., 2018; Söllinger et al., 2018). In Chapter 3, rumen fermentation characteristics were similar despite differences in the bacterial community structure produced by the intake of concentrates or forages. In Chapter 4, the intake of contrasting diets depicted changes in the bacteria and archaea community composition and resulting fermentation profiles, whilst differences in forage quality only affected the bacteria community and resulting fermentations. In Chapter 5, similar bacteria community composition produced different fermentation profiles, due to the increased H_2 partial pressure produced after the archaea community was changed using CH_4 inhibitors. However, results from the different trials in the present work did not elucidate how dietary rearing management affected the microbial community function-structure aspects impacting rumen ecology, identifying the microorganisms that participate in certain steps of the anaerobic degradation pathway. The necessity for understanding the complexity of the dynamic microbial ecosystem and their metabolism in the rumen requires the use of meta-omics techniques (Wallace et al., 2017). Current metagenomics technologies permit the identification of new bacterial genera (Henderson et al., 2019) and the type of genes that they possess and function (Seshadri et al., 2018; Söllinger et al., 2018). The use of

metagenomics and metatranscriptomics approaches have revealed high bacteria functional redundancy at several steps of the anaerobic degradation pathway in the rumen and functional guilds have shown high organismic diversity, with individual taxa being replaceable by others (Söllinger et al., 2018; Wirth et al., 2018). Therefore, gaining insight into the rumen's complex microbial community interactions and metabolism are important for improving our understanding of food digestion, animal production and reduction of greenhouse gas emissions. Further studies might require the use of meta-omics tools, i.e. metagenomics, metatranscriptomics and metabolomics, to increase the perceptions of the dynamic metabolism and interaction of the establishing rumen microbiota during dietary interventions and/or rumen development in young ruminants.

In Chapter 3, the correlation analysis between rumen bacteria and rumen morphology provided insights of potential associations during rumen development. A likely explanation to the observed results could be the indirect effect of the rumen bulk microbiota through the production of SCFA affecting the development of the ruminal epithelium. However, changes in the microbial composition of the rumen contents were not always related to shifts in rumen fermentation due to the functional redundancy of the ruminal microbial community (Weimer, 2015). Microbes attached to the rumen epithelium interact directly with the rumen epithelium (Chen and Oba, 2012) and changes in ruminal fermentation may affect such interactions (Chen et al., 2011). Future research may need to take place to determine whether changes in epimural microbiota might provide a better understanding of the association between its microbial composition and the rumen epithelial development and function. Additionally, it is of relevance to understand the epimural bacteria metagenome (shotgun metagenomics) and functionality (metatranscriptomics) and the interaction with host rumen epithelial transcripts (Sun et al., 2018) during rumen development and dietary interventions.

6.5 Conclusions

This thesis is one of the first studies to show that dietary manipulation in the young ruminant after colostrum intake from the mother does not produce long-term metabolic effects or leave an imprint in the rumen microbiota. Additionally, it determined that changes in the rumen microbial community are associated with dietary management, and consequently ruminal fermentation, with potential effects on rumen development and animal performance. The prokaryote community in the neonate ruminant has an adult-like

composition after separation from the mother during grazing conditions (first ~4 days of colostrum intake). The composition of the acquired microbial community is affected by the intake of starter diets during artificial rearing, with further compositional changes occurring after transition onto forage diets. Therefore, pre- and post-weaning dietary regimes are unable to leave a microbial imprint in the rumen of young ruminants after transitioning through different diets. The inclusion of the methanogen inhibitors in the starter diets reduces the proportions of abundant hydrogenotrophic archaea species, decreasing CH_4 emissions, but few changes are observed in the proportions of the abundant bacterial community, despite the intra-ruminal increase of the H_2 partial pressure. Changes in the proportions of the dominant archaea community and CH_4 emissions had a short-term lasting post-treatment effect, taking over the unoccupied H_2 sink and returning to control like-levels in the rumen a few wks after treatment termination. Changes in the proportions of the bacterial taxa from *Bacteroidetes* and *Firmicutes* are associated with variations in ruminal SCFA. In general, the rumen microbial community composition in the growing ruminant is diet dependent, with early life differences (from approximately 3-7 days of life) having only negligible effects on the microbiota of the growing ruminant. However, further studies are needed to elucidate the effect of the maternal microbial imprint in the long term for newborn ruminants, and the interactions of the rumen microbiota with the performance of the young ruminant.

The implementation of management strategies to modify the composition of the microbial community in the developing rumen relies on practical applicability, lasting effects in the microbiota and the economic impact generated. Based on the results of the present thesis, it is important to highlight that dietary management strategies, after colostrum intake, can produce short-term changes in the rumen microbiome. However, microbial changes, as a result of dietary interventions during the pre- and post-weaning of young ruminants, can be associated with changes in ruminal fermentation, and potential rumen development and animal performance. Further work needs to focus on interventions in the first few days of life, which may result in a lasting microbial imprint, i.e. modification of the maternal microbiota that is potentially imprinted to the offspring

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Appendix

Appendix A

Table A.1 Sequencing primers used to target theV3-V4 regions of the 16S rRNA gene

Name	Direction	Target	5'-3' Sequence
Ba9F	Forward	Universal bacteria	GAGTTTGATCMTGGCTCAG
Ba515Rmod1	Reverse		CCGCGGCKGCTGGCAC
Ar915aF	Forward	Universal archaea	AGGAATTGGCGGGGGAGCAC
Ar1386R	Reverse		GCGGTGTGTGCAAGGAGC

Appendix B

This appendix corresponds to additional tables and figures from Chapter 3

Table B.1 Effect of weaning age[1] at two sampling times[2] on the rumen bacteria community at the phyla level[3] in lambs. Results[4] are natural log back transformed means and standard error of the differences (SED), P-value for treatment effect (P-Tx), sampling time (P-Tm) and their interactions (P-Int).

	Treatment		Sampling time		Treatment x Time							
Bacteria phylum	**Ctrl**	**EW**	**Wk4**	**Wk16**	**Ctrl04**	**EW04**	**Ctrl16**	**EW16**	**SED**	**P-Tx**	**P-Tm**	**P-Int**
Actinobacteria	0.32	0.48	0.35	0.45	0.34	0.37	0.31	0.59	0.184	0.26	0.43	0.35
Armatimonadetes	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.011	0.44	0.40	0.31
Bacteroidetes	47.26	49.47	53.56	43.17	52.87	54.25	41.64	44.69	8.325	0.67	0.09	0.89
Chloroflexi	0.09	0.03	0.10	0.02	0.16	0.04	0.02	0.02	0.097	0.37	0.28	0.39
Cyanobacteria	0.10	0.20	0.14	0.16	0.11	0.17	0.10	0.22	0.105	0.22	0.76	0.70
Elusimicrobia	0.07	0.15	0.11	0.11	0.04	0.17	0.09	0.13	0.089	0.19	0.96	0.52
Fibrobacteres	0.47	0.44	0.63	0.28	0.72	0.55	0.22	0.34	0.517	0.95	0.35	0.69
Firmicutes	46.59	44.03	40.88	49.73	42.25	39.51	50.93	48.54	8.493	0.64	0.15	0.98
Omnitrophica	0.02	0.09	0.06	0.05	0.01	0.11	0.02	0.08	0.043	0.02	0.67	0.47
Planctomycetes	0.03	0.05	0.04	0.03	0.04	0.03	0.01	0.06	0.033	0.42	0.99	0.23
Proteobacteria	2.04	1.70	1.92	1.82	1.85	2.00	2.23	1.40	0.818	0.58	0.83	0.40
Saccharibacteria	0.02	0.13	0.07	0.08	0.04	0.11	0.01	0.15	0.091	0.12	0.92	0.62
Spirochaetae	1.57	0.96	0.33	2.20	0.34	0.32	2.80	1.60	0.983	0.36	0.01	0.40
SR1_(Absconditabacteria)	0.03	0.03	0.02	0.04	0.01	0.04	0.05	0.03	0.030	0.71	0.52	0.24
Synergistetes	0.08	0.06	0.06	0.08	0.07	0.05	0.09	0.07	0.031	0.35	0.28	0.98
Tenericutes	0.25	0.43	0.28	0.40	0.22	0.33	0.27	0.53	0.157	0.12	0.28	0.50
Verrucomicrobia	0.02	0.21	0.14	0.09	0.00	0.28	0.04	0.14	0.193	0.17	0.69	0.51
Other	1.04	1.53	1.30	1.27	0.93	1.68	1.16	1.39	0.365	0.07	0.88	0.33

[1] Treatment groups corresponded to early weaning (EW; weaning at wk 4) and control (Ctrl; weaning at wk 6).

[2] Rumen sampling times were at wk 4 (Wk04) and 16 (Wk16) of rearing.

[3] The bacteria community corresponded to 17 phyla.

[4] Effect of weaning age, sampling times and their interactions were evaluated using a linear mix effects model with treatment and sampling time as fixed effect, and animal as random effect.

Table B.2 Bacteria genera in different groups of lambs[1] at three rearing times[2]. The bacteria proportions are presented as the mean ± standard error of the difference.

	Wk00	Wk04		Wk16	
Genus	**Baseline**	**Ctrl**	**EW**	**Ctrl**	**EW**
Actinobacteria					
Acidaminococcaceae uncultured	0.00±0.00	0.00±0.00	0.00±0.00	0.02±0.01	0.02±0.01
Actinotalea	0.04±0.05	0.02±0.03	0.06±0.09	0.45±0.58	0.28±0.44
Aeriscardovia	0.00±0.00	0.00±0.00	0.00±0.00	0.01±0.01	0.01±0.01
Bifidobacterium	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Collinsella	0.00±0.00	0.01±0.01	0.01±0.01	0.07±0.05	0.04±0.03
Eggerthella	0.01±0.01	0.02±0.03	0.01±0.01	0.10±0.09	0.09±0.07
Gordonia	0.00±0.00	0.00±0.00	0.00±0.00	0.01±0.01	0.01±0.01
Nocardioides	0.00±0.00	0.00±0.00	0.02±0.03	0.01±0.02	0.01±0.02
Olsenella	0.01±0.01	0.01±0.01	0.03±0.03	0.22±0.46	0.07±0.02
OPB41 uncultured	0.00±0.00	0.00±0.00	0.01±0.02	0.00±0.00	0.00±0.00
Thermobispora	0.00±0.00	0.00±0.00	0.00±0.00	0.01±0.01	0.01±0.01
Trueperella	0.03±0.02	0.01±0.03	0.01±0.01	0.00±0.00	0.00±0.00
Armatimonadetes					
Armatimonas	0.01±0.02	0.01±0.01	0.01±0.01	0.00±0.00	0.00±0.00
Chthonomonadales uncultured	0.01±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Bacteroidetes					
Alistipes	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Alloprevotella	0.81±0.18	0.96±0.95	1.28±0.91	0.50±0.14	0.50±0.13
Arcicella	0.00±0.00	0.02±0.04	0.01±0.01	0.00±0.00	0.00±0.00
Bacteroidales S24-7 group uncultured	0.28±0.16	0.23±0.31	0.86±1.12	3.64±3.15	2.74±1.78
Bacteroides	0.14±0.01	0.69±1.05	0.17±0.11	0.06±0.04	0.06±0.05
Bacteroidetes vadinHA17 uncultured	0.24±0.12	0.14±0.20	0.49±0.33	0.29±0.40	0.32±0.46
BSV13	0.09±0.09	0.03±0.05	0.02±0.03	0.02±0.03	0.10±0.10
Cyclobacteriaceae uncultured	0.05±0.03	0.00±0.00	0.02±0.03	0.00±0.00	0.11±0.28
Flammeovirgaceae uncultured	0.03±0.04	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Fluviicola	0.07±0.09	1.67±2.91	2.95±7.69	0.00±0.00	0.02±0.01
KD1-131 uncultured	0.01±0.00	0.00±0.00	0.01±0.00	0.01±0.01	0.00±0.00
M2PB4-65 termite group ge	0.19±0.16	0.18±0.38	0.07±0.11	0.09±0.20	0.28±0.31
Mucilaginibacter	0.68±0.22	0.56±0.73	1.20±0.70	1.73±0.82	1.90±1.22
Muricauda	0.17±0.11	0.22±0.51	0.25±0.30	0.14±0.11	0.09±0.06
Nubsella	0.59±0.55	0.18±0.43	0.12±0.16	0.04±0.07	0.04±0.07
Odoribacter	0.03±0.01	0.03±0.03	0.13±0.28	0.50±1.06	0.10±0.15
Pedobacter	0.23±0.14	0.20±0.48	0.17±0.27	0.14±0.28	0.43±0.53
Porphyromonadaceae uncultured	0.33±0.06	0.17±0.20	0.40±0.30	0.58±0.59	0.68±0.54
Prevotella	0.14±0.05	0.31±0.74	0.34±0.57	0.01±0.01	0.03±0.04
Prevotella 1	50.0±11.69	48.6±27.63	40.4±20.4	32.7±9.81	29.7±11.23
Prevotella 6	0.13±0.10	0.07±0.14	0.12±0.10	0.03±0.04	0.01±0.01
Prevotella 7	2.73±1.94	1.79±1.52	1.46±1.25	1.58±1.68	0.84±0.22
Prevotella 9	0.14±0.07	0.28±0.47	0.13±0.18	0.07±0.08	0.10±0.18
Prevotellaceae UCG-003	0.69±0.30	0.41±0.59	0.39±0.45	1.22±1.71	2.23±2.93
Prolixibacter	0.13±0.08	0.03±0.08	0.06±0.11	0.02±0.01	0.02±0.02
Pseudopedobacter	0.00±0.00	0.00±0.00	0.00±0.00	0.02±0.05	0.00±0.00
Rikenellaceae RC9 gut group	6.48±0.50	2.50±3.23	7.67±9.69	2.78±0.62	3.29±1.44
Rikenellaceae uncultured	0.07±0.03	0.04±0.09	0.03±0.05	0.10±0.14	0.39±0.55
Tannerella	0.13±0.09	0.08±0.10	0.11±0.10	1.19±0.86	1.39±0.89
vadinBC27 wastewater-sludge group	0.13±0.03	0.07±0.11	0.05±0.05	0.10±0.01	0.09±0.06
WCHB1-32 uncultured	0.40±0.29	0.19±0.34	0.35±0.58	0.02±0.02	0.03±0.03

Table B.2 (Continued)

	Wk00	Wk04		Wk16	
Genus	**Baseline**	**Ctrl**	**EW**	**Ctrl**	**EW**
Chloroflexi					
Anaerolineaceae uncultured	0.09±0.01	0.05±0.08	0.11±0.09	0.07±0.03	0.13±0.09
Cyanobacteria					
FamilyI uncultured	0.31±0.17	0.09±0.15	0.10±0.14	0.00±0.00	0.00±0.00
Gastranaerophilales uncultured	0.18±0.07	0.06±0.10	0.09±0.14	0.01±0.01	0.01±0.01
Obscuribacterales uncultured	0.02±0.02	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Elusimicrobia					
Candidatus Endomicrobium	0.08±0.04	0.01±0.02	0.03±0.07	0.00±0.00	0.00±0.00
Elusimicrobium	0.05±0.02	0.01±0.03	0.03±0.03	0.00±0.00	0.00±0.00
Fibrobacteres					
Fibrobacter	3.37±1.93	1.26±1.93	1.30±1.29	0.66±0.58	0.85±0.94
Fibrobacteraceae uncultured	0.00±0.00	0.00±0.00	0.00±0.00	0.01±0.00	0.01±0.01
Firmicutes					
Acetivibrio	0.14±0.06	0.19±0.32	0.36±0.81	0.17±0.11	0.2±0.08
Acetobacterium	0.00±0.00	0.56±1.43	0.99±2.80	0.01±0.01	0.01±0.01
Acidaminobacter	0.04±0.02	0.01±0.03	0.02±0.02	0.10±0.07	0.14±0.09
Acidimicrobiaceae uncultured	0.01±0.01	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Anaerostipes	0.01±0.01	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Anaerovibrio	0.00±0.00	0.01±0.02	0.00±0.00	0.00±0.00	0.00±0.00
Anaerovorax	0.07±0.04	0.01±0.03	0.04±0.06	0.10±0.05	0.16±0.08
Bacillus	0.29±0.27	0.10±0.17	0.24±0.24	0.12±0.06	0.12±0.04
Blautia	0.13±0.08	0.06±0.08	0.71±1.18	0.09±0.09	0.09±0.08
Brassicibacter	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.01±0.01
Butyricicoccus	0.08±0.03	0.04±0.04	0.08±0.14	0.06±0.07	0.03±0.02
Butyrivibrio	0.06±0.02	0.01±0.01	0.02±0.02	0.04±0.03	0.04±0.02
Butyrivibrio 2	0.48±0.17	0.20±0.27	0.45±0.24	0.75±0.35	1.13±0.55
Caldibacillus	0.00±0.00	0.00±0.00	0.00±0.00	0.01±0.00	0.00±0.00
Caproiciproducens	0.11±0.15	0.02±0.04	0.02±0.03	0.06±0.06	0.05±0.04
Christensenellaceae R-7 group	3.33±0.28	1.74±2.29	3.28±2.56	3.50±1.48	4.44±1.82
Christensenellaceae uncultured	0.27±0.18	0.11±0.15	0.24±0.29	0.04±0.03	0.07±0.06
Clostridium sensu stricto 1	0.01±0.01	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Clostridium sensu stricto 13	0.03±0.02	0.01±0.02	0.01±0.01	0.01±0.01	0.00±0.00
Clostridium sensu stricto 7	0.16±0.21	0.03±0.06	0.04±0.06	0.00±0.00	0.00±0.00
Coprococcus 1	0.01±0.01	0.01±0.02	0.01±0.01	0.01±0.01	0.01±0.02
Coprococcus 2	0.02±0.01	0.01±0.01	0.03±0.05	0.03±0.02	0.03±0.02
Coprococcus 3	0.02±0.02	0.01±0.02	0.17±0.36	0.05±0.06	0.11±0.20
Dorea	0.00±0.00	0.01±0.02	0.01±0.03	0.01±0.01	0.00±0.00
Enterococcus	0.01±0.01	0.00±0.00	0.00±0.00	0.01±0.01	0.04±0.08
Erysipelatoclostridium	0.19±0.18	0.07±0.10	0.08±0.18	0.01±0.01	0.01±0.01
Erysipelothrix	0.09±0.03	0.02±0.04	0.04±0.04	0.04±0.02	0.06±0.04
Faecalibacterium	0.08±0.03	0.02±0.03	0.06±0.08	0.07±0.03	0.07±0.02
Faecalitalea	0.04±0.02	0.02±0.03	0.04±0.06	0.04±0.05	0.04±0.03
Family XIII AD3011 group	0.26±0.10	0.16±0.25	0.37±0.48	0.53±0.33	0.78±0.54
Family XIII uncultured	0.03±0.02	0.01±0.02	0.04±0.04	0.06±0.04	0.12±0.22
Family XIII uncultured	0.08±0.02	0.03±0.03	0.07±0.06	0.10±0.06	0.17±0.08
Fastidiosipila	0.02±0.01	0.02±0.04	0.03±0.03	0.02±0.04	0.09±0.12
Finegoldia	0.01±0.02	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Fusibacter	0.00±0.00	0.00±0.00	0.00±0.00	0.01±0.01	0.01±0.01
Fusicatenibacter	0.09±0.06	0.03±0.06	0.04±0.06	0.08±0.05	0.13±0.12

Table B.2 (Continued)

