The effect of an ionophore, essential oils and a probiotic on the rumen and jejunal microbiome of South African Bonsmara cattle raised under feedlot conditions

by

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Declaration

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I have not previously, in its entirety or in part, submitted this dissertation for a degree at any other tertiary institution.

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Date: September 2022

These wings came from pain
But they are wings headed for the light
Through it’s hard and it hurts
If I can fly, I will fly

A Supplementary Story: You Never Walk Alone, BTS

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Abstract

Feed additives are commonly used to modify the rumen microbiome towards more energy efficient pathways, such as the production of propionate. The urgency for the replacement or decrease in use of antibiotic growth promoters (AGP) and the reduction of methane emissions has necessitated the investigation of alternative feed additives, such as probiotics and essential oils, and its effect on the gastrointestinal tract microbiome. Microorganisms in the gastrointestinal tract of ruminants play an essential role in the health and production of the animal. The microbes in the rumen degrade feedstuff into energy the animal can utilize while the microorganisms in the small intestine can affect the health of the animal. The overall aim of this study was to investigate the effect of an ionophore, a probiotic and essential oils on the rumen and jejunal microbiome of Bonsmara cattle raised under feedlot conditions using 16S rRNA and internal transcriber spacer (ITS) amplicon sequencing. Forty-eight Bonsmara bull calves (228 ± 22 kg) were backgrounded and fed a starter, grower, and finisher diet for 120 days. The bulls were blocked by weight and randomly divided into four groups: a basal diet (CON) and the basal diet supplemented with monensin (MON), essential oils (EO) or a probiotic (PRO). Four animals from each group were selected at the start of the trial to have rumen content collected using a stomach tube in each phase. Rumen content and jejunum digesta samples were also collected from all the animals at slaughter. Extracted DNA was sent for 16S rRNA (V3-V4) and ITS1 sequencing. Quality control of the sequencing data was performed, and alpha and beta diversity was calculated. The Kruskal-Wallis and Dunn tests were used to determine significant differences in the relative abundance of the taxa and alpha diversity between the feedlot diets, the feed additive groups, and the sampling methods. The first objective of this study was to determine the changes in the rumen microbiome composition through the growth phases in cattle raised under feedlot conditions. There was a significant difference in the microbiome composition among the feedlot diets, with the alpha diversity of the rumen bacteria and the abundance of Bacteroidetes decreasing as the concentrate component in the feed increased from backgrounding to the finisher phase. The effect of feeding monensin was compared to the effect of essential oils and probiotics on the rumen microbiome composition of cattle fed feedlot diets. There was no substantial difference in the rumen microbiome composition of CON, EO or PRO compared to MON. The high abundance of Proteobacteria in the finisher phase has been associated with an imbalance in the rumen microbiome. The abundance of Proteobacteria in finisher phase of the PRO group was lower compared to the other groups while the alpha diversity was higher. The effect of monensin and essential oils on the rumen microbiome decreased as the feedlot period progressed from the starter to the finisher phases which could be an indication of the adaptation of the microbes to the feed additives. The effect of the different feed additives on the jejunum microbiome was investigated and it was observed that all three feed additives had a significant effect on the jejunum bacterial diversity and composition. A higher diversity was observed within the PRO and CON groups with higher abundances of beneficial microbes that has been associated with healthier animals such as *Roseburia*,

Blautia and *Eubacterium*, compared to MON and EO. The supplementation of probiotics has been reported to result in an increase in commensal and beneficial bacteria. The inclusion of monensin and essential oils decreased the diversity and the abundance of pathogenic and beneficial microbes. The microbial composition of rumen samples collected via different sampling techniques was studied and it was observed that samples collected via stomach tube differed significantly from those collected at slaughter. This might be due to the withdrawal of feed before the animals were slaughtered. Minimal significant differences were observed among monensin, essential oils and probiotics on the rumen microbiome of the cattle in this study, therefore essential oils and probiotics can be considered alternatives to the use of monensin. Large scale feeding studies are needed to validate the findings of this study.

Thesis outputs

Publications

Peer-review journals

Linde, D.A., van Marle-Köster, E., du Toit, C.J.L., Scholtz, M.M. & Schokker, D., 2022. Rumen microbial diversity of Bonsmara cattle using amplicon sequencing during a 120-day growth trial. S. Afr. J. Anim. Sci. 52, 148-161

Popular science articles

Linde, D.A., 2020. The role of the rumen microbiome in increasing animal efficiency. AFMA Matrix, July, pp. 45-49

*Received 1st price for a literature review in the AFMA Intervarsity Writer's Cup competition.