	Wk00	Wk04		Wk16	
Genus	**Baseline**	**Ctrl**	**EW**	**Ctrl**	**EW**
Geosporobacter	0.06±0.03	0.04±0.08	0.10±0.13	0.13±0.12	0.14±0.06
Howardella	0.03±0.01	0.04±0.05	0.02±0.02	0.12±0.14	0.08±0.06
Hydrogenoanaerobacterium	0.02±0.02	0.00±0.00	0.01±0.01	0.03±0.03	0.01±0.01
Incertae Sedis	0.01±0.00	0.01±0.01	0.01±0.01	0.02±0.03	0.01±0.01
Lachnoclostridium	0.01±0.01	0.11±0.17	0.08±0.15	0.02±0.01	0.02±0.01
Lachnoclostridium 12	0.02±0.01	0.01±0.01	0.01±0.01	0.03±0.02	0.04±0.03
Lachnoclostridium 5	0.07±0.04	0.02±0.03	0.03±0.04	0.03±0.02	0.04±0.03
Lachnospiraceae FCS020 group	0.01±0.01	0.02±0.02	0.06±0.07	0.03±0.02	0.04±0.02
Lachnospiraceae uncultured	0.74±0.65	0.49±0.74	0.30±0.22	1.16±0.60	1.40±0.45
Lachnospiraceae NK3A20 group	1.10±0.90	0.38±0.46	0.44±0.51	0.52±0.40	1.36±1.34
Lachnospiraceae NK4A136 group	0.52±0.16	0.54±1.07	1.48±3.00	0.84±0.69	1.68±2.06
Lachnospiraceae UCG-006	0.03±0.03	0.12±0.27	0.21±0.51	0.01±0.01	0.02±0.03
Lactobacillales uncultured	0.00±0.00	0.00±0.00	0.02±0.06	0.00±0.00	0.00±0.00
Leptospiraceae uncultured	0.06±0.02	0.01±0.02	0.04±0.05	0.02±0.02	0.03±0.03
Mitsuokella	0.03±0.03	0.09±0.21	0.16±0.29	0.58±1.11	0.33±0.45
Mobilitalea	0.02±0.01	0.00±0.00	0.00±0.00	0.00±0.00	0.03±0.02
Moryella	0.08±0.06	0.02±0.03	0.03±0.03	0.01±0.01	0.03±0.02
Oribacterium	0.16±0.14	0.04±0.05	0.31±0.52	0.11±0.12	0.18±0.21
Oscillospira	0.05±0.02	0.18±0.15	0.16±0.13	1.79±1.70	1.88±0.84
Paenibacillus	0.25±0.09	0.16±0.14	0.26±0.34	0.32±0.31	0.37±0.43
Parvimonas	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.03±0.06
Peptococcus	0.05±0.04	0.10±0.09	0.10±0.12	0.09±0.04	0.11±0.04
Pseudobutyrivibrio	0.78±0.39	0.50±0.59	0.41±0.29	3.48±2.92	4.36±2.31
Robinsoniella	0.00±0.00	0.07±0.18	0.14±0.40	0.00±0.00	0.00±0.00
Roseburia	0.25±0.06	0.57±0.48	0.95±1.30	0.98±0.75	0.87±0.39
Ruminiclostridium 1	0.01±0.01	0.00±0.00	0.01±0.01	0.02±0.02	0.05±0.05
Ruminiclostridium 6	0.24±0.09	0.09±0.16	0.18±0.20	0.27±0.20	0.35±0.11
Ruminococcaceae uncultured	1.03±0.14	0.37±0.56	0.39±0.42	0.66±0.23	0.84±0.54
Ruminococcaceae NK4A214 group	1.36±0.32	16.00±25.6	4.29±7.00	5.45±4.91	5.21±2.46
Ruminococcaceae UCG-002	0.63±0.15	0.47±0.57	1.37±1.92	2.16±0.73	3.68±2.73
Ruminococcaceae UCG-005	0.21±0.02	0.16±0.16	0.26±0.23	0.50±0.26	0.81±0.50
Ruminococcaceae UCG-008	0.01±0.00	0.06±0.09	0.03±0.04	0.05±0.08	0.03±0.02
Ruminococcaceae UCG-010	0.66±0.35	0.29±0.34	0.55±0.72	1.01±0.61	1.27±0.21
Ruminococcaceae UCG-014	0.35±0.34	0.22±0.29	0.46±0.73	0.47±0.26	0.75±0.66
Ruminococcaceae uncultured	0.36±0.23	0.55±1.14	4.3±11.71	0.3±0.24	0.30±0.11
Ruminococcus 1	2.55±1.32	1.95±2.31	1.14±0.96	5.19±2.03	6.71±2.54
Selenomonas	3.90±3.83	1.15±2.00	1.44±1.82	10.04±9.2	3.25±2.65
Selenomonas 1	0.20±0.13	0.28±0.20	0.22±0.21	2.33±1.98	3.08±2.72
Selenomonas 3	0.00±0.00	0.02±0.04	0.02±0.02	0.36±0.48	0.21±0.21
Selenomonas 4	0.63±1.09	0.01±0.02	0.00±0.00	0.00±0.00	0.00±0.00
Solobacterium	0.03±0.02	0.02±0.04	0.04±0.05	0.11±0.08	0.09±0.03
Stomatobaculum	0.03±0.01	0.01±0.01	0.01±0.02	0.02±0.01	0.02±0.01
Streptococcus	0.00±0.00	0.01±0.01	0.00±0.00	0.00±0.00	0.00±0.00
Subdoligranulum	0.59±0.62	0.45±0.71	0.31±0.46	0.71±0.76	0.57±0.75
Syntrophococcus	0.10±0.06	4.48±9.12	0.85±1.74	0.03±0.03	0.07±0.06
Thermoanaerobacter	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.01±0.01
Thermolithobacter	0.00±0.00	0.00±0.00	0.00±0.00	0.01±0.01	0.01±0.01
Tyzzerella	0.00±0.00	0.00±0.00	0.00±0.00	0.01±0.01	0.01±0.01
Veillonellaceae uncultured	0.07±0.05	0.04±0.07	0.06±0.06	0.55±0.88	0.42±0.40
Omnitrophica					
Omnitrophica uncultured	0.05±0.04	0.01±0.02	0.06±0.10	0.01±0.01	0.01±0.01

Table B.2 (Continued)

	Wk00	Wk04		Wk16	
Genus	**Baseline**	**Ctrl**	**EW**	**Ctrl**	**EW**
Planctomycetes					
Blastopirellula	0.01±0.00	0.00±0.00	0.03±0.06	0.00±0.00	0.00±0.00
Pirellula	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Singulisphaera	0.00±0.00	0.00±0.00	0.00±0.00	0.02±0.03	0.02±0.03
Proteobacteria					
Acidiphilium	0.01±0.01	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Anaplasma	0.01±0.01	0.00±0.00	0.01±0.01	0.00±0.00	0.00±0.00
bacteriap25 uncultured	0.14±0.15	0.01±0.03	0.03±0.04	0.00±0.00	0.00±0.00
Bradymonadales uncultured	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.01±0.01
Bradyrhizobium	0.02±0.01	0.00±0.00	0.01±0.01	0.00±0.00	0.00±0.00
Brenneria	0.04±0.06	1.89±3.77	5.1±13.23	0.01±0.01	0.01±0.01
Brevundimonas	0.98±0.63	0.48±0.84	0.64±0.91	0.54±0.79	0.23±0.21
Campylobacter	0.02±0.01	0.02±0.03	0.01±0.01	0.00±0.00	0.01±0.01
Candidatus Odyssella	0.02±0.02	0.00±0.00	0.00±0.00	0.01±0.01	0.01±0.01
Comamonas	0.00±0.00	0.03±0.06	0.01±0.02	0.00±0.00	0.00±0.00
Geobacter	0.00±0.00	0.00±0.00	0.00±0.00	0.01±0.01	0.01±0.01
Hoeflea	0.01±0.01	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Hyphomonadaceae uncultured	0.00±0.00	0.00±0.00	0.01±0.01	0.00±0.00	0.00±0.00
LWSR-14 uncultured	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.01±0.02
Mesorhizobium	0.10±0.12	0.03±0.08	0.02±0.04	0.00±0.00	0.01±0.01
Methylocystis	0.02±0.02	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
NB1-j uncultured	1.24±1.11	0.21±0.35	0.17±0.23	0.02±0.03	0.01±0.01
Neisseria	0.01±0.01	0.00±0.00	0.00±0.00	0.01±0.01	0.01±0.00
Nitrosomonadaceae uncultured	0.01±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
OM60(NOR5) clade	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Pararhodospirillum	0.02±0.02	0.01±0.03	0.02±0.04	0.01±0.01	0.01±0.01
Polyangiaceae uncultured	0.04±0.07	0.00±0.00	0.01±0.02	0.00±0.00	0.00±0.00
Pseudohongiella	0.37±0.15	0.08±0.14	0.18±0.25	0.03±0.03	0.04±0.06
Reyranella	0.04±0.05	0.00±0.00	0.01±0.01	0.00±0.00	0.00±0.00
Rhodobacteraceae uncultured	0.02±0.02	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Rhodocyclaceae uncultured	0.01±0.02	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Rhodopseudomonas	0.03±0.05	0.01±0.01	0.00±0.00	0.00±0.00	0.00±0.00
Rhodospirillaceae uncultured	0.01±0.01	0.00±0.00	0.01±0.01	0.01±0.01	0.01±0.01
SAR324 clade(Marine group B) uncultured	0.02±0.02	0.01±0.02	0.02±0.03	0.00±0.00	0.00±0.00
Schlegelella	0.03±0.03	0.00±0.00	0.01±0.02	0.00±0.00	0.00±0.00
Shewanella	0.01±0.00	0.01±0.02	0.02±0.04	0.01±0.01	0.01±0.01
SM2D12 uncultured	0.13±0.15	0.03±0.03	0.03±0.03	0.06±0.05	0.10±0.11
Sva0485 uncultured	0.00±0.00	0.00±0.00	0.00±0.00	0.02±0.03	0.00±0.00
Volucribacter	0.13±0.08	0.03±0.06	0.06±0.09	0.00±0.00	0.00±0.00
Saccharibacteria					
Candidatus Saccharimonas	0.42±0.31	0.10±0.22	0.13±0.27	0.04±0.06	0.38±0.49
Spirochaetae					
Lachnospiraceae uncultured	0.34±0.09	0.49±0.77	0.44±0.59	0.40±0.17	0.46±0.23
Sphaerochaeta	0.00±0.00	0.00±0.00	0.01±0.03	0.00±0.00	0.00±0.00
Spirochaeta 2	0.01±0.00	0.01±0.01	0.01±0.02	0.00±0.00	0.00±0.00
Termite Treponema cluster	0.01±0.01	0.00±0.00	0.01±0.01	0.05±0.09	0.00±0.00
Treponema	0.01±0.01	0.01±0.02	0.04±0.10	0.07±0.08	0.02±0.03
Treponema 2	0.40±0.20	0.26±0.20	0.66±0.84	1.18±1.42	0.46±0.35

Table B.2 (Continued)

	Wk00	Wk04		Wk16	
Genus	**Baseline**	**Ctrl**	**EW**	**Ctrl**	**EW**
SR1_(Absconditabacteria)					
SR1 (Absconditabacteria) uncultured	0.15±0.05	0.13±0.24	0.04±0.07	0.08±0.12	0.11±0.12
Synergistetes					
Candidatus Tammella	0.00±0.00	0.01±0.01	0.10±0.26	0.00±0.00	0.00±0.00
Fretibacterium	0.03±0.00	0.02±0.02	0.05±0.08	0.22±0.47	0.31±0.21
Synergistaceae uncultured	0.01±0.00	0.03±0.06	0.04±0.05	0.00±0.00	0.01±0.00
Tenericutes					
Acholeplasma	0.08±0.07	0.09±0.17	0.03±0.04	0.03±0.04	0.12±0.21
Anaeroplasma	0.10±0.10	0.02±0.05	0.03±0.04	0.01±0.02	0.01±0.01
Candidatus Phytoplasma	0.45±0.22	0.11±0.26	0.17±0.22	0.06±0.05	0.12±0.11
Mycoplasma	0.04±0.06	0.01±0.02	0.01±0.02	0.00±0.00	0.02±0.03
NB1-n uncultured	0.05±0.03	0.01±0.02	0.01±0.02	0.05±0.03	0.02±0.01
Verrucomicrobia					
FukuN18 freshwater group uncultured	0.00±0.00	0.00±0.00	0.13±0.37	0.00±0.00	0.00±0.00

[1] Group of lambs corresponded to baseline (at arrival; wk 0), early weaning (EW; weaning at wk 4) and control (Ctrl; weaning at wk 6).

[2] Rearing times corresponded to lambs separated from their dams wks 0 (Wk00), reared in pens and with access to concentrates and hay at wk 4 (Wk04) and grazed in paddocks in a mixed sward of forages at wk 16 (Wk16) of rearing.

Table B.3 Effect of weaning age[1] at two sampling times[2] on the rumen abundant bacteria community[3] at the genus level in lambs. Results[4] are natural log back transformed means and standard error of the differences (SED), P-value for treatment effect (P-Tx), sampling time (P-Tm) and their interactions (P-Int).

	Treatment		Sampling time		Treatment x Time							
Bacteria genus	**Ctrl**	**EW**	**Wk4**	**Wk16**	**Ctrl04**	**EW04**	**Ctrl16**	**EW16**	**SED**	**P-Tx**	**P-Tm**	**P-Int**
Bacteroidetes												
Prevotella 1	40.63	35.07	44.51	31.19	48.62	40.40	32.65	29.74	9.888	0.46	0.07	0.71
Bacteroidales S24-7 group	1.93	1.80	0.54	3.19	0.23	0.86	3.64	2.74	0.986	0.78	0.00	0.28
Rikenellaceae RC9 gut group	2.64	5.48	5.09	3.04	2.50	7.67	2.78	3.29	2.850	0.15	0.30	0.26
Mucilaginibacter	1.14	1.55	0.88	1.82	0.56	1.20	1.73	1.90	0.466	0.25	0.01	0.48
Prevotella 7	1.69	1.15	1.63	1.21	1.79	1.46	1.58	0.84	0.682	0.29	0.39	0.67
Prevotellaceae UCG-003	0.81	1.31	0.40	1.73	0.41	0.39	1.22	2.23	0.897	0.50	0.04	0.43
Tannerella	0.63	0.75	0.09	1.29	0.08	0.11	1.19	1.39	0.322	0.76	0.00	0.70
Alloprevotella	0.73	0.89	1.12	0.50	0.96	1.28	0.50	0.50	0.357	0.47	0.02	0.53
Fluviicola	0.84	1.48	2.31	0.01	1.67	2.95	0.00	0.02	2.269	0.65	0.16	0.70
Fibrobacteres												
Fibrobacter	0.96	1.07	1.28	0.76	1.26	1.30	0.66	0.85	0.676	0.79	0.29	0.88
Firmicutes												
Butyrivibrio 2	0.48	0.79	0.33	0.94	0.20	0.45	0.75	1.13	0.193	0.04	0.00	0.66
Christensenellaceae R-7 group	2.62	3.86	2.51	3.97	1.74	3.28	3.50	4.44	1.106	0.13	0.07	0.70
Lachnospiraceae uncultured	0.83	0.85	0.40	1.28	0.49	0.30	1.16	1.40	0.278	0.97	0.00	0.30
Lachnospiraceae NK3A20 group	0.45	0.90	0.41	0.94	0.38	0.44	0.52	1.36	0.405	0.16	0.07	0.19
Lachnospiraceae NK4A136 group	0.69	1.58	1.01	1.26	0.54	1.48	0.84	1.68	1.041	0.24	0.74	0.95
Oscillospira	0.98	1.02	0.17	1.83	0.18	0.16	1.79	1.88	0.492	0.95	0.00	0.88
Pseudobutyrivibrio	1.99	2.39	0.45	3.92	0.50	0.41	3.48	4.36	0.976	0.70	0.00	0.49

Table B.3 (Continued)

	Treatment		Sampling time		Treatment x Time							
Bacteria genus	**Ctrl**	**EW**	**Wk4**	**Wk16**	**Ctrl04**	**EW04**	**Ctrl16**	**EW16**	**SED**	**P-Tx**	**P-Tm**	**P-Int**
Roseburia	0.78	0.91	0.76	0.92	0.57	0.95	0.98	0.87	0.441	0.66	0.63	0.44
Ruminococcaceae uncultured	0.52	0.62	0.38	0.75	0.37	0.39	0.66	0.84	0.241	0.62	0.04	0.65
Ruminococcaceae NK4A214 group	10.70	4.75	10.12	5.33	15.96	4.29	5.45	5.21	7.011	0.24	0.36	0.26
Ruminococcaceae UCG-002	1.31	2.53	0.92	2.92	0.47	1.37	2.16	3.68	0.914	0.09	0.00	0.64
Ruminococcaceae UCG-010	0.65	0.91	0.42	1.14	0.29	0.55	1.01	1.27	0.274	0.23	0.00	1.00
Ruminococcaceae uncultured	0.43	2.29	2.42	0.30	0.55	4.29	0.30	0.30	3.273	0.40	0.35	0.43
Ruminococcus 1	3.57	3.92	1.55	5.95	1.95	1.14	5.19	6.71	1.062	0.83	0.00	0.13
Selenomonas	5.59	2.35	1.30	6.65	1.15	1.44	10.04	3.26	2.570	0.08	0.01	0.06
Selenomonas 1	1.30	1.65	0.25	2.71	0.28	0.22	2.33	3.08	0.869	0.69	0.00	0.52
Subdoligranulum	0.58	0.44	0.38	0.64	0.45	0.31	0.71	0.57	0.354	0.57	0.31	1.00
Syntrophococcus	2.26	0.46	2.67	0.05	4.48	0.85	0.03	0.07	2.401	0.31	0.14	0.29
Proteobacteria												
Brenneria	0.95	2.56	3.50	0.01	1.89	5.11	0.01	0.01	3.809	0.51	0.20	0.56
Brevundimonas	0.51	0.44	0.56	0.39	0.48	0.64	0.54	0.23	0.394	0.83	0.52	0.40
Spirochaetae												
Treponema 2	0.72	0.56	0.46	0.82	0.26	0.66	1.18	0.46	0.446	0.64	0.29	0.09

[1] Treatment groups corresponded to early weaning (EW; weaning at wk 4) and control (Ctrl; weaning at wk 6).

[2] Rumen sampling times were at wk 4 (Wk04) and 16 (Wk16) of rearing.

[3] The abundant bacteria community corresponded to 31 genera (≥ 0.5% in at least one of the sampled groups of lambs).

[4] Effect of weaning age, sampling times and their interactions were evaluated using a linear mix effects model with treatment and sampling time as fixed effect, and animal as random effect.

Table B.4 Archaea species in different groups of lambs[1] at three rearing times[2]. The archaea proportions are presented as the mean ± standard error of the difference.

Archaea Species	Wk00	Wk04		Wk16	
	Baseline	Ctrl	EW	Ctrl	EW
Methanobrevibacter gottschalkii clade	73.14	80.86	75.53	81.60	81.32
Methanobrevibacter ruminantium clade	12.58	9.74	15.85	11.61	13.08
Methanosphaera sp. Group5	4.95	2.51	2.48	2.17	2.64
Methanomassiliicoccales Group10 sp.	2.64	3.54	1.12	0.55	0.76
Methanosphaera sp. ISO3-F5	2.60	0.50	1.07	2.11	1.01
Methanomassiliicoccales Group9 sp.	2.68	0.91	1.88	0.06	0.20
Methanosphaera cuniculi	0.45	0.88	0.54	1.02	0.63
Methanomassiliicoccales Group12 sp. ISO4-H5	0.30	0.37	0.93	0.00	0.01
Methanobrevibacter sp. 30Y	0.15	0.11	0.21	0.80	0.30
Methanomassiliicoccales Group4 sp. MpT1	0.27	0.03	0.19	0.00	0.01
Methanomassiliicoccales Group11 sp. ISO4-G11	0.07	0.01	0.06	0.00	0.01
Methanobrevibacter smithii strain TS96A	0.01	0.00	0.01	0.06	0.02
Methanobrevibacter acididurans	0.01	0.00	0.10	0.00	0.00
Methanomassiliicoccales Group8	0.07	0.00	0.00	0.00	0.00
Methanomassiliicoccales Group3b sp.	0.02	0.00	0.01	0.00	0.00
Methanobrevibacter smithii strain ALI	0.01	0.00	0.00	0.01	0.00
Others	0.07	0.52	0.02	0.01	0.00

[1] Group of lambs corresponded to baseline (at arrival; wk 0), early weaning (EW; weaning at wk 4) and control (Ctrl; weaning at wk 6).

[2] Rearing times corresponded to lambs separated from their dams at wk 0 (Wk00), reared in pens and with access to concentrates and hay at wk 4 (Wk04) and grazed in paddocks in a mixed sward of forages at wk 16 (Wk16) of rearing.

Table B.5 Effect of weaning age[1] at two sampling times[2] on the rumen abundant archaea community at the spices level in lambs[3]. Results[4] are natural log back transformed means and standard error of the differences (SED), P-value for treatment effect (P-Tx), time of rearing (P-Tm) and their interactions (P-Int).

	Treatment		Time of rearing		Treatment x Time							
Archaea species	**Ctrl**	**EW**	**Wk04**	**Wk16**	**Ctrl04**	**EW04**	**Ctrl16**	**EW16**	**SED**	**P-Tx**	**P-Tm**	**P-Int**
Methanobrevibacter gottschalkii clade	81.23	78.43	78.20	81.46	80.86	75.53	81.60	81.32	8.104	0.63	0.55	0.66
Methanobrevibacter ruminantium clade	10.67	14.46	12.79	12.35	9.74	15.85	11.61	13.08	7.074	0.46	0.90	0.65
Methanosphaera sp. Group5	2.34	2.56	2.50	2.41	2.51	2.48	2.17	2.64	0.872	0.72	0.90	0.68
Methanomassiliicoccaceae Group10 sp.	2.05	0.94	2.33	0.65	3.54	1.12	0.55	0.76	1.812	0.40	0.22	0.31
Methanosphaera sp. ISO3-F5	1.30	1.04	0.79	1.56	0.50^{a}	1.07^{ab}	2.11^{b}	1.01^{ab}	0.546	0.51	0.07	0.04
Methanomassiliicoccaceae Group9 sp.	0.49	1.04	1.40	0.13	0.91	1.88	0.06	0.20	0.758	0.31	0.02	0.45
Methanosphaera cuniculi	0.95	0.58	0.71	0.82	0.88	0.54	1.02	0.63	0.542	0.35	0.78	0.96

[1] Lambs were allocated in early weaning (EW; weaning at wk 4) and control (Ctrl; weaning at wk 6) groups.

[2.] Rumen sampled at wk 4 (Wk04) and 16 (Wk16) of rearing (time of rearing).

[3] Measured effect corresponded to the 7 ruminal archaea species (≥ 1.0% in at least one of the sampled groups of lambs).

[4] Effect of weaning age, sampling times and their interactions were evaluated using a linear mix effects model with treatment and time of rearing as fixed effect, and animal as random effect

Table B.6 Number identification (ID) for archaea and bacteria taxa.

ID	Taxa	ID	Taxa
A001	Methanomassiliicoccaceae Group10 sp	**B096**	Mucilaginibacter
A007	Methanomassiliicoccaceae Group9 sp	**B111**	Oscillospira
A009	Methanobrevibacter gottschalkii clade	**B119**	Prevotella 1
A010	Methanobrevibacter ruminantium clade	**B121**	Prevotella 7
A014	Methanosphaera cuniculi	**B123**	Prevotellaceae UCG-003
A015	Methanosphaera sp Group5	**B125**	Pseudobutyrivibrio
A016	Methanosphaera sp ISO3-F5	**B131**	Rikenellaceae RC9 gut group
		B133	Roseburia
B002	Acetobacterium	**B136**	Ruminococcaceae unclassified
B009	Alloprevotella	**B137**	Ruminococcaceae NK4A214 group
B019	Bacteroidales S24-7 group ge	**B138**	Ruminococcaceae UCG-002
B028	Brenneria	**B141**	Ruminococcaceae UCG-010
B029	Brevundimonas	**B142**	Ruminococcaceae UCG-014
B033	Butyrivibrio 2	**B143**	Ruminococcus 1
B042	Christensenellaceae R-7 group	**B146**	Selenomonas
B060	Family XIII AD3011 group	**B147**	Selenomonas 1
B064	Fibrobacter	**B159**	Subdoligranulum
B066	Fluviicola	**B161**	Syntrophococcus
B084	Lachnospiraceae ge	**B162**	Tannerella
B085	Lachnospiraceae NK3A20 group	**B168**	Treponema 2
B086	Lachnospiraceae NK4A136 group	**B171**	Ruminococcaceae uncultured

Appendix C

In Chapter 3, the third specific objective was to explore the association between the predominant bacteria and archaea in the rumen and rumen fermentation profiles, morphology and blood metabolites, in lambs that are transitioning physiologically into a ruminant.

To evaluate the correlations between ruminal microbiota and rumen morphology and blood metabolites the following procedures were followed:

Blood metabolites

The blood metabolites used for analysis in the current study correspond to the samples taken at wk 4 of the trial. The detailed methodology used to analyze the blood metabolites is described in a companion study (McCoard et al. unpublished). Lambs were fed in pairs, at two-minute intervals, to enable timed collection of the blood 2h post MR feeding. Blood samples were collected by jugular venipuncture into potassium-EDTA containing vacutainers. Collected samples were put on ice for 40 to 60 minutes and then centrifuged for 15 minutes at 1000 x g, and plasma stored at -20°C. Plasma β-hydroxybutyrate (BHBA), blood urea (BU), triglycerides (TG), glucose (GLU), non-esterified fatty acids (NEFA) and total plasma protein (TPP) were analyzed by the New Zealand Veterinary Pathology Laboratory (Palmerston North, New Zealand) using the Roche modular platform P800 module (UV method).

Rumen tissues sampling

Rumen tissues samples were obtained at slaughter. Euthanasia was performed by captive bolt stunning and exsanguination. Five animals were randomly euthanized at wk 0 (arrival; Wk00), eight animals per weaning group were euthanized at wk 4 (Wk04; last day of MR feeding for the EW lambs) and wk 16 (Wk16) of the trial. From each animal at each slaughter, rumen tissue samples, approximately 4 x 4 cm, were dissected from the ventral sac (VS), dorsal sac (DS), ventral blind sac (VBS) and dorsal blind sac (DBS) of 4 and 16 wks lambs, rinsed in PBS and fixed in 4% paraformaldehyde.

Rumen morphology - tissue preparation and measurements

Post-mortem, the rumen was collected, and full and empty weights were recorded. Histology measurements were made of the VS, DS, VBS, and DBS of the rumen of 4- and 16-wk lambs. A square centimeter of tissue was dissected from each ruminal sac and papillae density counted. Histology slices were prepared and dyed with Mayer's-Harris

haematoxylin mixture and eosin. Papillae length, papillae width, muscular layer thickness and mucosal epithelium thickness were measured for each ruminal sac. Histology preparation and histomorphology measurements are described in **Chapter 2**.

Statistical analysis

Canonical correlation analysis (CCA) was used to explore potential associations between the microbial community and rumen morphology and blood metabolites (Henderson et al., 2015).

RESULTS

Correlations between abundant rumen microbes and rumen morphology of both groups of lambs are shown in **Figure C.1**. Bacterial genera from the phylum *Bacteroidetes* and *Fibrobacteres* were positively correlated with papillae density in all four sites of the rumen, while bacterial genera from the phyla *Firmicutes* and *Spirochaetae* were positively correlated with live weight, rumen empty and full weight, papillae length (in all four rumen sites), muscle thickness (DS, VS and VBS) and papillae width (VS and VBS) (**Figure C.1a**). For the archaea species *Mmc.* Group 10 sp, *Mmc.* Group 9 sp., and *Mph.* sp. ISO3-F5 were positively correlated with papillae density, and *Mbb. gottschalkii* clade and *Mph. cuniculi* were positively correlated with live weight, full and empty rumen weight, and papilla length (**Figure C.1b**).

The CCA of the most abundant bacterial genera and archaea species with blood metabolites at Wk04 is shown in **Figure C.2**. For bacteria, *Brenneria* and *Fibrobacter* were positively correlated to BHBA and triglyceride concentrations, while *Rikenellaceae* RC9 gut group, Lachnospiraceae NK4A136 group, *Alloprevotella*, *Fluviicola*, *Roseburia*, *Bacteroidales* S24-7 group, *Christensenellaceae* R-7 group and *Prevotella* 7 were positively correlated to TPP, NEFA and BUN (**Figure C.2a**). For the Archaea *Mbb. ruminantium* had a positive correlation with BUN, GLU and NEFA, while these were negatively correlated to *Mbb. gottschalkii* (**Figure C.2b**).

Figure C.1 Canonical correlation analysis clustering image maps of the associations between abundant rumen microbes and the rumen histomorphology of lambs reared at wk 4 and 16 of rearing. Rumen microbes and rumen histomorphology were sampled at wk 4 (Wk04) and 16 (Wk16) of rearing. Abundant rumen microbes corresponded to 31 bacteria genera and 7 archaea species with a relative abundance of ≥ 1.00% and ≥ 0.50% in average across treatment groups and sampling times. a) Canonical correlation analysis (CCA) between abundant bacteria genus and rumen histomorphology. b) CCA between abundant archaea species and rumen histomorphology. The dark red and blue indicate positive and negative correlation coefficient values, respectively, whereas white color indicate zero correlation coefficient values. Microbial groups are identified by ID numbers in Supplementary Table 3.6.

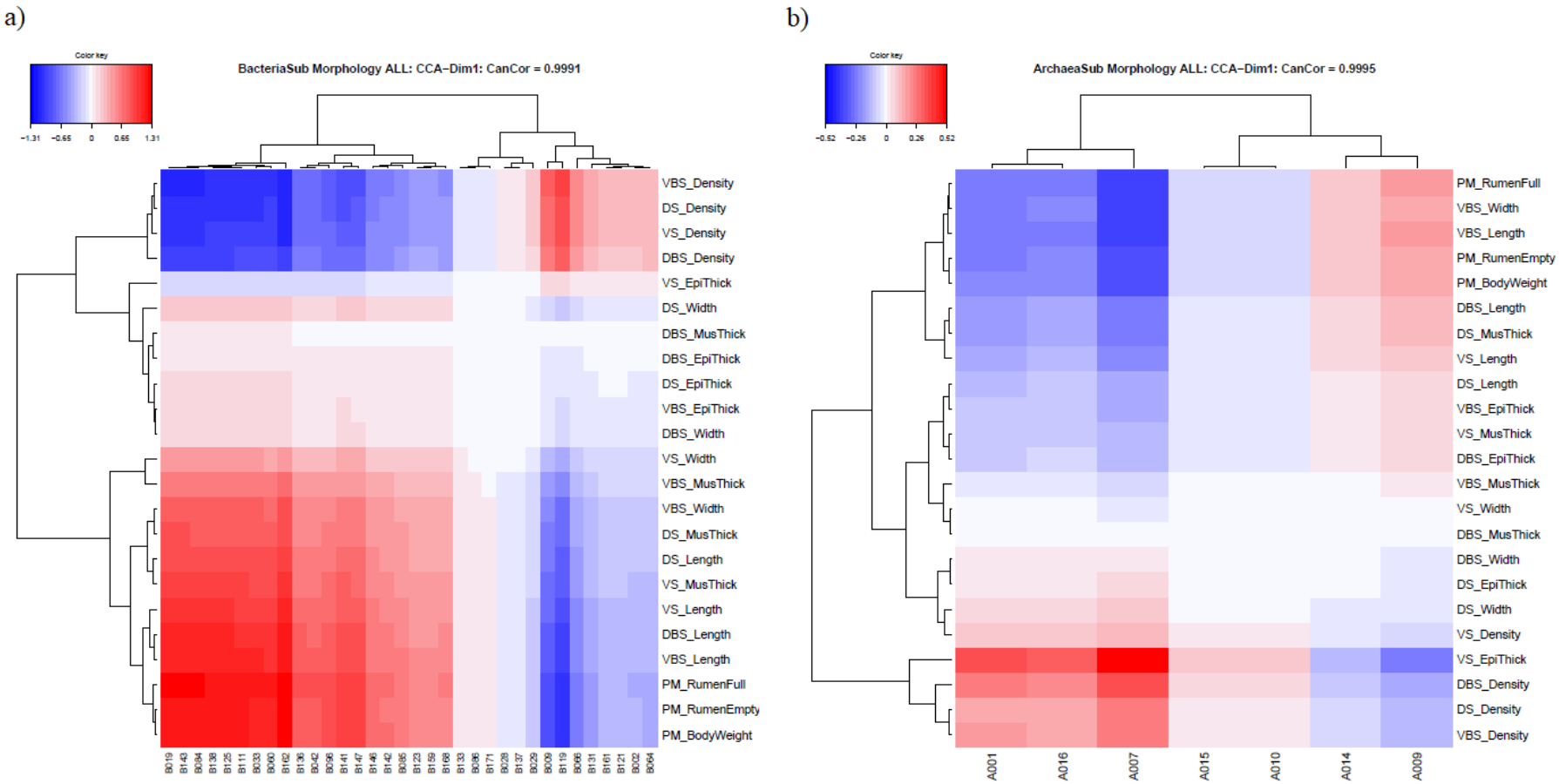
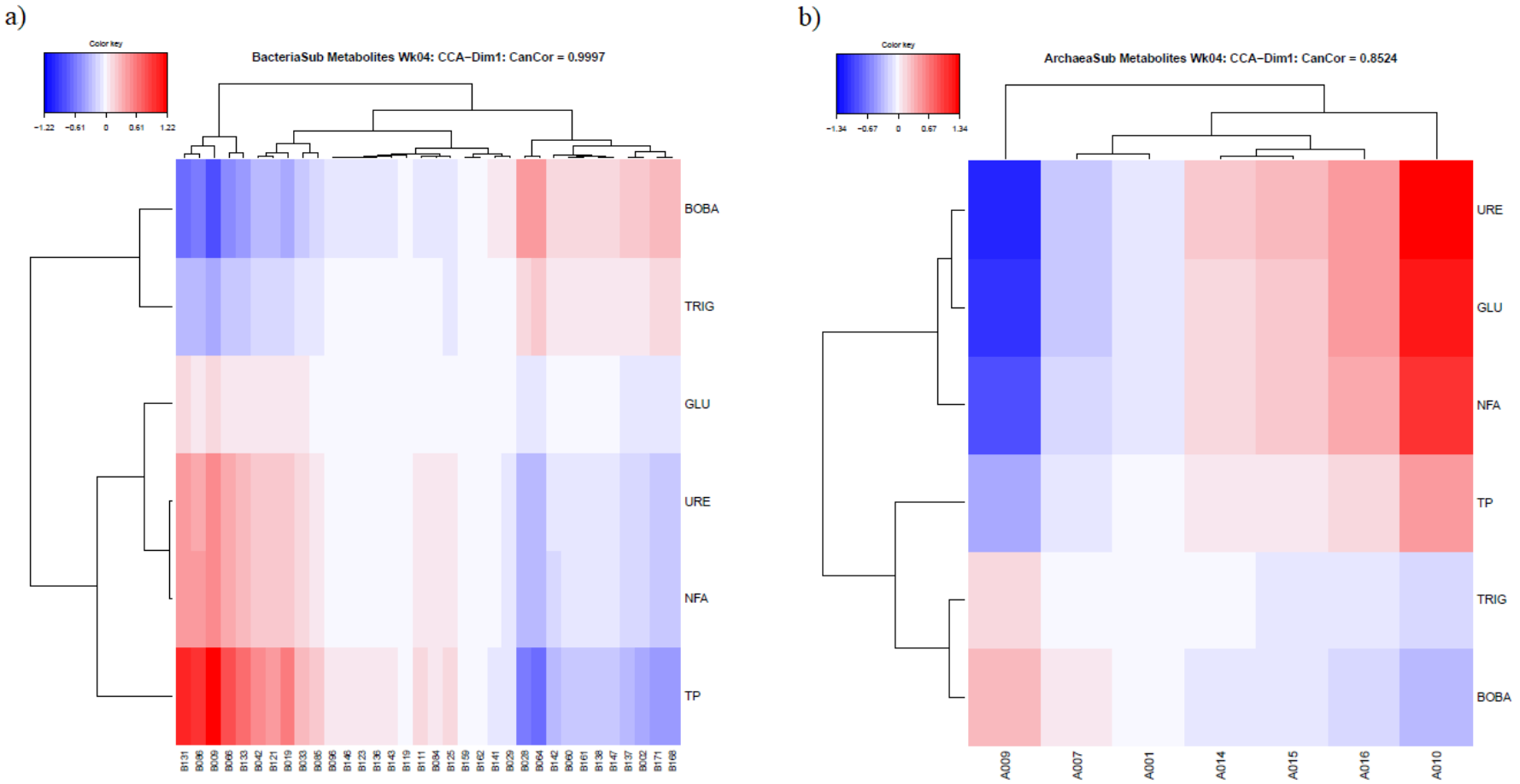


Figure C.2 Canonical correlation analysis clustering image maps of the associations between abundant rumen microbes and blood metabolites of lambs reared at wk 4 of rearing. Rumen microbes and blood metabolites were sampled at wk 4 (Wk04) of rearing. Abundant rumen microbes represented 31 bacteria genera and 7 archaea species with a relative abundance of ≥ 1.00% and ≥ 0.50% in average across treatment groups and sampling times. a) Canonical correlation analysis (CCA) between abundant bacteria genus and blood metabolites. b) CCA between abundant archaea species and blood metabolites. The dark red and blue indicate positive and negative correlation coefficient values, respectively, whereas white color indicate zero correlation coefficient values. Microbial groups are identified by ID numbers in Supplementary Table 3.6.



DISCUSION

Development of the rumen papillae was strongly associated with the proportion of bacteria from the genera *Firmicutes*. Genera such as *Butyrivibrio* and *Pseudobutyrivibrio* produce butyrate, which is known to be a substrate used for rumen epithelial cells metabolism, stimulating papillae growth (Niwińska et al., 2017). On the other hand, papillae density was associated with the relative abundance of *Prevotella*, which belongs to the phylum *Bacteroidetes*. However, these associations may be a confounded effect, e.g. the transitioning from concentrate onto forage diets was associated with reductions in the proportion of *Prevotella*, which coincide with the reduction in papillae density as the ruminal volume growth; whilst the genera *Butyrivibrio* and *Pseudobutyrivibrio* increases in forage diets, which coincided with the larger papillae observed at 16 wks, but the production of butyrate remained similar at 4 and 16 wks. To gain better insights into the association between rumen microbiota and rumen morphology it is necessary to focus on the epimural microbes. These microbiota lives attached to the surface of the rumen epithelium and may interact more closely with the metabolism of the epithelial cells.

High proportions of *Prevotella* were positively correlated with BHBA and triglyceride concentrations in young ruminants, an association that was likely to be due to high SCFA concentrations in the rumen and the absorption of these across the rumen epithelium (Baldwin et al., 2004). Positive correlations of *Firmicutes* genera with plasma protein and serum urea were observed when genera from this phylum increased their relative abundance. However, serum urea concentrations depend on microbial protein, ammonia production (absorbed by the rumen epithelium) and protein that escape rumen degradation (Lewis, 1957; Lewis et al., 1957). Therefore, the observed association between genera from *Firmicutes* and serum urea and plasma protein may be a confounded effect of increased protein in the diet.

CONCLUSIONS

Associations between the 16s rRNA of the rumen microbiota and rumen development and blood metabolites showed confounding effects that require further evaluation. A different approach needs to be considered in future research such as to correlate the functionality of the ruminal microbial genetics (microbiome). the rumen metabolome and gene expression in ruminal epithelial cells. The correlation of the ruminal

microbiota and blood metabolites is likely inappropriate since plasma metabolites are end products of the liver metabolism.

Appendix D

This appendix corresponds to additional tables and figures from Chapter 4

Table D.1 Analysis of variance of ruminal bacteria at the phylum level during the three-rearing period[1]. Results[2] are natural log back transformed means and standard error of the differences (SED), P-value for treatment effect for FO *vs.* CO (P-1), treatment effect for HQ *vs.* LQ (P-2) and their interactions (P-int).

	P01				**P02**								**P03**							
Phylum	**FO**	**CO**	**SED**	**P-1**	**CO**	**FO**	**HQ**	**LQ**	**SED**	**P-1**	**P-2**	**P-int**	**CO**	**FO**	**HQ**	**LQ**	**SED**	**P-1**	P-2	P-int
Acidobacteria	0.00	0.00	0.000	0.24	0.00	0.00	0.00	0.00	0.000	0.34	0.20	0.34	0.00	0.00	0.00	0.00	0.002	0.13	0.34	0.82
Actinobacteria	1.29	1.01	0.316	0.55	0.80	1.19	1.14	0.85	0.153	0.09	0.21	0.73	0.69	0.65	0.78	0.55	0.126	0.74	0.08	0.29
Armatimonadetes	0.01	0.00	0.003	0.10	0.02	0.01	0.01	0.02	0.015	0.50	<0.01	0.53	0.04	0.03	0.04	0.03	0.007	0.51	0.74	0.68
Bacteroidetes	53.80	44.19	5.978	0.12	55.65	55.81	53.85	57.61	1.985	0.96	0.20	0.69	54.49	53.33	53.44	54.37	2.152	0.60	0.67	0.30
Candidate division SR1	0.04	0.00	0.024	0.28	0.31	0.15	0.29	0.17	0.101	0.14	0.24	0.82	0.34	0.33	0.34	0.33	0.054	0.84	0.94	0.20
Chlamydiae	0.00	0.00	0.002	0.21	0.01	0.01	0.00	0.02	0.002	0.47	<0.01	0.55	0.02	0.02	0.03	0.02	0.006	0.57	0.08	0.63
Chloroflexi	0.05	0.04	0.008	0.22	0.09	0.12	0.10	0.11	0.019	0.16	0.58	0.17	0.14	0.16	0.14	0.15	0.026	0.47	0.66	0.38
Cyanobacteria	0.34	0.33	0.103	0.92	1.07	0.76	0.38	1.45	0.209	0.15	<0.01	0.31	0.40	0.36	0.39	0.36	0.072	0.60	0.66	0.54
Elusimicrobia	0.02	0.00	0.003	<0.01	0.06	0.05	0.01	1.00	0.022	0.52	<0.01	0.56	0.03	0.05	0.05	0.02	0.021	0.26	0.15	0.14
Fibrobacteres	0.93	0.06	0.294	0.05	0.93	0.94	0.28	1.59	0.304	0.96	<0.01	0.72	1.18	1.17	1.17	1.18	0.214	0.96	0.96	0.88
Firmicutes	41.28	52.61	4.250	0.07	36.22	36.28	39.33	33.17	2.260	0.98	0.01	0.35	37.15	38.84	38.12	37.86	1.927	0.39	0.90	0.23
Fusobacteria	0.00	0.00	0.000	0.93	0.00	0.01	0.01	0.00	0.004	0.22	0.13	0.27	0.00	0.00	0.00	0.00	0.001	0.99	0.78	0.31
Gemmatimonadetes	0.00	0.00	0.001	0.31	0.01	0.01	0.01	0.01	0.003	0.56	0.14	0.98	0.00	0.00	0.00	0.00	0.001	0.39	0.24	0.58
JL-ETNP-Z39	0.00	0.00	0.000	0.33	0.00	0.00	0.00	0.00	0.002	0.16	0.06	0.16	0.00	0.00	0.00	0.00	0.000	0.78	0.15	0.63
Lentisphaerae	0.05	0.00	0.012	<0.01	0.31	0.32	0.07	0.56	0.043	0.86	<0.01	0.89	0.43	0.42	0.42	0.43	0.091	0.94	0.84	0.13
Planctomycetes	0.01	0.00	0.002	<0.01	0.03	0.04	0.01	0.06	0.013	0.65	<0.01	0.95	0.05	0.05	0.05	0.05	0.013	0.91	0.94	0.34
Proteobacteria	0.47	0.83	0.242	0.31	1.23	1.50	1.31	1.42	0.362	0.47	0.78	0.07	1.27	1.17	1.39	1.06	0.178	0.57	0.08	0.99
Saccharibacteria	0.16	0.00	0.022	<0.01	0.50	0.36	0.61	0.26	0.112	0.22	<0.01	0.47	0.64	0.60	0.62	0.63	0.106	0.66	0.93	0.77
SHA-109	0.16	0.00	0.054	0.04	0.04	0.03	0.02	0.03	0.012	0.32	0.67	0.30	0.08	0.09	0.08	0.09	0.025	0.64	0.48	0.37

Table D.1 (Continued)

	P01				P02								P03							
Phylum	**FO**	**CO**	**SED**	**P-1**	**CO**	**FO**	**HQ**	**LQ**	**SED**	**P-1**	**P-2**	**P-int**	**CO**	**FO**	**HQ**	**LQ**	**SED**	**P-1**	P-2	P-int
Spirochaetae	0.58	0.12	0.088	<0.01	0.60	0.61	0.70	0.51	0.167	0.96	0.27	0.97	0.50	0.44	0.47	0.47	0.070	0.41	0.91	0.63
Synergistetes	0.01	0.02	0.003	0.20	0.04	0.03	0.02	0.05	0.010	0.37	0.02	0.10	0.02	0.01	0.01	0.01	0.003	0.51	0.98	0.82
Tenericutes	0.97	0.44	0.108	<0.01	1.90	1.56	1.66	1.80	0.252	0.20	0.58	0.53	2.32	2.07	2.22	2.16	0.207	0.24	0.77	0.79
Verrucomicrobia	0.00	0.00	0.000	0.33	0.00	0.00	0.00	0.01	0.000	0.80	<0.01	0.72	0.03	0.02	0.03	0.02	0.010	0.56	0.51	0.01

[1] Dietary treatments corresponded to: Period 1 (P01) concentrate (CO) *vs.* pasture (FO) diets, and Period 2 (P02) high quality (HQ) *vs.* low quality (LQ) pastures, with measurements in P01 (9 wks), P02 (19 wks) and Period 3 (P03; 41 wks) when all calves were offered a common pasture diet. Measured data corresponded to the 24 ruminal bacteria phyla or main community.

[2] Dietary treatments in each period were evaluated as follow: a one-way ANOVA in P01 to analyze FO *vs*. CO diets, and a 2 x 2 factorial ANOVA in P02 and P03 to evaluate FO *vs.* CO and HQ *vs.* LQ dietary treatment effects and their interaction

Table D.2 Analysis of variance of ruminal bacteria at the genus level during the three-rearing period[1]. Results[2] are natural log back transformed means and standard error of the differences (SED), P-value for treatment effect for FO *vs.* CO (P-1), treatment effect for HQ *vs*. LQ (P-2) and their interactions (P-int).

	P01				P02								P03							
Genus	**CO**	**FO**	**SED**	**P-1**	**FO**	**CO**	**HQ**	**LQ**	**SED**	**P-1**	**P-2**	**P-int**	**FO**	**CO**	**HQ**	**LQ**	**SED**	**P-1**	**P-2**	**P-int**
Atopobium	0.79	0.83	0.413	0.92	0.89	0.59	0.85	0.63	0.196	0.14	0.27	0.83	0.36	0.32	0.42	0.26	0.074	0.64	0.05	0.12
Olsenella	0.36	0.01	0.108	0.00	0.01	0.01	0.02	0.01	0.003	0.62	0.01	0.06	0.01	0.01	0.01	0.01	0.003	0.97	0.14	0.75
VC2.1 Bac22 (Bacteroidetes)	0.00	0.25	0.058	0.00	0.07	0.14	0.10	0.11	0.046	0.16	0.87	0.23	0.15	0.17	0.15	0.17	0.047	0.79	0.77	0.96
Bacteroides	0.05	0.16	0.036	0.01	0.40	0.30	0.25	0.45	0.085	0.25	0.03	0.90	0.18	0.17	0.16	0.18	0.023	0.69	0.44	0.79
BS11 (Bacteroidales)	0.03	2.06	0.489	0.00	3.90	3.52	2.06	5.37	0.872	0.67	0.00	0.95	3.75	3.94	3.78	3.91	0.443	0.67	0.78	0.25
RF16 (Bacteroidales)	0.00	1.06	0.174	0.00	1.33	1.14	0.84	1.63	0.361	0.62	0.04	0.79	1.01	0.85	0.91	0.95	0.194	0.43	0.83	0.05
S24-7 (Bacteroidales)	0.31	1.87	0.343	0.00	1.72	1.33	1.00	2.06	0.433	0.38	0.02	1.00	3.84	3.62	3.78	3.68	0.514	0.67	0.86	0.18
UCG-001 (Bacteroidales)	0.00	0.16	0.070	0.04	0.29	0.36	0.15	0.49	0.128	0.62	0.01	0.48	0.42	0.48	0.48	0.42	0.084	0.47	0.43	0.06
Alloprevotella	0.05	0.28	0.102	0.03	0.17	0.18	0.20	0.14	0.056	0.80	0.30	0.20	0.07	0.09	0.09	0.07	0.022	0.56	0.37	0.16
Paraprevotella	0.00	0.01	0.008	0.23	0.03	0.10	0.07	0.06	0.049	0.16	0.80	0.72	0.01	0.02	0.02	0.01	0.012	0.57	0.61	0.81
Prevotella 1	7.80	41.01	6.197	0.00	35.94	36.64	38.35	34.23	3.620	0.85	0.27	0.52	34.12	33.32	33.24	34.21	2.710	0.77	0.73	0.95
Prevotella 7	31.77	0.08	5.928	0.00	0.12	0.09	0.17	0.04	0.057	0.63	0.03	0.58	0.07	0.10	0.10	0.07	0.026	0.25	0.23	0.79
Prevotella 9	0.30	0.05	0.059	0.00	0.02	0.03	0.04	0.01	0.011	0.37	0.01	0.21	0.01	0.02	0.01	0.02	0.005	0.72	0.64	0.46
Prevotella	1.50	0.00	0.379	0.00	0.00	0.00	0.00	0.00	0.390	0.41	0.60	0.01	0.00	0.00	0.00	0.00	0.001	0.43	0.58	0.34
Ga6A1 group (Prevotellaceae)	0.00	0.01	0.007	0.07	0.16	0.18	0.25	0.09	0.079	0.88	0.06	0.83	0.03	0.03	0.04	0.02	0.008	0.45	0.01	0.54
NK3B31 group (Prevotellaceae)	0.00	0.53	0.405	0.21	0.47	0.59	0.35	0.70	0.291	0.68	0.24	0.95	0.50	0.58	0.55	0.53	0.083	0.32	0.79	0.85
UCG-001 (Prevotellaceae)	1.94	1.12	0.898	0.37	2.29	3.77	3.67	2.38	0.875	0.11	0.16	0.21	2.73	2.66	2.59	2.80	0.331	0.82	0.54	0.17
UCG-003 (Prevotellaceae)	0.01	1.42	0.280	0.00	3.23	2.10	1.71	3.62	0.497	0.03	0.00	0.14	2.78	2.55	2.75	2.58	0.382	0.54	0.68	0.85
UCG-004 (Prevotellaceae)	0.03	0.06	0.016	0.10	0.06	0.08	0.05	0.09	0.038	0.52	0.40	0.59	0.11	0.11	0.11	0.12	0.018	0.98	0.90	0.76
YAB2003 group (Prevotellaceae)	0.01	0.11	0.025	0.00	0.14	0.10	0.20	0.04	0.040	0.37	0.00	0.91	0.18	0.16	0.19	0.16	0.075	0.77	0.72	0.14
Unclassified[a] (Prevotellaceae)	0.01	0.07	0.022	0.02	0.20	0.08	0.12	0.16	0.070	0.11	0.64	0.30	0.26	0.29	0.27	0.27	0.070	0.72	0.99	0.01
RC9 gut group (Rikenellaceae)	0.26	1.93	0.329	0.00	4.96	4.62	3.96	5.61	0.657	0.61	0.02	0.30	3.78	3.76	3.70	3.84	0.323	0.97	0.66	0.89
Unclassified (SR1)	0.00	0.04	0.034	0.28	0.15	0.31	0.29	0.17	0.101	0.14	0.24	0.82	0.34	0.33	0.34	0.33	0.054	0.84	0.94	0.20
Unclassified Anaerolineaceae	0.03	0.04	0.008	0.22	0.11	0.08	0.08	0.11	0.018	0.07	0.14	0.29	0.13	0.15	0.13	0.15	0.026	0.55	0.56	0.40

Table D.2 (Continued)

	P01				P02								P03							
Genus	**CO**	**FO**	**SED**	**P-1**	**FO**	**CO**	**HQ**	**LQ**	**SED**	**P-1**	**P-2**	**P-int**	**FO**	**CO**	**HQ**	**LQ**	**SED**	**P-1**	**P-2**	**P-int**
Unclassified Gastranaerophilales	0.32	0.32	0.146	0.96	0.74	1.05	0.35	1.44	0.210	0.15	0.00	0.33	0.40	0.34	0.37	0.35	0.073	0.72	0.77	0.61
Fibrobacter	0.06	0.93	0.415	0.05	0.94	0.93	0.28	1.59	0.304	0.96	0.00	0.72	1.18	1.17	1.17	1.18	0.214	0.96	0.96	0.88
Bacillus	0.00	0.00	0.000	0.93	0.09	0.03	0.11	0.01	0.042	0.15	0.04	0.17	0.00	0.01	0.00	0.00	0.001	0.45	0.64	0.79
Streptococcus	0.03	0.19	0.007	0.02	0.35	0.22	0.33	0.23	0.124	0.32	0.42	0.10	0.17	0.26	0.24	0.19	0.083	0.29	0.52	0.59
R-7 group (Christensenellaceae)	0.13	3.65	0.731	0.00	8.32	6.73	5.91	9.14	0.853	0.08	0.00	0.05	7.48	9.33	8.97	7.83	0.717	0.02	0.13	0.25
UCG-011 (Defluviitaleaceae)	0.02	0.07	0.015	0.01	0.09	0.10	0.08	0.11	0.025	0.66	0.19	0.18	0.11	0.11	0.11	0.11	0.020	0.72	0.96	0.29
Pseudoramibacter	0.12	0.00	0.018	0.00	0.00	0.00	0.01	0.00	0.003	0.45	0.06	0.42	0.01	0.01	0.01	0.01	0.005	0.33	0.16	0.15
Nodatum group [Eubacterium]	0.24	0.10	0.037	0.00	0.13	0.13	0.16	0.10	0.022	0.93	0.02	0.48	0.19	0.22	0.19	0.23	0.025	0.29	0.12	0.74
Anaerovorax	0.00	0.09	0.020	0.00	0.15	0.17	0.13	0.19	0.030	0.61	0.04	0.32	0.35	0.37	0.36	0.36	0.048	0.62	0.91	0.45
AD3011 group (Family XIII)	0.02	0.15	0.025	0.00	0.26	0.21	0.29	0.19	0.027	0.06	0.00	0.74	0.45	0.49	0.46	0.49	0.061	0.51	0.67	0.64
UCG-002 (Family XIII)	0.00	0.02	0.007	0.01	0.06	0.06	0.06	0.06	0.009	0.79	0.74	0.90	0.10	0.12	0.11	0.11	0.019	0.29	0.73	0.11
Mogibacterium	0.10	0.20	0.029	0.00	0.46	0.38	0.56	0.29	0.053	0.18	0.00	0.40	0.95	1.08	1.03	1.00	0.136	0.35	0.82	0.96
Cellulosolvens group [Eubacterium]	0.00	0.17	0.043	0.00	0.03	0.04	0.07	0.00	0.018	0.57	0.00	0.49	0.23	0.23	0.21	0.25	0.095	0.98	0.68	0.25
Hallii group [Eubacterium]	0.21	0.30	0.056	0.12	0.33	0.26	0.41	0.17	0.051	0.18	0.00	0.74	0.16	0.20	0.17	0.19	0.023	0.12	0.35	0.11
Ruminantium group [Eubacterium]	0.25	1.05	0.312	0.02	0.34	0.40	0.40	0.34	0.072	0.36	0.37	0.11	0.83	0.78	0.74	0.87	0.124	0.65	0.30	0.02
Ventriosum group [Eubacterium]	0.00	0.03	0.005	0.00	0.14	0.14	0.08	0.20	0.072	0.97	0.11	0.88	0.07	0.06	0.07	0.06	0.009	0.67	0.26	0.80
Gauvreauii group [Ruminococcus]	0.54	0.31	0.106	0.05	0.24	0.15	0.24	0.14	0.041	0.04	0.03	0.94	0.39	0.37	0.37	0.39	0.037	0.64	0.58	0.02
Acetitomaculum	1.21	0.33	0.535	0.12	0.49	0.39	0.45	0.43	0.078	0.20	0.79	0.10	0.36	0.38	0.37	0.37	0.041	0.63	0.96	0.46
Blautia	0.10	0.08	0.018	0.54	0.15	0.13	0.15	0.12	0.038	0.59	0.44	0.88	0.18	0.18	0.17	0.19	0.033	0.89	0.65	0.73
Butyrivibrio 2	0.02	0.44	0.053	0.00	1.21	1.33	1.88	0.66	0.509	0.81	0.03	0.69	0.65	0.69	0.73	0.61	0.131	0.80	0.41	0.96
Coprococcus 1	0.16	0.23	0.067	0.27	0.12	0.09	0.13	0.07	0.012	0.02	0.00	0.08	0.13	0.15	0.15	0.13	0.020	0.28	0.48	0.98
Coprococcus 2	0.00	0.22	0.050	0.00	0.03	0.06	0.05	0.03	0.013	0.02	0.20	0.20	0.02	0.03	0.02	0.04	0.012	0.52	0.17	0.23
Incertae Sedis	0.03	0.36	0.095	0.00	0.04	0.14	0.15	0.03	0.058	0.09	0.06	0.12	0.11	0.12	0.11	0.12	0.023	0.86	0.57	0.27
Lachnospira	0.11	0.01	0.023	0.00	0.03	0.03	0.05	0.00	0.011	0.56	0.00	0.70	0.03	0.02	0.03	0.02	0.009	0.58	0.63	0.17
AC2044 group (Lachnospiraceae)	0.00	0.69	0.346	0.06	0.61	0.49	0.55	0.56	0.125	0.33	0.94	0.32	0.74	0.68	0.67	0.75	0.066	0.31	0.24	0.00

Table D.2 (Continued)

	P01				P02								P03							
Genus	**CO**	**FO**	**SED**	**P-1**	**FO**	**CO**	**HQ**	**LQ**	**SED**	**P-1**	**P-2**	**P-int**	**FO**	**CO**	**HQ**	**LQ**	**SED**	**P-1**	**P-2**	**P-int**
FCS020 group (Lachnospiraceae)	0.00	0.05	0.020	0.03	0.19	0.13	0.13	0.19	0.059	0.30	0.32	0.61	0.13	0.10	0.13	0.11	0.015	0.11	0.18	0.05
ND3007 group (Lachnospiraceae)	0.01	0.14	0.033	0.00	0.24	0.66	0.61	0.30	0.236	0.09	0.21	0.21	0.48	0.40	0.42	0.47	0.061	0.20	0.43	0.92
NK3A20 group (Lachnospiraceae)	11.07	2.42	3.261	0.01	2.30	2.05	2.33	2.02	0.347	0.49	0.38	0.44	0.70	0.92	0.78	0.84	0.102	0.05	0.53	0.68
NK4A136 group (Lachnospiraceae)	0.85	0.78	0.422	0.88	0.35	0.59	0.49	0.45	0.133	0.09	0.74	0.79	0.28	0.27	0.25	0.29	0.042	0.81	0.34	0.08
UCG-005 (Lachnospiraceae)	1.11	0.01	0.820	0.20	0.04	0.02	0.05	0.01	0.036	0.42	0.33	0.23	0.03	0.03	0.03	0.03	0.005	0.92	0.63	0.57
UCG-006 (Lachnospiraceae)	0.00	0.01	0.010	0.00	0.09	0.07	0.06	0.11	0.015	0.30	0.00	0.66	0.14	0.15	0.14	0.16	0.026	0.64	0.51	0.41
UCG-008 (Lachnospiraceae)	0.01	0.11	0.024	0.00	0.18	0.18	0.13	0.24	0.032	0.90	0.00	0.82	0.21	0.26	0.23	0.24	0.024	0.03	0.64	0.25
XPB1014 group (Lachnospiraceae)	0.00	0.66	0.201	0.00	1.51	0.59	1.63	0.47	0.503	0.08	0.03	1.00	0.32	0.33	0.32	0.33	0.036	0.63	0.68	0.28
Marvinbryantia	0.01	0.14	0.037	0.00	0.19	0.16	0.19	0.16	0.023	0.17	0.34	0.16	0.15	0.19	0.18	0.16	0.018	0.01	0.37	0.13
Oribacterium	0.09	0.36	0.080	0.00	0.33	0.28	0.43	0.18	0.056	0.47	0.00	0.58	0.50	0.49	0.51	0.48	0.071	0.92	0.69	0.12
probable genus 10	0.00	0.12	0.052	0.03	0.18	0.19	0.17	0.20	0.043	0.73	0.49	0.79	0.20	0.22	0.19	0.23	0.028	0.39	0.13	0.70
Pseudobutyrivibrio	0.28	0.83	0.132	0.00	1.37	1.26	1.79	0.84	0.341	0.76	0.01	0.36	1.09	0.98	1.13	0.95	0.143	0.45	0.21	0.03
Roseburia	8.87	0.88	2.178	0.00	0.40	0.66	0.92	0.14	0.184	0.18	0.00	0.19	0.36	0.29	0.31	0.43	0.048	0.19	0.44	0.09
Shuttleworthia	0.03	0.15	0.038	0.00	0.08	0.05	0.12	0.02	0.025	0.27	0.00	0.36	0.11	0.11	0.10	0.12	0.028	0.82	0.44	0.11
Syntrophococcus	2.05	0.11	0.541	0.00	0.14	0.11	0.15	0.10	0.024	0.16	0.04	0.99	0.05	0.06	0.05	0.06	0.007	0.20	0.59	0.93
Unclassified Lachnospiraceae	0.13	0.16	0.032	0.50	0.29	0.29	0.28	0.30	0.038	0.82	0.51	0.80	0.26	0.29	0.27	0.28	0.023	0.12	0.63	0.11
Coprostanoligenes group [Eubacterium]	0.36	1.00	0.128	0.00	1.21	1.14	1.08	1.27	0.132	0.62	0.17	0.03	1.29	1.30	1.19	1.39	0.176	0.97	0.28	0.43
Anaerotruncus	0.03	0.05	0.017	0.14	0.08	0.09	0.05	0.11	0.017	0.41	0.00	0.34	0.26	0.24	0.23	0.26	0.032	0.50	0.42	0.43
Intestinimonas	0.00	0.12	0.069	0.09	0.00	0.00	0.00	0.00	0.001	0.18	0.27	0.44	0.00	0.00	0.00	0.00	0.000	0.93	0.58	0.59
Papillibacter	0.00	0.04	0.011	0.00	0.11	0.13	0.05	0.19	0.022	0.55	0.00	0.93	0.15	0.15	0.16	0.14	0.029	0.75	0.67	0.59
Ruminiclostridium 5	0.00	0.18	0.027	0.00	0.23	0.35	0.23	0.34	0.049	0.02	0.04	0.18	0.23	0.19	0.20	0.21	0.030	0.20	0.59	0.69
Ruminiclostridium 9	0.01	5.22	2.023	0.02	0.93	1.29	1.85	0.37	0.371	0.34	0.00	0.39	0.16	0.20	0.18	0.18	0.071	0.54	0.99	0.09
NK4A214 group (Ruminococcaceae)	0.10	2.14	0.356	0.00	2.26	2.05	2.53	1.78	0.345	0.55	0.04	0.77	2.94	3.06	3.16	2.84	0.291	0.69	0.28	0.99
UCG-002 (Ruminococcaceae)	0.02	0.29	0.082	0.00	0.11	0.22	0.22	0.11	0.032	0.00	0.00	0.45	0.21	0.22	0.23	0.20	0.028	0.95	0.19	0.97
UCG-005 (Ruminococcaceae)	0.03	0.45	0.061	0.00	0.31	0.48	0.36	0.43	0.094	0.08	0.48	0.79	0.91	1.10	1.05	0.95	0.190	0.31	0.61	0.75

Table D.2 (Continued)

	P01				P02								P03							
Genus	**CO**	**FO**	**SED**	**P-1**	**FO**	**CO**	**HQ**	**LQ**	**SED**	**P-1**	**P-2**	**P-int**	**FO**	**CO**	**HQ**	**LQ**	**SED**	**P-1**	**P-2**	**P-int**
UCG-007 (Ruminococcaceae)	0.00	0.15	0.093	0.12	0.08	0.09	0.10	0.06	0.020	0.42	0.07	0.26	0.08	0.03	0.08	0.02	0.055	0.38	0.32	0.31
UCG-010 (Ruminococcaceae)	0.01	0.23	0.027	0.00	0.36	0.51	0.29	0.58	0.061	0.03	0.00	0.06	0.61	0.67	0.63	0.66	0.082	0.49	0.72	0.66
UCG-013 (Ruminococcaceae)	0.01	0.06	0.018	0.01	0.06	0.12	0.06	0.11	0.017	0.00	0.01	0.93	0.08	0.08	0.08	0.07	0.014	0.82	0.40	1.00
Thalassospira	0.00	0.24	0.058	0.00	0.32	0.42	0.14	0.59	0.098	0.32	0.00	0.21	0.22	0.16	0.21	0.16	0.080	0.47	0.54	0.86
Sutterella	0.01	0.01	0.005	0.27	0.01	0.03	0.02	0.02	0.008	0.10	0.84	0.74	0.17	0.15	0.17	0.15	0.036	0.52	0.69	0.55
UCG-014 (Ruminococcaceae)	1.76	1.06	0.380	0.08	1.19	1.48	1.28	1.39	0.207	0.18	0.61	0.71	0.72	0.64	0.67	0.69	0.103	0.44	0.87	0.94
Ruminococcus 1	1.01	2.42	0.520	0.01	1.04	1.20	0.98	1.27	0.152	0.30	0.07	0.39	2.17	1.94	1.84	2.27	0.363	0.52	0.25	0.07
Ruminococcus 2	0.01	0.81	0.102	0.00	0.39	0.46	0.40	0.46	0.103	0.51	0.56	0.08	0.27	0.25	0.29	0.23	0.041	0.73	0.13	0.23
Saccharofermentans	0.01	1.00	0.268	0.00	0.72	0.85	0.65	0.92	0.115	0.27	0.03	0.15	1.04	1.04	0.98	1.09	0.105	0.99	0.33	0.14
UCG-001(Erysipelotrichaceae)	0.89	0.01	0.235	0.00	0.05	0.03	0.05	0.03	0.010	0.04	0.13	0.69	0.00	0.00	0.00	0.00	0.001	0.26	0.59	0.09
UCG-002 (Erysipelotrichaceae)	7.89	2.30	2.749	0.05	0.02	0.02	0.03	0.01	0.010	0.92	0.01	0.42	0.01	0.00	0.01	0.00	0.002	0.09	0.05	0.04
UCG-004 (Erysipelotrichaceae)	0.01	0.12	0.015	0.00	0.69	0.34	0.71	0.32	0.288	0.24	0.19	0.13	0.51	0.45	0.50	0.46	0.083	0.51	0.61	0.11
UCG-006 (Erysipelotrichaceae)	0.37	0.00	0.084	0.00	0.00	0.00	0.00	0.00	0.001	0.68	0.72	0.51	0.00	0.00	0.00	0.00	0.000	0.93	0.04	0.94
UCG-007 (Erysipelotrichaceae)	0.76	0.00	0.399	0.07	0.00	0.00	0.00	0.00	0.000	0.23	0.72	0.74	0.00	0.00	0.00	0.00	0.000	0.67	0.36	0.40
UCG-009 (Erysipelotrichaceae)	0.00	0.10	0.015	0.00	0.18	0.15	0.28	0.06	0.046	0.47	0.00	0.75	0.41	0.40	0.47	0.34	0.061	0.91	0.06	0.75
Kandleria	0.01	4.40	1.364	0.00	0.15	2.02	2.15	0.01	1.187	0.13	0.09	0.13	0.26	0.26	0.19	0.33	0.112	0.95	0.24	0.34
Sharpea	1.76	0.01	0.747	0.03	0.00	0.00	0.00	0.00	0.001	0.16	0.16	0.24	0.00	0.00	0.00	0.00	0.000	0.97	0.97	0.17
Solobacterium	2.28	0.17	0.477	0.00	0.34	0.42	0.24	0.52	0.096	0.37	0.01	0.44	0.18	0.22	0.19	0.22	0.038	0.31	0.44	0.16
Unclassified Erysipelotrichaceae	0.01	0.04	0.009	0.00	0.06	0.06	0.06	0.07	0.011	0.71	0.31	0.09	0.14	0.13	0.14	0.13	0.016	0.34	0.38	0.54
Acidaminococcus	0.74	0.00	0.117	0.00	0.00	0.00	0.00	0.00	0.001	0.28	0.20	0.38	0.00	0.00	0.00	0.00	0.001	0.35	0.36	0.30
Succiniclasticum	2.55	1.02	0.392	0.00	1.74	1.44	1.21	1.97	0.321	0.36	0.03	0.55	2.72	2.43	2.42	2.73	0.351	0.42	0.38	0.08
Dialister	0.73	0.00	0.341	0.04	0.00	0.00	0.00	0.00	0.000	0.52	0.04	0.53	0.00	0.00	0.00	0.00	0.000	0.24	0.68	0.71
Megasphaera	0.52	0.00	0.118	0.00	0.00	0.00	0.00	0.00	0.000	0.68	0.69	0.78	0.00	0.00	0.00	0.00	0.000	0.20	0.77	0.82
Mitsuokella	0.18	0.00	0.033	0.00	0.00	0.00	0.00	0.00	0.001	0.40	0.40	0.26	0.00	0.00	0.00	0.00	0.000	0.28	0.24	0.59
Selenomonas 1	0.02	0.95	0.100	0.00	0.87	0.51	1.07	0.31	0.213	0.11	0.00	0.13	1.12	0.88	0.91	1.09	0.285	0.40	0.54	0.17

Table D.2 (Continued)

Genus	P01				P02								P03							
	CO	FO	SED	P-1	FO	CO	HQ	LQ	SED	P-1	P-2	P-int	FO	CO	HQ	LQ	SED	P-1	P-2	P-int
Selenomonas	0.50	0.00	0.263	0.07	0.03	0.01	0.03	0.01	0.017	0.24	0.31	0.13	0.00	0.00	0.00	0.00	0.001	0.50	0.68	0.72
Unclassified Veillonellaceae	1.38	0.04	0.226	0.00	0.03	0.02	0.01	0.05	0.005	0.02	0.00	0.43	0.04	0.04	0.05	0.02	0.013	0.97	0.09	0.03
UCG-001 (Veillonellaceae)	0.04	0.09	0.019	0.01	0.09	0.14	0.09	0.15	0.042	0.25	0.13	0.12	0.08	0.07	0.08	0.07	0.013	0.76	0.65	0.93
RFP12 gut group (Lentisphaerae)	0.00	0.02	0.005	0.00	0.09	0.12	0.03	0.17	0.020	0.10	0.00	0.04	0.09	0.08	0.08	0.09	0.014	0.63	0.76	0.22
Victivallis	0.00	0.01	0.004	0.02	0.17	0.14	0.02	0.28	0.032	0.33	0.00	0.38	0.26	0.25	0.25	0.25	0.072	0.91	0.98	0.18
Rhizobium	0.00	0.01	0.001	0.03	0.07	0.04	0.07	0.04	0.020	0.17	0.21	0.30	0.10	0.09	0.12	0.07	0.031	0.63	0.16	0.98
Desulfovibrio	0.13	0.02	0.030	0.00	0.03	0.03	0.04	0.03	0.005	0.94	0.06	0.11	0.04	0.04	0.04	0.04	0.008	0.94	0.74	0.20
GR-WP33-58 (Desulfuromonadales)	0.00	0.04	0.015	0.01	0.07	0.09	0.06	0.10	0.014	0.10	0.00	0.29	0.08	0.09	0.09	0.09	0.018	0.60	0.86	0.65
UCG-001 (Succinivibrionaceae)	0.64	0.00	0.343	0.08	0.00	0.00	0.00	0.00	0.000	0.96	0.96	0.17	--	--	--	--	--	--	--	--
Pantoea	0.00	0.00	0.001	0.71	0.38	0.10	0.41	0.08	0.190	0.17	0.10	0.26	0.01	0.01	0.01	0.01	0.005	0.60	0.31	0.88
Candidatus Saccharimonas	0.00	0.16	0.031	0.00	0.36	0.50	0.61	0.26	0.112	0.22	0.01	0.48	0.63	0.58	0.60	0.60	0.102	0.62	0.94	0.74
Unclassified SHA-109	0.00	0.16	0.077	0.04	0.02	0.04	0.03	0.03	0.012	0.31	0.67	0.30	0.08	0.09	0.08	0.09	0.025	0.64	0.48	0.37
Sphaerochaeta	0.08	0.14	0.041	0.17	0.05	0.07	0.04	0.08	0.015	0.22	0.01	0.66	0.06	0.03	0.05	0.04	0.008	0.01	0.22	0.77
Treponema 2	0.04	0.42	0.121	0.00	0.50	0.46	0.60	0.36	0.156	0.79	0.14	0.92	0.36	0.33	0.34	0.35	0.069	0.64	0.91	0.53
Anaeroplasma	0.00	0.08	0.031	0.03	0.20	0.28	0.21	0.27	0.050	0.17	0.30	0.71	0.68	0.55	0.69	0.54	0.132	0.34	0.25	0.82
RF9 (Mollicutes)	0.43	0.87	0.141	0.01	1.29	1.58	1.38	1.49	0.231	0.21	0.66	0.59	1.52	1.40	1.41	1.51	0.197	0.58	0.64	0.87

[1]Dietary treatments corresponded to: Period 1 (P01) concentrate (CO) *vs*. pasture (FO) diets, and Period 2 (P02) high quality (HQ) *vs*. low quality (LQ) pastures, with measurements in P01 (9 wks), P02 (19 wks) and Period 3 (P03; 41 wks) when all calves were offered a common pasture diet. Measured data corresponded to the 115 ruminal bacteria genus that had a relative abundance ≥ 0.01%.

[2]Dietary treatments in each period were evaluated as follow: a one-way ANOVA in P01 to analyze FO *vs.* CO diets, and a 2 x 2 factorial ANOVA in P02 and P03 to evaluate FO *vs.* CO and HQ *vs.* LQ dietary treatment effects and their interactions.

The bacteria sequences that integrate the above 115 groups have a relative abundance of > 0.1% in at least one of the groups of calves during one of the three rearing periods. These bacterial taxa represent 96.68±0.826 of the total sequences analyzed in all the groups of calves during the three rearing periods.

[a] Indicates the group contains sequences not classified down to the genus level.

Table D.3 Analysis of variance of ruminal archaea at the species level during the three-rearing period[1]. Results[2] are natural log back transformed means and standard error of the differences (SED), P-value for treatment effect for FO *vs.* CO (P-1), treatment effect for HQ vs. LQ (P-2) and their interactions (P-int).

	P01				P02								P03							
Species	CO	FO	SED	P-1	CO	FO	HQ	LQ	SED	P-1	P-2	P-Int	CO	FO	HQ	LQ	SED	P-1	P-2	P-Int
Methanomassiliicoccales																				
Group10 sp	0.01	0.91	0.212	<0.01	3.47	2.80	1.21	5.06	0.926	0.48	<0.01	0.57	5.66	5.45	4.18	6.93	1.282	0.88	0.05	0.97
Group11 sp BRNA1	0.00	0.00	0.001	0.30	0.09	0.02	0.00	0.10	0.052	0.19	0.08	0.21	0.05	0.02	0.03	0.04	0.021	0.09	0.75	0.99
Group12 sp ISO4 H5	0.01	0.06	0.021	0.01	0.34	0.15	0.04	0.45	0.134	0.17	0.01	0.20	0.68	0.44	0.61	0.51	0.128	0.07	0.44	0.41
Group4 sp MpT1	0.00	0.08	0.026	0.01	0.43	0.32	0.14	0.61	0.132	0.42	0.00	0.09	1.80	1.39	1.82	1.37	0.330	0.23	0.18	0.70
Group8 sp WGK1	0.00	0.02	0.009	0.11	0.45	0.41	0.04	0.82	0.211	0.84	0.00	0.68	0.34	0.44	0.38	0.40	0.129	0.43	0.85	0.88
Group9 sp ISO4_G1	0.00	0.11	0.022	<0.01	0.95	0.75	0.32	1.38	0.459	0.68	0.03	0.88	0.23	0.17	0.14	0.26	0.057	0.27	0.05	0.26
Methanobacterium alkaliphilum	0.00	0.00	0.001	0.16	0.00	0.13	0.00	0.13	0.121	0.29	0.30	0.29	1.69	1.09	1.09	1.69	0.534	0.27	0.27	0.37
Methanobrevibacter																				
acididurans	0.00	0.18	0.051	0.02	0.00	0.00	0.00	0.00	0.001	1.00	0.17	1.00	0.00	0.01	0.00	0.00	0.003	0.49	1.00	0.44
boviskoreani clade	71.76	0.05	6.465	<0.01	1.21	0.04	0.04	1.21	1.160	0.32	0.33	0.33	0.05	0.07	0.07	0.04	0.025	0.43	0.30	0.08
gottschalkii clade	4.90	60.89	5.286	<0.01	60.60	66.30	59.50	67.40	5.190	0.29	0.14	0.55	64.90	64.40	63.20	66.10	2.470	0.82	0.24	0.89
…ruminantium clade	7.84	29.29	7.232	<0.01	13.90	12.50	12.10	14.30	3.620	0.70	0.55	0.77	12.88	12.67	15.57	9.97	1.441	0.89	<0.01	1.00
…smithii	0.17	0.17	0.072	0.96	0.19	0.15	0.23	0.11	0.059	0.62	0.19	0.28	0.09	0.20	0.19	0.10	0.092	0.27	0.30	0.36
…wolinii clade	0.24	0.00	0.163	0.16	0.00	0.00	0.00	0.00	0.000	0.33	0.33	0.33	-	-	-	-	-	-	-	-
Methanosphaera																				
cuniculi	0.13	0.25	0.087	0.20	1.30	0.56	1.71	0.15	0.397	0.08	<0.01	0.04	0.00	0.01	0.01	0.00	0.004	0.54	0.28	0.50
sp A4	9.83	0.03	1.498	<0.01	0.21	0.07	0.19	0.08	0.077	0.08	0.18	0.76	0.01	0.01	0.01	0.01	0.005	0.82	0.61	0.08
sp Group5	4.92	2.58	1.009	0.03	12.20	9.70	16.90	5.10	1.720	0.32	<0.01	0.71	3.61	2.84	2.92	3.53	0.844	0.37	0.48	0.22
sp ISO3 F5	0.19	5.37	0.908	<0.01	4.47	6.03	7.55	2.96	1.228	0.38	0.02	0.32	7.95	10.79	9.75	8.99	1.471	0.07	0.61	0.64

[1] Dietary treatments corresponded to: Period 1 (P01) concentrate (CO) *vs.* pasture (FO) diets, and Period 2 (P02) high quality (HQ) *vs.* low quality (LQ) pastures, with measurements in P01 (9 wks), P02 (19 wks) and Period 3 (P03; 41 wks) when all calves were offered a common pasture diet. Measured data corresponded to the 17 ruminal archaea species or main community.

[2] Dietary treatments in each period were evaluated as follow: a one-way ANOVA in P01 to analyze FO *vs.* CO diets, and a 2 x 2 factorial ANOVA in P02 and P03 to evaluate FO *vs.* CO and HQ *vs.* LQ dietary treatment effects and their interactions.

Table D.4 Effect of dietary treatments[1] on the highly abundant archaea species[2] during the three measurement periods[3]. Results are natural log back transformed means and standard error of the differences (SED), P-value for treatment effect for FO *vs.* CO (P-1), treatment effect for HQ *vs.* LQ (P-2) and their interactions (P-int).

	P01				P02								P03							
	FO	CO	SED	P-Val	FO	CO	HQ	LQ	SED	P-1	P-2	P-int	FO	CO	HQ	LQ	SED	P-1	P-2	P-int
Mmc. Group10 sp.	0.61	0.15	0.306	0.073	1.47	1.69	0.56	4.41	0.912	0.761	< < 0.001	0.966	4.60	4.65	3.55	6.03	1.219	0.966	0.052	0.882
Mbb. boviskoreani clade	0.05	64.03	6.851	< 0.001	0.03	0.06	0.03	0.05	0.029	0.294	0.564	0.586	0.03	0.04	0.05	0.03	0.013	0.628	0.113	0.016
Mbb. gottschalkii clade	57.4	2.53	13.552	< 0.001	65.36	58.91	58.16	66.21	5.748	0.274	0.176	0.787	64.22	64.56	62.89	65.93	2.506	0.892	0.239	0.937
Mbb. ruminantium clade	24.36	0.81	10.256	< 0.001	10.35	10.25	10.61	10.00	3.868	0.981	0.875	0.847	11.99	11.94	15.35	9.32	1.552	0.975	0.001	0.833
Mph. sp. A4	0.05	8.58	2.322	< 0.001	0.06	0.08	0.13	0.03	0.045	0.577	0.036	0.327	0.03	0.04	0.04	0.03	0.036	0.982	0.860	0.674
Mph. sp. Group5	1.73	4.35	0.990	0.009	5.76	9.03	14.94	3.48	3.157	0.240	0.001	0.373	2.44	3.01	2.40	3.06	0.659	0.393	0.324	0.200
Mph. sp. ISO3 F5	4.35	0.07	1.176	< 0.001	3.36	3.34	6.13	1.83	1.458	0.991	0.005	0.323	10.27	6.92	8.93	7.96	1.818	0.077	0.595	0.668

[1] Dietary treatments corresponded to: Period 1 (P01) concentrate (CO) *vs.* pastures (FO) diets, and Period 2 (P02) high quality (HQ) *vs*. low quality (LQ) pastures, with measurements in P01 (9 wks), P02 (19 wks) and Period 3 (P03; 41 wks) when all calves were offered a common pasture diet.

[2] Measured effect corresponded to the 7 abundant ruminal archaea species.

[3] Dietary treatments in each period were evaluated as follow: a one-way ANOVA in P01 to analyze FO *vs.* CO diets, and a 2 x 2 factorial ANOVA in P02 and P03 to evaluate FO *vs.* CO and HQ *vs.* LQ dietary treatment effects and their interactions.

Mbb. – *Methanobrevibacter*

Mmc. – *Methanomassiliicoccales*

Mph. – *Methanosphaera*

Appendix E

This appendix corresponds to additional tables and figures from Chapter 5

Table E.1 Phyla composition of the ruminal bacteria across the different sampling periods. Bacterial phyla highlighted in bold are the most abundant in the rumen of calves.

Time (weeks)	**1**		**2**		**3**		**4**		**5**		**6**		**7**		**8**	
Treatment	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**
Firmicutes	48.13	41.12	40.47	31.18	43.07	46.95	38.34	49.42	47.37	42.83	49.16	45.11	53.60	51.26	31.60	30.49
Bacteroidetes	39.62	39.18	42.67	46.80	39.38	38.26	40.38	37.53	37.08	43.84	39.36	43.46	40.54	42.59	59.10	59.89
Proteobacteria	9.92	16.89	13.01	16.77	5.04	8.44	11.87	6.36	8.52	4.25	5.05	5.32	0.74	0.57	2.53	2.24
Spirochaetae	0.68	1.24	1.35	2.47	7.81	3.33	6.11	3.06	2.13	3.40	0.89	1.88	1.00	1.08	0.97	1.12
Tenericutes	0.22	0.19	0.40	0.63	0.55	0.79	0.61	1.06	0.79	0.90	1.70	1.97	1.46	1.55	1.35	1.42
Fibrobacteres	0.04	0.15	0.12	0.27	1.19	0.12	0.83	0.84	1.72	2.33	0.91	0.80	0.55	0.91	1.23	1.92
Actinobacteria	1.14	0.96	1.18	1.61	1.02	0.49	0.77	0.76	0.71	1.80	0.66	0.66	0.51	0.57	0.51	0.39
Cyanobacteria	0.03	0.02	0.02	0.01	0.14	0.11	0.24	0.07	0.41	0.08	0.84	0.15	0.42	0.35	0.98	1.00
SHA 109	0.01	0.01	0.23	0.03	0.98	1.16	0.17	0.43	0.58	0.14	0.13	0.05	0.19	0.09	0.05	0.05
Saccharibacteria	0.00	0.00	0.00	0.00	0.09	0.02	0.06	0.09	0.14	0.11	0.11	0.20	0.32	0.44	0.49	0.41
Lentisphaerae	0.02	0.01	0.05	0.02	0.13	0.03	0.15	0.05	0.14	0.04	0.56	0.06	0.10	0.12	0.30	0.27
Elusimicrobia	0.04	0.01	0.29	0.00	0.17	0.01	0.08	0.00	0.09	0.00	0.14	0.01	0.03	0.02	0.04	0.05
Chloroflexi	0.01	0.00	0.05	0.02	0.14	0.03	0.09	0.05	0.07	0.05	0.06	0.03	0.05	0.04	0.08	0.07
Synergistetes	0.02	0.06	0.03	0.05	0.07	0.01	0.07	0.04	0.05	0.01	0.09	0.01	0.02	0.01	0.04	0.04
Candidate division SR1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.10	0.06	0.20	0.18
Planctomycetes	0.00	0.00	0.01	0.00	0.04	0.03	0.04	0.02	0.02	0.01	0.03	0.02	0.01	0.02	0.04	0.02
Armatimonadetes	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.01	0.02	0.02
Chlamydiae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.01	0.01

Table E.2 Class composition of the ruminal bacteria across the different sampling periods. Bacterial classes highlighted in bold are the most abundant in the rumen of calves.

Time (weeks)	**1**		**2**		**3**		**4**		**5**		**6**		**7**		**8**	
Treatment	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**
Bacteroidia	39.62	39.18	42.67	46.80	39.38	38.25	40.36	37.52	37.06	43.82	39.27	43.45	40.41	42.47	58.97	59.81
Clostridia	33.62	26.53	31.94	22.06	37.97	41.15	32.45	41.62	33.73	32.31	44.08	37.23	41.49	40.26	27.33	26.80
Gammaproteobacteria	9.39	14.78	12.67	15.49	4.60	8.01	11.40	5.88	7.98	3.80	3.85	4.33	0.20	0.13	0.90	0.92
Erysipelotrichia	9.07	10.34	5.30	4.43	3.11	2.77	3.65	5.03	11.85	5.47	3.31	4.22	6.38	5.09	1.00	0.78
Negativicutes	5.37	4.18	3.19	4.65	1.93	2.89	2.19	2.67	1.51	2.98	1.37	1.95	5.31	5.21	3.13	2.81
Spirochaetes	0.68	1.24	1.35	2.47	7.81	3.33	6.11	3.06	2.13	3.40	0.89	1.88	1.00	1.08	0.97	1.12
Mollicutes	0.22	0.19	0.40	0.63	0.55	0.79	0.61	1.06	0.79	0.90	1.70	1.97	1.46	1.55	1.35	1.42
Fibrobacteria	0.04	0.15	0.12	0.27	1.19	0.12	0.83	0.84	1.72	2.33	0.91	0.80	0.55	0.91	1.23	1.92
Coriobacteriia	1.13	0.95	1.17	1.61	1.00	0.47	0.75	0.74	0.69	1.77	0.63	0.62	0.48	0.54	0.45	0.32
Alphaproteobacteria	0.04	0.01	0.04	0.09	0.28	0.17	0.32	0.36	0.43	0.28	1.11	0.89	0.42	0.30	1.24	0.96
Bacilli	0.07	0.08	0.04	0.04	0.06	0.15	0.05	0.10	0.27	2.08	0.40	1.71	0.42	0.70	0.13	0.10
Deltaproteobacteria	0.32	1.97	0.23	1.08	0.11	0.18	0.09	0.08	0.04	0.08	0.05	0.07	0.07	0.07	0.20	0.17
Melainabacteria	0.03	0.01	0.02	0.01	0.14	0.11	0.23	0.06	0.40	0.07	0.82	0.13	0.36	0.30	0.98	1.00
SHA 109 *	0.01	0.01	0.23	0.03	0.98	1.16	0.17	0.43	0.58	0.14	0.13	0.05	0.19	0.09	0.05	0.05
Saccharibacteria *	0.00	0.00	0.00	0.00	0.09	0.02	0.06	0.09	0.14	0.11	0.11	0.20	0.32	0.44	0.49	0.41
Betaproteobacteria	0.08	0.08	0.04	0.06	0.03	0.08	0.04	0.04	0.05	0.08	0.03	0.03	0.04	0.06	0.17	0.20
Lentisphaeria	0.00	0.00	0.00	0.00	0.06	0.00	0.09	0.00	0.09	0.00	0.40	0.00	0.05	0.06	0.13	0.12
Elusimicrobia	0.04	0.01	0.29	0.00	0.17	0.01	0.08	0.00	0.09	0.00	0.14	0.01	0.03	0.02	0.04	0.05
Anaerolineae	0.01	0.00	0.05	0.02	0.14	0.03	0.09	0.05	0.07	0.05	0.06	0.03	0.04	0.04	0.06	0.06
Lentisphaerae RFP12 gut group	0.02	0.00	0.04	0.01	0.06	0.02	0.06	0.05	0.05	0.03	0.12	0.05	0.03	0.03	0.09	0.09
Synergistia	0.02	0.06	0.03	0.05	0.07	0.01	0.07	0.04	0.05	0.01	0.09	0.01	0.02	0.01	0.04	0.04
Candidate division SR1 *	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.10	0.06	0.20	0.18
Actinobacteria	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.03	0.03	0.04	0.03	0.03	0.06	0.07

Table E.2 (Continued)

Time (weeks)	**1**		**2**		**3**		**4**		**5**		**6**		**7**		**8**	
Treatment	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**
Bacteroidetes VC2.1 Bac22	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.01	0.00	0.01	0.00	0.05	0.07	0.11	0.06
Planctomycetacia	0.00	0.00	0.01	0.00	0.04	0.03	0.04	0.02	0.02	0.01	0.03	0.02	0.01	0.02	0.04	0.02
Epsilonproteobacteria	0.07	0.05	0.03	0.04	0.02	0.01	0.03	0.01	0.02	0.01	0.01	0.00	0.00	0.01	0.01	0.00
Oligosphaeria	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.02	0.03	0.07	0.05
Bacteroidetes BD2-2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.07	0.01	0.06	0.03	0.01	0.02
Chloroplast	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.06	0.04	0.01	0.01
Armatimonadetes *	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.01	0.02	0.02
Flavobacteriia	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.01	0.01	0.00	0.00	0.01	0.01	0.00	0.00
Dehalococcoidia	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.02	0.01
WCHB1-25	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00
Chlamydiae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.01	0.01
Other classes	0.14	0.17	0.13	0.14	0.17	0.22	0.19	0.21	0.18	0.20	0.30	0.22	0.35	0.32	0.44	0.41

* Unclassified class

Table E.3 Order composition of the ruminal bacteria across the different sampling periods. Bacterial order highlighted in bold are the most abundant in the rumen of calves.

Periods	**1**		**2**		**3**		**4**		**5**		**6**		**7**		**8**	
Treatment	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**
Bacteroidales	39.62	39.18	42.67	46.80	39.38	38.25	40.36	37.52	37.06	43.82	39.27	43.45	40.41	42.47	58.97	59.81
Clostridiales	33.62	26.53	31.94	22.06	37.97	41.15	32.45	41.62	33.73	32.31	44.08	37.23	41.49	40.26	27.33	26.80
Aeromonadales	9.38	14.75	12.66	15.47	4.58	7.97	11.37	5.83	7.93	3.75	3.82	4.30	0.11	0.07	0.16	0.17
Erysipelotrichales	9.07	10.34	5.30	4.43	3.11	2.77	3.65	5.03	11.85	5.47	3.31	4.22	6.38	5.09	1.00	0.78
Selenomonadales	5.37	4.18	3.19	4.65	1.93	2.89	2.19	2.67	1.51	2.98	1.37	1.95	5.31	5.21	3.13	2.81
Spirochaetales	0.68	1.24	1.35	2.47	7.81	3.33	6.11	3.06	2.13	3.40	0.89	1.88	1.00	1.08	0.97	1.12
Fibrobacterales	0.04	0.15	0.12	0.27	1.19	0.12	0.83	0.84	1.72	2.33	0.91	0.80	0.55	0.91	1.23	1.92
Coriobacteriales	1.13	0.95	1.17	1.61	1.00	0.47	0.75	0.74	0.69	1.77	0.63	0.62	0.48	0.54	0.45	0.32
Mollicutes RF9	0.08	0.16	0.30	0.60	0.39	0.72	0.57	1.02	0.78	0.87	1.50	1.93	1.16	1.21	0.91	0.87
Lactobacillales	0.07	0.07	0.04	0.04	0.06	0.15	0.05	0.10	0.27	2.08	0.40	1.70	0.42	0.70	0.12	0.09
Rhodospirillales	0.04	0.00	0.04	0.09	0.27	0.15	0.31	0.34	0.41	0.24	1.06	0.81	0.37	0.25	1.01	0.74
Gastranaerophilales	0.03	0.01	0.02	0.01	0.14	0.11	0.23	0.06	0.40	0.07	0.82	0.13	0.36	0.30	0.98	1.00
SHA 109 *	0.01	0.01	0.23	0.03	0.98	1.16	0.17	0.43	0.58	0.14	0.13	0.05	0.19	0.09	0.05	0.05
Desulfovibrionales	0.31	1.97	0.20	1.08	0.05	0.10	0.03	0.04	0.01	0.01	0.01	0.01	0.01	0.02	0.03	0.03
Saccharibacteria *	0.00	0.00	0.00	0.00	0.09	0.02	0.06	0.09	0.14	0.11	0.11	0.20	0.32	0.44	0.49	0.41
Anaeroplasmatales	0.05	0.00	0.01	0.01	0.08	0.04	0.04	0.02	0.01	0.03	0.18	0.04	0.28	0.32	0.39	0.51
Enterobacteriales	0.01	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.02	0.01	0.64	0.67
Victivallales	0.00	0.00	0.00	0.00	0.06	0.00	0.09	0.00	0.09	0.00	0.40	0.00	0.05	0.06	0.13	0.12
Anaerolineales	0.01	0.00	0.05	0.02	0.14	0.03	0.09	0.05	0.07	0.05	0.06	0.03	0.04	0.04	0.06	0.06
Lentisphaerae RFP12 gut group *	0.02	0.00	0.04	0.01	0.06	0.02	0.06	0.05	0.05	0.03	0.12	0.05	0.03	0.03	0.09	0.09
Burkholderiales	0.02	0.04	0.01	0.03	0.01	0.05	0.02	0.02	0.03	0.06	0.02	0.02	0.04	0.05	0.15	0.17
Desulfuromonadales	0.01	0.00	0.03	0.00	0.06	0.08	0.06	0.03	0.02	0.06	0.03	0.06	0.06	0.05	0.06	0.09
Lineage I (Endomicrobia)	0.01	0.00	0.28	0.00	0.11	0.00	0.05	0.00	0.06	0.00	0.11	0.00	0.00	0.00	0.01	0.01
Synergistales	0.02	0.06	0.03	0.05	0.07	0.01	0.07	0.04	0.05	0.01	0.09	0.01	0.02	0.01	0.04	0.04
Candidate division SR1 *	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.10	0.06	0.20	0.18
NB1-n	0.06	0.03	0.09	0.02	0.08	0.00	0.01	0.01	0.01	0.00	0.02	0.01	0.02	0.01	0.05	0.04
Rhizobiales	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.02	0.02	0.03	0.02	0.02	0.15	0.15

Table E.3 (Continued)

Periods	**1**		**2**		**3**		**4**		**5**		**6**		**7**		**8**	
Treatment	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**
Bacteroidetes VC2.1 Bac22 *	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.01	0.00	0.01	0.00	0.05	0.07	0.11	0.06
Pseudomonadales	0.01	0.01	0.00	0.01	0.01	0.02	0.01	0.02	0.01	0.02	0.01	0.01	0.06	0.03	0.07	0.06
Planctomycetales	0.00	0.00	0.01	0.00	0.04	0.03	0.04	0.02	0.02	0.01	0.03	0.02	0.01	0.02	0.04	0.02
Campylobacterales	0.07	0.05	0.03	0.04	0.02	0.01	0.03	0.01	0.02	0.01	0.01	0.00	0.00	0.01	0.01	0.00
Elusimicrobiales	0.04	0.01	0.00	0.00	0.05	0.01	0.02	0.00	0.03	0.00	0.03	0.01	0.02	0.01	0.03	0.04
Neisseriales	0.06	0.04	0.02	0.03	0.01	0.02	0.02	0.01	0.01	0.02	0.00	0.01	0.01	0.01	0.01	0.01
Corynebacteriales	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.03	0.02	0.02	0.02	0.02	0.04	0.05
Oligosphaerales	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.02	0.03	0.07	0.05
Bacteroidetes BD2-2 *	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.07	0.01	0.06	0.03	0.01	0.02
Chloroplast *	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.06	0.04	0.01	0.01
Pasteurellales	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.01	0.02	0.02	0.03	0.02
Sphingomonadales	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.02	0.02	0.03	0.03	0.02	0.02
Oligoflexales	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.04
Rickettsiales	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.02	0.00	0.00	0.05	0.04
Micrococcales	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.00	0.01	0.01	0.02	0.01	0.01	0.02	0.02
Acholeplasmatales	0.03	0.00	0.00	0.00	0.00	0.03	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00
Armatimonadetes *	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.01	0.02	0.02
UCT N117	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.00	0.01	0.00	0.01	0.00	0.00	0.00	0.01	0.02
Flavobacteriales	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.01	0.01	0.00	0.00	0.01	0.01	0.00	0.00
Caulobacterales	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.00	0.01	0.01	0.00	0.01	0.00	0.00	0.00	0.00
MSBL5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.02	0.01
WCHB1-25 *	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00
Desulfobacterales	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00
Order Incertae Sedis	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.00
Bacillales	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.02
Chlamydiales	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.01	0.01
na	0.14	0.17	0.13	0.14	0.17	0.22	0.19	0.21	0.18	0.20	0.30	0.22	0.35	0.32	0.44	0.41

* Unclassified order

Table E.4 Family composition of the ruminal bacteria across the different sampling periods. Bacteria family highlighted in bold are the most abundant in the rumen of calves.

Time (weeks)	**1**		**2**		**3**		**4**		**5**		**6**		**7**		**8**	
Treatment	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**
Prevotellaceae	32.71	31.69	26.45	36.22	23.73	17.87	23.41	16.15	23.79	27.48	25.39	23.57	32.83	34.02	46.78	47.21
Ruminococcaceae	12.72	10.11	9.34	6.50	19.44	16.51	18.27	16.19	18.27	15.65	25.77	15.83	19.34	19.28	10.46	10.39
Lachnospiraceae	18.21	14.89	18.73	13.90	11.39	17.46	8.05	14.12	7.91	8.34	10.20	11.42	16.63	14.72	10.06	9.86
Succinivibrionaceae	9.38	14.75	12.66	15.47	4.58	7.97	11.37	5.83	7.93	3.75	3.82	4.30	0.11	0.07	0.16	0.17
Christensenellaceae	1.97	0.92	2.62	1.27	6.08	6.37	5.41	10.25	6.85	7.49	5.43	9.07	4.20	4.84	5.58	5.43
Erysipelotrichaceae	9.07	10.34	5.30	4.43	3.11	2.77	3.65	5.03	11.85	5.47	3.31	4.22	6.38	5.09	1.00	0.78
Rikenellaceae	1.44	3.54	2.47	3.12	3.81	6.24	3.89	8.41	3.61	6.72	4.47	9.99	2.20	2.56	4.98	4.73
Bacteroidales BS11 gut group	2.70	0.56	9.45	2.62	4.53	2.12	3.28	2.83	3.16	2.38	4.35	2.33	1.83	2.06	2.92	3.20
Bacteroidales S24-7 group	0.59	0.84	3.48	2.34	6.06	3.75	5.33	3.39	3.38	3.25	1.86	2.89	2.23	2.34	2.03	2.54
Spirochaetaceae	0.68	1.24	1.35	2.47	7.81	3.32	6.11	3.04	2.12	3.39	0.89	1.87	0.99	1.07	0.95	1.10
Acidaminococcaceae	1.73	2.56	1.62	3.18	1.18	2.52	1.67	2.15	0.92	2.24	0.76	1.24	1.03	1.23	1.71	1.52
p-2534-18B5 gut group	0.02	0.08	0.03	1.25	0.09	7.38	3.26	5.51	2.09	3.00	0.67	3.23	0.22	0.32	0.02	0.03
Veillonellaceae	3.64	1.63	1.57	1.47	0.74	0.37	0.51	0.52	0.59	0.74	0.62	0.71	4.28	3.99	1.43	1.28
Fibrobacteraceae	0.04	0.15	0.12	0.27	1.19	0.12	0.83	0.84	1.72	2.33	0.91	0.80	0.55	0.91	1.23	1.92
Coriobacteriaceae	1.13	0.95	1.17	1.61	1.00	0.47	0.75	0.74	0.69	1.77	0.63	0.62	0.48	0.54	0.45	0.32
Mollicutes RF9 *	0.08	0.16	0.30	0.60	0.39	0.72	0.57	1.02	0.78	0.87	1.50	1.93	1.16	1.21	0.91	0.87
Family XIII	0.14	0.11	0.25	0.25	0.42	0.55	0.44	0.59	0.50	0.51	0.62	0.55	1.21	1.31	1.04	0.92
Bacteroidales RF16 group	0.02	0.03	0.00	0.13	0.16	0.25	0.36	0.37	0.57	0.20	1.40	0.15	0.65	0.77	1.55	1.47
Bacteroidaceae	1.75	1.84	0.29	0.59	0.41	0.17	0.18	0.12	0.15	0.17	0.39	0.21	0.13	0.14	0.31	0.33
Rhodospirillaceae	0.04	0.00	0.04	0.09	0.27	0.15	0.31	0.34	0.41	0.24	1.06	0.81	0.37	0.25	1.01	0.74
Streptococcaceae	0.02	0.03	0.01	0.02	0.03	0.12	0.03	0.08	0.22	2.04	0.37	1.67	0.35	0.69	0.12	0.09
Gastranaerophilales *	0.03	0.01	0.02	0.01	0.14	0.11	0.23	0.06	0.40	0.07	0.82	0.13	0.36	0.30	0.98	1.00
Bacteroidales UCG-001	0.01	0.02	0.04	0.24	0.36	0.32	0.45	0.47	0.17	0.54	0.18	0.95	0.18	0.13	0.20	0.16
SHA 109 *	0.01	0.01	0.23	0.03	0.98	1.16	0.17	0.43	0.58	0.14	0.13	0.05	0.19	0.09	0.05	0.05

Table E.4 (Continued)

Time (weeks)	**1**		**2**		**3**		**4**		**5**		**6**		**7**		**8**	
Treatment	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**
Desulfovibrionaceae	0.31	1.97	0.20	1.08	0.05	0.10	0.03	0.04	0.01	0.01	0.01	0.01	0.01	0.02	0.03	0.03
Clostridiales vadinBB60 group	0.15	0.10	0.95	0.10	0.49	0.21	0.18	0.41	0.16	0.24	0.37	0.25	0.05	0.03	0.08	0.09
Clostridiaceae 1	0.40	0.36	0.02	0.00	0.11	0.01	0.00	0.00	0.00	0.00	1.65	0.08	0.00	0.00	0.01	0.01
Saccharibacteria *	0.00	0.00	0.00	0.00	0.09	0.02	0.06	0.09	0.14	0.11	0.11	0.20	0.32	0.44	0.49	0.41
Anaeroplasmataceae	0.05	0.00	0.01	0.01	0.08	0.04	0.04	0.02	0.01	0.03	0.18	0.04	0.28	0.32	0.39	0.51
Porphyromonadaceae	0.26	0.29	0.06	0.11	0.03	0.04	0.04	0.15	0.05	0.05	0.12	0.08	0.04	0.07	0.09	0.06
Enterobacteriaceae	0.01	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.02	0.01	0.64	0.67
Victivallaceae	0.00	0.00	0.00	0.00	0.06	0.00	0.09	0.00	0.09	0.00	0.40	0.00	0.05	0.06	0.13	0.12
Bacteroidales Incertae Sedis	0.06	0.23	0.19	0.13	0.08	0.06	0.06	0.04	0.03	0.02	0.01	0.03	0.03	0.02	0.00	0.00
Anaerolineaceae	0.01	0.00	0.05	0.02	0.14	0.03	0.09	0.05	0.07	0.05	0.06	0.03	0.04	0.04	0.06	0.06
Lentisphaerae RFP12 gut group *	0.02	0.00	0.04	0.01	0.06	0.02	0.06	0.05	0.05	0.03	0.12	0.05	0.03	0.03	0.09	0.09
RH-aaj90h05	0.04	0.01	0.19	0.05	0.10	0.05	0.07	0.06	0.05	0.02	0.02	0.01	0.02	0.01	0.01	0.00
GR-WP33-58	0.01	0.00	0.03	0.00	0.06	0.08	0.06	0.03	0.02	0.06	0.03	0.06	0.06	0.05	0.06	0.09
Alcaligenaceae	0.01	0.02	0.01	0.02	0.01	0.05	0.01	0.02	0.03	0.06	0.02	0.02	0.03	0.05	0.15	0.17
Lineage I (Endomicrobia) *	0.01	0.00	0.28	0.00	0.11	0.00	0.05	0.00	0.06	0.00	0.11	0.00	0.00	0.00	0.01	0.01
Synergistaceae	0.02	0.06	0.03	0.05	0.07	0.01	0.07	0.04	0.05	0.01	0.09	0.01	0.02	0.01	0.04	0.04
Candidate division SR1 *	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.10	0.06	0.20	0.18
Defluviitaleaceae	0.01	0.00	0.00	0.00	0.00	0.01	0.05	0.04	0.02	0.07	0.01	0.02	0.05	0.06	0.07	0.08
Lactobacillaceae	0.05	0.04	0.03	0.02	0.03	0.03	0.01	0.02	0.05	0.04	0.02	0.04	0.07	0.01	0.00	0.00
Marinilabiaceae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.38	0.00	0.02	0.01	0.02	0.02
NB1-n *	0.06	0.03	0.09	0.02	0.08	0.00	0.01	0.01	0.01	0.00	0.02	0.01	0.02	0.01	0.05	0.04
Bacteroidetes VC2.1 Bac22 *	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.01	0.00	0.01	0.00	0.05	0.07	0.11	0.06
Planctomycetaceae	0.00	0.00	0.01	0.00	0.04	0.03	0.04	0.02	0.02	0.01	0.03	0.02	0.01	0.02	0.04	0.02
Campylobacteraceae	0.07	0.05	0.03	0.04	0.02	0.01	0.03	0.01	0.02	0.01	0.01	0.00	0.00	0.01	0.01	0.00

Table E.4 (Continued)

Time (weeks)	1		2		3		4		5		6		7		8	
Treatment	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**
Elusimicrobiaceae	0.04	0.01	0.00	0.00	0.05	0.01	0.02	0.00	0.03	0.00	0.03	0.01	0.02	0.01	0.03	0.04
Neisseriaceae	0.06	0.04	0.02	0.03	0.01	0.02	0.02	0.01	0.01	0.02	0.00	0.01	0.01	0.01	0.01	0.01
Bacteroidales *	0.01	0.06	0.01	0.01	0.02	0.01	0.01	0.03	0.01	0.00	0.03	0.01	0.02	0.02	0.02	0.02
Rhizobiaceae	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.01	0.09	0.08
Oligosphaeraceae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.02	0.03	0.07	0.05
Bacteroidetes BD2-2 *	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.07	0.01	0.06	0.03	0.01	0.02
Pseudomonadaceae	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.01	0.01	0.00	0.01	0.05	0.02	0.06	0.04
Eubacteriaceae	0.02	0.03	0.03	0.03	0.03	0.02	0.02	0.02	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00
Chloroplast *	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.06	0.04	0.01	0.01
Pasteurellaceae	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.01	0.02	0.02	0.03	0.02
Methylobacteriaceae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.06	0.07
Sphingomonadaceae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.02	0.02	0.03	0.03	0.02	0.02
Peptococcaceae	0.00	0.00	0.00	0.00	0.01	0.00	0.02	0.00	0.01	0.00	0.03	0.00	0.01	0.01	0.02	0.02
Oligoflexales *	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.04
Corynebacteriaceae	0.01	0.01	0.00	0.01	0.00	0.01	0.01	0.02	0.01	0.02	0.01	0.01	0.01	0.01	0.00	0.00
Nocardiaceae	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.01	0.02	0.01	0.04	0.04
Rickettsiales Incertae Sedis	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.02	0.00	0.00	0.05	0.04
Microbacteriaceae	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.00	0.01	0.01	0.02	0.01	0.01	0.02	0.02
Moraxellaceae	0.01	0.00	0.00	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.01
Acholeplasmataceae	0.03	0.00	0.00	0.00	0.00	0.03	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00
PL-11B10	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.02	0.01	0.01	0.00	0.01	0.00	0.00	0.01	0.02
PeH15	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.04
Comamonadaceae	0.01	0.02	0.00	0.00	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Armatimonadetes *	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.01	0.02	0.02

Table E.4 (Continued)

Time (weeks)	**1**		**2**		**3**		**4**		**5**		**6**		**7**		**8**	
Treatment	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**
UCT N117 *	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.00	0.01	0.00	0.01	0.00	0.00	0.00	0.01	0.02
Flavobacteriaceae	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.01	0.01	0.00	0.00	0.01	0.01	0.00	0.00
Caulobacteraceae	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.00	0.01	0.01	0.00	0.01	0.00	0.00	0.00	0.00
MSBL5 *	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.02	0.01
WCHB1-25 *	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00
Desulfobulbaceae	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00
Family Incertae Sedis	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.00
Bacillaceae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.02
WA-aaa01f12	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.01	0.01
na	0.14	0.17	0.13	0.14	0.17	0.22	0.19	0.21	0.18	0.20	0.30	0.22	0.35	0.32	0.44	0.41

* Unclassified family

Table E.5 Genus composition of the ruminal bacteria across the different sampling periods. Bacterial genera highlighted in bold are the most abundant in the rumen of calves.

Time (weeks)	**1**		**2**		**3**		**4**		**5**		**6**		**7**		**8**	
Treatment	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**
Prevotella 1	24.11	25.41	21.49	29.31	21.30	15.09	20.88	13.83	19.24	25.01	20.91	21.17	28.64	29.72	40.01	40.22
Christensenellaceae R-7 group	1.96	0.91	2.60	1.26	6.07	6.36	5.39	10.25	6.81	7.49	5.40	9.07	4.19	4.83	5.57	5.42
Rikenellaceae RC9 gut group	1.02	3.41	2.21	2.98	3.38	6.13	3.71	8.29	3.53	6.64	4.44	9.88	2.16	2.51	4.91	4.59
Ruminococcus 2	5.98	5.24	2.24	1.66	7.14	8.15	6.66	4.12	8.03	4.97	6.56	3.22	0.48	0.59	0.31	0.27
Sharpea	8.85	10.08	2.45	4.20	2.04	2.34	3.13	4.65	7.69	4.61	0.44	0.99	0.01	0.01	0.00	0.01
Bacteroidales BS11 gut group *	2.70	0.56	9.45	2.62	4.53	2.12	3.28	2.83	3.16	2.38	4.35	2.33	1.83	2.06	2.92	3.20
OBacteroidales S24-7 group *	0.59	0.84	3.48	2.34	6.06	3.75	5.33	3.39	3.38	3.25	1.86	2.89	2.23	2.34	2.03	2.54
Ruminobacter	0.02	0.12	4.45	2.95	0.13	7.20	7.95	3.14	7.03	2.96	3.38	3.96	0.03	0.02	0.03	0.03
Ruminiclostridium 9	0.05	0.10	0.09	0.13	0.76	1.39	1.35	2.95	1.68	3.55	7.47	5.08	6.49	7.67	0.40	0.37
Lachnospiraceae UCG-005	10.25	4.99	8.43	0.43	0.03	3.19	0.02	2.84	0.01	0.03	0.01	0.01	0.02	0.01	0.05	0.03
Treponema 2	0.11	0.94	0.98	2.03	7.23	2.92	4.37	2.23	1.48	1.93	0.53	1.01	0.87	0.93	0.80	0.95
Roseburia	3.34	5.53	1.77	7.28	0.90	4.79	0.08	0.31	0.10	0.17	0.35	0.18	1.74	1.28	0.53	0.42
p-2534-18B5 gut group *	0.02	0.08	0.03	1.25	0.09	7.38	3.26	5.51	2.09	3.00	0.67	3.23	0.22	0.32	0.02	0.03
Lachnospiraceae NK3A20 group	0.02	0.10	0.15	1.06	0.32	4.04	0.81	2.27	2.02	2.28	3.65	5.33	1.27	1.28	1.24	1.18
Succiniclasticum	0.95	1.90	1.49	2.89	1.17	2.51	1.66	2.13	0.92	2.23	0.75	1.24	1.03	1.23	1.71	1.52
Succinivibrio	3.06	9.48	5.54	3.82	1.69	0.14	0.28	0.03	0.70	0.32	0.10	0.06	0.00	0.00	0.02	0.02
Ruminococcus 1	0.46	0.37	0.71	0.59	1.43	0.80	1.61	1.29	2.06	1.64	1.99	1.58	3.37	2.55	1.79	1.80
Ruminococcaceae UCG-014	1.42	1.55	0.74	1.74	1.20	2.69	1.55	1.82	2.01	1.16	2.26	1.09	1.28	1.29	1.03	0.96
Succinivibrionaceae UCG-002	3.09	0.11	1.68	7.97	2.68	0.41	2.86	2.55	0.15	0.32	0.32	0.21	0.07	0.03	0.08	0.09
Ruminococcaceae NK4A214 group	1.49	0.17	0.44	0.37	0.93	0.74	0.99	0.74	0.96	0.62	0.91	0.71	2.89	2.64	2.46	2.80
Ruminiclostridium 5	1.77	0.91	2.55	0.15	3.68	0.19	2.32	1.76	0.66	0.71	1.94	0.29	0.31	0.34	0.25	0.25
Prevotellaceae UCG-001	1.32	0.95	1.53	1.32	1.03	0.43	0.60	0.43	0.77	0.48	1.55	0.40	1.00	1.28	1.83	2.27
Prevotellaceae UCG-003	0.25	0.32	0.41	0.57	0.63	0.49	0.82	0.59	2.16	0.61	1.67	0.66	0.93	1.43	2.84	2.53
Selenomonas 1	0.87	0.69	0.32	0.60	0.27	0.19	0.19	0.40	0.28	0.66	0.38	0.60	3.74	3.36	0.97	0.90

Table E.5 (Continued)

Time (weeks)	**1**		**2**		**3**		**4**		**5**		**6**		**7**		**8**	
Treatment	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**
Fibrobacter	0.04	0.15	0.12	0.27	1.19	0.12	0.83	0.84	1.72	2.33	0.91	0.80	0.55	0.91	1.23	1.92
Pseudobutyrivibrio	0.51	0.45	0.64	0.37	0.32	0.21	0.25	0.45	0.23	0.35	1.01	0.69	2.02	2.54	1.52	1.52
Mollicutes RF9 *	0.08	0.16	0.30	0.60	0.39	0.72	0.57	1.02	0.78	0.87	1.50	1.93	1.16	1.21	0.91	0.87
Prevotella 7	6.08	2.51	1.87	0.85	0.05	0.08	0.02	0.03	0.07	0.02	0.01	0.01	0.50	0.30	0.04	0.03
Kandleria	0.00	0.00	0.00	0.00	0.01	0.00	0.05	0.04	1.34	0.38	0.10	0.16	5.56	4.33	0.29	0.05
Succinimonas	3.19	5.02	0.96	0.71	0.06	0.22	0.26	0.10	0.04	0.13	0.00	0.04	0.00	0.00	0.01	0.01
Ruminococcaceae UCG-005	0.22	0.07	0.94	0.15	0.88	0.29	0.96	1.21	0.60	0.99	0.93	0.65	0.64	0.68	0.63	0.49
[Eubacterium] coprostanoligenes group	0.06	0.05	0.21	0.19	0.74	0.33	0.79	0.45	0.77	0.39	1.14	0.58	1.04	1.10	1.02	0.95
Erysipelotrichaceae UCG-002	0.00	0.00	1.11	0.00	0.54	0.00	0.20	0.02	2.40	0.03	2.33	2.66	0.26	0.11	0.00	0.00
Atopobium	0.61	0.24	0.56	0.94	0.87	0.15	0.67	0.60	0.62	1.69	0.58	0.55	0.32	0.35	0.33	0.24
Lachnospiraceae **	0.25	0.28	0.34	0.20	3.81	0.51	1.20	0.58	0.22	0.26	0.20	0.36	0.23	0.23	0.27	0.26
Prevotellaceae NK3B31 group	0.02	1.46	0.31	2.47	0.29	0.36	0.50	0.20	0.90	0.14	0.72	0.08	0.54	0.31	0.31	0.29
Sphaerochaeta	0.57	0.30	0.37	0.45	0.58	0.40	1.74	0.81	0.63	1.47	0.34	0.86	0.11	0.13	0.06	0.03
Lachnospiraceae NK4A136 group	0.19	0.45	0.73	0.32	0.50	0.64	0.46	0.46	0.43	0.40	0.70	0.98	0.71	0.61	0.46	0.41
Ruminococcaceae UCG-002	0.22	0.83	0.37	0.73	0.50	0.78	0.30	0.75	0.30	0.60	0.39	0.91	0.53	0.42	0.38	0.38
Bacteroidales RF16 group *	0.02	0.03	0.00	0.13	0.16	0.25	0.36	0.37	0.57	0.20	1.40	0.15	0.65	0.77	1.55	1.47
Alloprevotella	0.64	0.83	0.65	1.26	0.34	1.19	0.40	0.70	0.24	0.64	0.13	0.42	0.11	0.13	0.15	0.11
[Eubacterium] ventriosum group	0.95	0.06	3.58	2.40	0.27	0.02	0.09	0.10	0.09	0.03	0.06	0.05	0.04	0.04	0.06	0.05
Saccharofermentans	0.02	0.00	0.10	0.01	0.41	0.37	0.99	0.41	0.57	0.36	1.11	0.33	1.20	1.03	0.49	0.47
[Eubacterium] oxidoreducens group	0.00	0.00	0.01	0.00	0.50	0.04	1.75	2.18	1.23	1.25	0.41	0.17	0.01	0.01	0.03	0.03
Bacteroides	1.75	1.84	0.29	0.59	0.41	0.17	0.18	0.12	0.15	0.17	0.39	0.21	0.13	0.14	0.31	0.33
[Eubacterium] ruminantium group	0.12	0.06	0.07	0.31	0.13	0.16	0.51	0.28	0.35	0.15	0.28	0.32	1.53	0.94	0.34	0.36
Streptococcus	0.02	0.03	0.01	0.02	0.03	0.12	0.03	0.08	0.22	2.04	0.37	1.67	0.35	0.69	0.12	0.09
[Eubacterium] hallii group	0.06	0.33	0.13	0.15	0.40	0.55	0.32	1.02	0.46	0.61	0.23	0.38	0.33	0.36	0.24	0.22
Thalassospira	0.03	0.00	0.03	0.09	0.25	0.14	0.27	0.34	0.39	0.23	0.97	0.77	0.35	0.24	0.96	0.69

Table E.5 (Continued)

Time (weeks)	1		2		3		4		5		6		7		8	
Treatment	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**
Butyrivibrio 2	0.01	0.01	0.02	0.00	0.04	0.01	0.04	0.12	0.07	0.18	0.27	0.20	1.21	1.14	1.24	1.18
[Ruminococcus] gauvreauii group	0.40	0.18	0.88	0.13	0.80	0.26	0.24	0.19	0.77	0.13	0.36	0.06	0.34	0.36	0.17	0.17
Gastranaerophilales *	0.03	0.01	0.02	0.01	0.14	0.11	0.23	0.06	0.40	0.07	0.82	0.13	0.36	0.30	0.98	1.00
Bacteroidales UCG-001 *	0.01	0.02	0.04	0.24	0.36	0.32	0.45	0.47	0.17	0.54	0.18	0.95	0.18	0.13	0.20	0.16
Acetitomaculum	0.02	0.00	0.19	0.12	1.04	0.35	0.48	0.54	0.19	0.27	0.21	0.22	0.15	0.17	0.22	0.20
Blautia	0.70	0.98	0.31	0.25	0.22	0.31	0.12	0.26	0.14	0.40	0.07	0.08	0.10	0.10	0.15	0.14
Ruminococcaceae UCG-010	0.04	0.01	0.03	0.03	0.28	0.10	0.15	0.14	0.14	0.19	0.26	1.05	0.28	0.33	0.66	0.63
SHA 109 *	0.01	0.01	0.23	0.03	0.98	1.16	0.17	0.43	0.58	0.14	0.13	0.05	0.19	0.09	0.05	0.05
Erysipelotrichaceae UCG-004	0.18	0.21	1.48	0.08	0.25	0.20	0.08	0.08	0.16	0.10	0.14	0.05	0.18	0.15	0.28	0.30
Desulfovibrio	0.31	1.97	0.20	1.08	0.05	0.10	0.03	0.04	0.01	0.01	0.01	0.01	0.01	0.02	0.03	0.03
Christensenellaceae *	0.15	0.10	0.95	0.10	0.49	0.21	0.18	0.41	0.16	0.24	0.37	0.25	0.05	0.03	0.08	0.09
Lachnospiraceae AC2044 group	0.00	0.00	0.05	0.00	0.04	0.01	0.04	0.08	0.05	0.04	0.20	0.57	0.46	1.07	0.52	0.54
Prevotellaceae **	0.02	0.08	0.00	0.09	0.00	0.15	0.01	0.14	0.01	0.15	0.04	0.53	0.18	0.10	0.96	1.05
Mogibacterium	0.06	0.06	0.10	0.17	0.14	0.30	0.11	0.27	0.11	0.20	0.14	0.17	0.46	0.51	0.36	0.31
Coprococcus 2	0.10	0.14	0.15	0.03	0.06	0.05	0.24	0.28	0.24	0.33	0.33	0.25	0.65	0.23	0.05	0.04
Olsenella	0.48	0.69	0.57	0.61	0.08	0.26	0.05	0.06	0.04	0.02	0.00	0.00	0.01	0.01	0.01	0.01
Lachnospiraceae ND3007 group	0.00	0.00	0.03	0.01	0.00	0.02	0.01	0.01	0.01	0.01	0.01	0.05	1.19	0.76	0.34	0.41
Oribacterium	0.09	0.03	0.04	0.07	0.13	0.04	0.06	0.03	0.05	0.03	0.06	0.03	0.41	0.43	0.63	0.67
Lachnospiraceae XPB1014 group	0.00	0.00	0.00	0.00	0.01	0.00	0.02	0.04	0.02	0.03	0.09	0.02	1.32	0.47	0.36	0.37
Clostridium sensu stricto 1	0.40	0.36	0.02	0.00	0.11	0.01	0.00	0.00	0.00	0.00	1.65	0.08	0.00	0.00	0.01	0.01
Syntrophococcus	0.15	0.23	0.40	0.25	0.23	0.18	0.11	0.09	0.15	0.19	0.10	0.11	0.05	0.11	0.07	0.07
Candidatus Saccharimonas	0.00	0.00	0.00	0.00	0.09	0.02	0.06	0.09	0.14	0.11	0.11	0.20	0.32	0.44	0.49	0.41
Anaerosporobacter	0.02	0.00	0.14	0.01	0.68	0.15	0.18	1.04	0.07	0.04	0.06	0.02	0.00	0.02	0.00	0.00
Lachnoclostridium	0.68	0.69	0.23	0.22	0.07	0.16	0.03	0.06	0.02	0.03	0.02	0.03	0.05	0.04	0.03	0.02
Anaerotruncus	0.05	0.05	0.46	0.09	0.88	0.09	0.10	0.06	0.05	0.05	0.12	0.04	0.08	0.09	0.08	0.07

Table E.5 (Continued)

Time (weeks)	**1**		**2**		**3**		**4**		**5**		**6**		**7**		**8**	
Treatment	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**
Anaerovibrio	0.36	0.10	0.48	0.23	0.16	0.04	0.11	0.02	0.06	0.03	0.06	0.03	0.08	0.12	0.20	0.16
Family XIII AD3011 group	0.03	0.00	0.07	0.01	0.14	0.06	0.15	0.06	0.16	0.07	0.26	0.08	0.31	0.32	0.25	0.21
Anaeroplasma	0.05	0.00	0.01	0.01	0.08	0.04	0.04	0.02	0.01	0.03	0.18	0.04	0.28	0.32	0.39	0.51
Incertae Sedis	0.01	0.01	0.02	0.01	0.05	0.03	0.02	0.05	0.03	0.04	0.36	0.03	0.62	0.40	0.13	0.10
Prevotellaceae YAB2003 group	0.04	0.03	0.02	0.04	0.01	0.01	0.01	0.01	0.01	0.03	0.13	0.06	0.70	0.43	0.16	0.19
Shuttleworthia	0.01	0.07	0.01	0.02	0.00	0.86	0.07	0.02	0.02	0.01	0.09	0.02	0.36	0.22	0.05	0.05
Marvinbryantia	0.01	0.00	0.01	0.02	0.17	0.25	0.15	0.07	0.18	0.18	0.13	0.13	0.10	0.09	0.13	0.15
Ruminiclostridium 6	0.02	0.02	0.05	0.06	0.16	0.03	0.15	0.07	0.11	0.07	0.16	0.07	0.20	0.13	0.22	0.24
Coprococcus 1	0.07	0.04	0.05	0.04	0.07	0.05	0.20	0.13	0.21	0.05	0.08	0.01	0.22	0.26	0.11	0.11
[Eubacterium] nodatum group	0.01	0.01	0.01	0.03	0.06	0.13	0.09	0.17	0.12	0.16	0.10	0.20	0.17	0.18	0.12	0.09
Megasphaera	0.38	0.13	0.28	0.31	0.12	0.07	0.08	0.00	0.12	0.00	0.12	0.00	0.00	0.00	0.00	0.00
Prevotellaceae Ga6A1 group	0.01	0.00	0.00	0.02	0.00	0.01	0.06	0.05	0.33	0.19	0.15	0.10	0.04	0.13	0.19	0.27
Selenomonas	1.43	0.01	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Acidaminococcus	0.56	0.51	0.13	0.28	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ruminococcaceae **	0.17	0.27	0.08	0.17	0.09	0.07	0.05	0.07	0.03	0.06	0.04	0.03	0.12	0.08	0.07	0.08
Veillonellaceae UCG-001	0.08	0.23	0.05	0.08	0.09	0.02	0.07	0.07	0.07	0.04	0.04	0.06	0.09	0.09	0.12	0.11
Prevotellaceae UCG-004	0.01	0.00	0.02	0.04	0.07	0.02	0.11	0.10	0.04	0.17	0.05	0.10	0.06	0.08	0.20	0.15
Ruminococcaceae UCG-004	0.09	0.08	0.08	0.07	0.08	0.09	0.05	0.11	0.07	0.08	0.06	0.06	0.07	0.07	0.06	0.06
Schwartzia	0.21	0.28	0.15	0.12	0.00	0.03	0.01	0.01	0.01	0.01	0.01	0.03	0.07	0.18	0.02	0.02
Prevotella 9	0.20	0.06	0.15	0.23	0.01	0.01	0.00	0.01	0.01	0.01	0.00	0.00	0.13	0.10	0.10	0.10
Lachnospiraceae UCG-008	0.02	0.01	0.01	0.01	0.05	0.04	0.07	0.06	0.08	0.08	0.08	0.10	0.10	0.10	0.13	0.14
SP3-e08	0.08	0.01	0.10	0.03	0.34	0.02	0.13	0.08	0.06	0.04	0.01	0.05	0.03	0.02	0.02	0.03
Lachnospiraceae FCS020 group	0.00	0.00	0.02	0.00	0.01	0.01	0.01	0.01	0.02	0.03	0.05	0.34	0.14	0.18	0.09	0.08
Victivallis	0.00	0.00	0.00	0.00	0.06	0.00	0.09	0.00	0.09	0.00	0.40	0.00	0.05	0.06	0.13	0.12
Anaerovorax	0.01	0.00	0.01	0.02	0.02	0.04	0.05	0.06	0.05	0.05	0.06	0.07	0.12	0.13	0.15	0.14

Table E.5 (Continued)

Time (weeks)	1		2		3		4		5		6		7		8	
Treatment	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**
Phocaeicola	0.06	0.23	0.19	0.13	0.08	0.06	0.06	0.04	0.03	0.02	0.01	0.03	0.03	0.02	0.00	0.00
Erysipelotrichaceae UCG-009	0.00	0.00	0.00	0.01	0.05	0.01	0.02	0.04	0.02	0.05	0.04	0.06	0.20	0.26	0.10	0.11
Erysipelotrichaceae **	0.01	0.01	0.12	0.06	0.08	0.12	0.05	0.07	0.03	0.10	0.03	0.05	0.04	0.06	0.06	0.07
Ruminiclostridium	0.14	0.20	0.12	0.11	0.02	0.11	0.02	0.01	0.02	0.00	0.03	0.00	0.01	0.01	0.06	0.08
Pantoea	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.00	0.00	0.00	0.44	0.44
Lachnospiraceae FE2018 group	0.01	0.02	0.02	0.01	0.13	0.11	0.07	0.12	0.03	0.12	0.12	0.14	0.00	0.00	0.01	0.01
[Eubacterium] cellulosolvens group	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.39	0.43	0.05	0.03
Lachnospiraceae UCG-001	0.00	0.01	0.04	0.01	0.05	0.03	0.12	0.13	0.08	0.14	0.10	0.07	0.02	0.02	0.03	0.02
Mitsuokella	0.27	0.15	0.17	0.09	0.05	0.01	0.03	0.01	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ruminococcaceae UCG-013	0.17	0.06	0.02	0.02	0.02	0.04	0.03	0.03	0.04	0.03	0.07	0.06	0.06	0.04	0.08	0.09
probable genus 10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.08	0.11	0.11	0.23	0.14	0.15
Solobacterium	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.01	0.04	0.16	0.07	0.08	0.09	0.16	0.14
Anaerolineaceae **	0.01	0.00	0.05	0.02	0.14	0.03	0.09	0.05	0.07	0.05	0.06	0.03	0.04	0.04	0.06	0.06
Lentisphaerae RFP12 gut group *	0.02	0.00	0.04	0.01	0.06	0.02	0.06	0.05	0.05	0.03	0.12	0.05	0.03	0.03	0.09	0.09
Moryella	0.01	0.05	0.04	0.02	0.08	0.12	0.07	0.05	0.06	0.07	0.05	0.02	0.02	0.03	0.04	0.02
RH-aaj90h05 *	0.04	0.01	0.19	0.05	0.10	0.05	0.07	0.06	0.05	0.02	0.02	0.01	0.02	0.01	0.01	0.00
GR-WP33-58 *	0.01	0.00	0.03	0.00	0.06	0.08	0.06	0.03	0.02	0.06	0.03	0.06	0.06	0.05	0.06	0.09
Tyzzerella 3	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.00	0.04	0.00	0.11	0.13	0.17	0.19
Sutterella	0.01	0.02	0.01	0.02	0.01	0.05	0.01	0.02	0.03	0.06	0.02	0.02	0.03	0.05	0.15	0.17
U29-B03	0.07	0.01	0.15	0.08	0.06	0.07	0.01	0.02	0.01	0.02	0.01	0.02	0.01	0.02	0.04	0.09
Lineage I (Endomicrobia) *	0.01	0.00	0.28	0.00	0.11	0.00	0.05	0.00	0.06	0.00	0.11	0.00	0.00	0.00	0.01	0.01
[Anaerorhabdus] furcosa group	0.01	0.00	0.06	0.01	0.05	0.03	0.05	0.04	0.07	0.03	0.02	0.03	0.05	0.05	0.07	0.08
Howardella	0.07	0.06	0.05	0.05	0.04	0.04	0.04	0.06	0.03	0.08	0.02	0.05	0.01	0.01	0.01	0.02
Quinella	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.27	0.20	0.08	0.06
Papillibacter	0.00	0.00	0.01	0.00	0.02	0.00	0.07	0.02	0.08	0.01	0.10	0.02	0.02	0.02	0.13	0.11

Table E.5 (Continued)

Time (weeks)	**1**		**2**		**3**		**4**		**5**		**6**		**7**		**8**	
Treatment	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**
Candidate division SR1 *	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.10	0.06	0.20	0.18
Erysipelotrichaceae UCG-001	0.01	0.00	0.05	0.03	0.06	0.04	0.03	0.04	0.07	0.10	0.02	0.10	0.00	0.00	0.00	0.00
Lachnospiraceae UCG-006	0.01	0.00	0.01	0.00	0.02	0.01	0.06	0.01	0.05	0.03	0.04	0.02	0.03	0.05	0.09	0.09
Family XIII UCG-001	0.01	0.02	0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.02	0.02	0.02	0.08	0.09	0.08	0.08
Ruminococcaceae UCG-007	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.00	0.01	0.01	0.13	0.13	0.13	0.07
Defluviitaleaceae UCG-011	0.01	0.00	0.00	0.00	0.00	0.01	0.05	0.04	0.02	0.07	0.01	0.02	0.05	0.06	0.07	0.08
Lactobacillus	0.05	0.04	0.03	0.02	0.03	0.03	0.01	0.02	0.05	0.04	0.02	0.04	0.07	0.01	0.00	0.00
Marinilabiaceae **	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.38	0.00	0.02	0.01	0.02	0.02
Alistipes	0.27	0.11	0.02	0.02	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00
Ruminococcaceae UCG-003	0.02	0.04	0.01	0.13	0.01	0.16	0.01	0.03	0.00	0.00	0.01	0.01	0.00	0.00	0.01	0.01
NB1-n *	0.06	0.03	0.09	0.02	0.08	0.00	0.01	0.01	0.01	0.00	0.02	0.01	0.02	0.01	0.05	0.04
Subdoligranulum	0.25	0.07	0.04	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lachnospira	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.01	0.00	0.00	0.00	0.01	0.06	0.06	0.11	0.14
Coriobacteriaceae **	0.03	0.01	0.03	0.02	0.03	0.02	0.02	0.04	0.02	0.01	0.01	0.01	0.04	0.05	0.03	0.03
Phascolarctobacterium	0.22	0.15	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lachnospiraceae UCG-002	0.01	0.00	0.01	0.01	0.02	0.02	0.02	0.03	0.03	0.02	0.03	0.03	0.02	0.02	0.05	0.05
Rhodospirillaceae **	0.01	0.00	0.01	0.00	0.02	0.00	0.03	0.00	0.02	0.01	0.09	0.04	0.01	0.01	0.05	0.06
Ruminococcaceae V9D2013 group	0.00	0.00	0.01	0.00	0.14	0.00	0.02	0.00	0.01	0.00	0.06	0.00	0.01	0.01	0.04	0.05
Anaerostipes	0.01	0.00	0.00	0.01	0.02	0.01	0.02	0.04	0.02	0.07	0.02	0.05	0.03	0.02	0.03	0.02
Porphyromonadaceae **	0.00	0.00	0.01	0.01	0.01	0.00	0.03	0.01	0.02	0.00	0.05	0.04	0.01	0.04	0.06	0.05
Butyricimonas	0.12	0.13	0.02	0.06	0.01	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Dorea	0.05	0.04	0.05	0.03	0.02	0.10	0.01	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Parabacteroides	0.12	0.13	0.01	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00
Bacteroidetes VC2.1 Bac22 *	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.01	0.00	0.01	0.00	0.05	0.07	0.11	0.06
Proteiniphilum	0.00	0.00	0.00	0.00	0.00	0.02	0.01	0.13	0.02	0.04	0.02	0.03	0.03	0.02	0.02	0.01

Table E.5 (Continued)

Time (weeks)	1		2		3		4		5		6		7		8	
Treatment	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**
Pyramidobacter	0.01	0.04	0.02	0.04	0.04	0.01	0.03	0.00	0.02	0.00	0.04	0.00	0.01	0.01	0.02	0.02
[Eubacterium] brachy group	0.03	0.00	0.03	0.01	0.04	0.00	0.02	0.01	0.02	0.01	0.02	0.01	0.03	0.04	0.02	0.02
p-1088-a5 gut group	0.00	0.00	0.01	0.00	0.04	0.03	0.04	0.02	0.02	0.01	0.03	0.02	0.01	0.02	0.04	0.02
Campylobacter	0.07	0.05	0.03	0.04	0.02	0.01	0.03	0.01	0.02	0.01	0.01	0.00	0.00	0.01	0.01	0.00
Senegalimassilia	0.00	0.01	0.01	0.01	0.01	0.02	0.01	0.02	0.01	0.02	0.02	0.02	0.04	0.04	0.04	0.03
Prevotella 2	0.02	0.03	0.01	0.03	0.00	0.03	0.00	0.07	0.01	0.03	0.03	0.03	0.01	0.01	0.01	0.01
Elusimicrobium	0.04	0.01	0.00	0.00	0.05	0.01	0.02	0.00	0.03	0.00	0.03	0.01	0.02	0.01	0.03	0.04
Oscillospira	0.00	0.00	0.00	0.01	0.01	0.04	0.01	0.07	0.01	0.05	0.01	0.02	0.00	0.00	0.05	0.02
Enterobacter	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.12	0.15
Bacteroidales **	0.01	0.06	0.01	0.01	0.02	0.01	0.01	0.03	0.01	0.00	0.03	0.01	0.02	0.02	0.02	0.02
Lachnospiraceae NK4B4 group	0.00	0.00	0.00	0.00	0.05	0.00	0.02	0.02	0.05	0.02	0.04	0.03	0.03	0.02	0.01	0.00
Ruminococcaceae UCG-001	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.04	0.06	0.03	0.01	0.03	0.02	0.03	0.04
Spirochaeta 2	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.00	0.02	0.00	0.01	0.01	0.09	0.12
Lachnospiraceae UCG-009	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.02	0.04	0.07	0.04	0.03	0.01	0.01	0.02
Rhizobium	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.01	0.09	0.08
Asteroleplasma	0.01	0.03	0.02	0.05	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.03	0.00	0.00	0.01	0.01
Neisseriaceae **	0.05	0.04	0.02	0.03	0.01	0.02	0.02	0.01	0.01	0.01	0.00	0.00	0.01	0.01	0.01	0.01
Lachnobacterium	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.13	0.10	0.01	0.01
Oligosphaeraceae *	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.02	0.03	0.07	0.05
Lachnospiraceae *	0.01	0.01	0.00	0.01	0.01	0.01	0.00	0.01	0.01	0.05	0.03	0.05	0.01	0.01	0.01	0.01
Bacteroidetes BD2-2 *	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.07	0.01	0.06	0.03	0.01	0.02
Lachnospiraceae NC2004 group	0.00	0.00	0.00	0.00	0.03	0.01	0.01	0.00	0.00	0.00	0.03	0.00	0.05	0.04	0.03	0.03
Lachnoclostridium 1	0.00	0.00	0.04	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.06	0.04	0.01	0.01
Pseudomonas	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.01	0.01	0.00	0.01	0.05	0.02	0.06	0.04
hoa5-07d05 gut group	0.00	0.00	0.00	0.02	0.02	0.01	0.04	0.02	0.01	0.02	0.02	0.03	0.00	0.00	0.01	0.01

Table E.5 (Continued)

Time (weeks)	**1**		**2**		**3**		**4**		**5**		**6**		**7**		**8**	
Treatment	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**
Family XIII UCG-002	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.04	0.06	0.05
Veillonellaceae **	0.01	0.01	0.02	0.00	0.05	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.02	0.02	0.03	0.02
Cloacibacillus	0.01	0.01	0.01	0.01	0.03	0.01	0.02	0.03	0.02	0.01	0.05	0.00	0.00	0.00	0.01	0.01
Chloroplast *	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.06	0.04	0.01	0.01
Christensenellaceae **	0.00	0.00	0.01	0.00	0.01	0.00	0.02	0.00	0.03	0.00	0.03	0.00	0.02	0.02	0.02	0.01
Ruminococcaceae UCG-012	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.00	0.07	0.00	0.01	0.02	0.02	0.03
Methylobacterium	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.06	0.07
Sphingomonas	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.02	0.02	0.03	0.03	0.02	0.02
Lachnospiraceae UCG-010	0.01	0.02	0.00	0.02	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.02	0.01	0.00	0.01	0.01
Succinivibrionaceae **	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.01	0.01	0.01
Ruminococcaceae UCG-009	0.00	0.00	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Enterorhabdus	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.01	0.01	0.01	0.04	0.04	0.01	0.01
Peptococcaceae **	0.00	0.00	0.00	0.00	0.01	0.00	0.02	0.00	0.01	0.00	0.03	0.00	0.01	0.01	0.02	0.02
Oligoflexales *	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.04
Ruminiclostridium 1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.03	0.02	0.04	0.04
Pseudoramibacter	0.01	0.00	0.02	0.02	0.03	0.02	0.02	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Corynebacterium 1	0.01	0.01	0.00	0.01	0.00	0.01	0.01	0.02	0.01	0.02	0.01	0.01	0.01	0.01	0.00	0.00
Rhodococcus	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.01	0.02	0.01	0.04	0.04
Candidatus Hepatincola	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.02	0.00	0.00	0.05	0.04
Oscillibacter	0.00	0.00	0.00	0.02	0.00	0.01	0.01	0.04	0.00	0.02	0.00	0.01	0.00	0.00	0.00	0.00
Allisonella	0.03	0.03	0.02	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.00
Lachnospiraceae UCG-004	0.01	0.00	0.02	0.01	0.01	0.01	0.00	0.00	0.00	0.00	0.01	0.00	0.02	0.02	0.01	0.01
Erysipelothrix	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.03	0.02	0.01	0.01	0.00	0.00	0.00	0.00
Lachnoclostridium 10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.04	0.04
Lachnoclostridium 12	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.01	0.01	0.02

Table E.5 (Continued)

Time (weeks)	1		2		3		4		5		6		7		8	
Treatment	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**
Acinetobacter	0.01	0.00	0.00	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.01
Tyzzerella	0.00	0.01	0.00	0.01	0.00	0.01	0.01	0.01	0.00	0.01	0.00	0.02	0.00	0.00	0.00	0.00
Anaerobiospirillum	0.00	0.01	0.02	0.01	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01
Ruminococcaceae UCG-008	0.00	0.00	0.00	0.01	0.01	0.02	0.00	0.02	0.00	0.02	0.01	0.00	0.00	0.00	0.00	0.00
Acholeplasma	0.03	0.00	0.00	0.00	0.00	0.03	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00
Erysipelotrichaceae UCG-008	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.00	0.01	0.01	0.02	0.02
PL-11B10 *	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.02	0.01	0.01	0.00	0.01	0.00	0.00	0.01	0.02
Collinsella	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.00	0.02	0.00	0.01	0.00	0.01	0.00	0.00
Mannheimia	0.00	0.01	0.00	0.01	0.00	0.01	0.00	0.00	0.01	0.01	0.00	0.00	0.01	0.00	0.01	0.00
Denitrobacterium	0.00	0.00	0.00	0.02	0.00	0.01	0.00	0.01	0.00	0.00	0.00	0.01	0.01	0.02	0.00	0.00
Faecalibacterium	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Eubacterium	0.02	0.03	0.01	0.01	0.00	0.01	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00
Erwinia	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.03	0.03
Parvibacter	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.03	0.01	0.01
Other F_PeH15	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.04
Comamonas	0.01	0.02	0.00	0.00	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Odoribacter	0.01	0.01	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.01	0.00	0.00	0.00	0.00
Curtobacterium	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.00	0.00	0.01	0.01
Porphyromonas	0.01	0.03	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
possible genus Sk018	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.01	0.01	0.01
Armatimonadetes *	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.01	0.02	0.02
Fretibacterium	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.01	0.00	0.00	0.00	0.01	0.00	0.01	0.01
UCT N117 *	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.00	0.01	0.00	0.01	0.00	0.00	0.00	0.01	0.02
Intestinimonas	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.01	0.00	0.00
Haemophilus	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.01	0.01	0.00	0.01	0.00	0.00	0.00	0.00

Table E.5 (Continued)

Time (weeks)	**1**		**2**		**3**		**4**		**5**		**6**		**7**		**8**	
Treatment	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**	**Ctrl**	**Trt**
Coprococcus 3	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00
Flavobacterium	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.01	0.01	0.00	0.00	0.01	0.01	0.00	0.00
Candidatus Soleaferrea	0.01	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00
Microbacterium	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.01	0.01	0.01
Tyzzerella 4	0.01	0.00	0.01	0.00	0.01	0.00	0.00	0.01	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00
Bibersteinia	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.02	0.01
Christensenella	0.01	0.01	0.01	0.01	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Brevundimonas	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.00	0.01	0.01	0.00	0.01	0.00	0.00	0.00	0.00
MSBL5 *	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.02	0.01
Neisseria	0.01	0.01	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00
WCHB1-25 *	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00
Desulfobulbus	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00
Marinicella	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.00
Fusicatenibacter	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00
Bacillus	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.02
Rahnella	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.02
Catonella	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.02	0.00	0.00
Hafnia	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.03
Tatumella	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.02
WA-aaa01f12 *	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.01	0.01
na	0.14	0.17	0.13	0.14	0.17	0.22	0.19	0.21	0.18	0.20	0.30	0.22	0.35	0.32	0.44	0.41

* Unclassified genera

** Uncultured genera