Congresses

National

Linde, D.A., du Toit, C.J.L., Scholtz, M.M. & van Marle-Köster, E., 2021. Methane emissions measured with a Laser Methane Detector in Bonsmara cattle fed different feed additives in a feedlot growth trial. 52nd South African Society for Animal Science (SASAS) Congress, 10 – 12 August 2021.

Linde, D.A., du Toit, C.J.L. & van Marle-Köster, E., 2021. Die samestelling van die rumen mikrobioom van Suid-Afrikaanse voerkraal beeste met verwysing na bakteriee, archaea en fungi (*The composition of the rumen microbiome of South African feedlot cattle with reference to bacteria, archaea and fungi*). Studentesimposium in die Natuurwetenskappe/ Student symposium in the Natural Sciences, Potchefstroom, 28-29 October 2021.

International

Linde, D.A., du Toit, C.J.L., Schokker, D. & van Marle-Köster, E., 2022. The effect of probiotic supplementation on the alpha diversity and Proteobacteria ratio in South African feedlot cattle in the finisher phase. Proceedings of the American Society for Animal science (ASAS) Annual Meeting, Oklahoma, United States of America, 26-30 June.

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Reports to industry

Progress report on the study investigating the microbiome of feedlot cattle, Sernick Feedlot, Edenville, Free State, 1 June 2021

Table of Contents

List of figures

List of tables

Pages

Chapter 2 Literature review

Chapter 3 Rumen microbial diversity of Bonsmara cattle using amplicon sequencing during a 120-day growth trial

Chapter 4 A comparison of the effect of a probiotic and essential oils to an ionophore on the rumen microbiome composition of Bonsmara cattle raised under feedlot conditions using 16S rRNA and ITS amplicon sequencing

Chapter 5 The effect of monensin, a Bacillus-probiotic, and essential oils on the bacterial and fungal composition in the jejunum of South African Bonsmara cattle

Chapter 6 Non-invasive approaches for sample collection in rumen microbiome studies

List of supplementary tables

List of abbreviations

ADG	Average daily gain
AGP	Antibiotic growth promoter
ANOVA	Analysis of variance
ANOSIM	Analysis of similarities
ASV	Amplicon sequence variant
ATP	Adenosine triphosphate
bp	base pairs
CON	Control
DFI	Daily feed intake
DFM	Direct fed microbials
DNA	Deoxyribonucleic acid
EO	Essential oil
EU	European Union
FCR	Feed conversion ratio
FI	Feed intake
ITS	Internal transcribed spacer
KEGG	Kyoto encyclopaedia of genes and genomes
kg	Kilogram
l	Litre
LAB	Lactic acid producing bacteria
LW	Live weight
mg	Milligram
ml	Millilitre
mmol	Millimole
MON	Monensin
mRNA	Messenger Ribonucleic acid
NGS	Next generation sequencing
OTU	Operational taxonomic unit
PCoA	Principal coordinate analysis
PERMANOVA	Permutation multivariate analysis of variance
PRO	Probiotic
QIIME	Quantitative insights into microbial ecology
qPCR	Quantitative polymerase chain reaction
RFI	Residual feed intake

RFLP	Restriction fragment length polymorphism
rRNA	Ribosomal Ribonucleic acid
rpm	Revolutions per minute
SCFA	Short chain volatile fatty acids
tVFA	Total volatile fatty acids
USA	United States of America
VFA	Volatile fatty acid

Chapter 1
Introduction

1.1 Overview

Approximately 75% of beef is produced under feedlot systems in South Africa (DAFF, 2019) which require sustainable genetic potential for growth, optimal feeding regimes, feeding facilities and management. Consumers are demanding the formulation of diets and the production of cattle without antibiotic growth promotors (AGP), such as ionophores, and several studies have been performed on potential feed additives to replace AGPs (Markowiak & Śliżewska, 2018).

Increasing the efficiency of the animal has become one of the primary goals in livestock production. An efficient animal, in present times, yields more product with a lower feed intake, emit less methane, and has higher microbial fermentation. It has been reported that an improvement of 10% in cattle efficiency resulted in 43% increase in net income (Fox *et al.*, 2001). This is due to the fact that feed costs are the largest expenditure in a ruminant production system (Clemmons *et al.*, 2019). Methods are continuously being discovered and used to increase the efficiency of ruminant production and decrease the adverse effects of the livestock sector on the environment. Modern day livestock production methods should take into consideration sustainability, in terms of environment and economy, to combat contributions to climate change and meet the demand of a growing population for animal-based products.

Microorganisms found in the digestive tract of ruminants have been reported to influence their health and efficiency (Ley *et al.*, 2008). The microorganisms that are housed in the rumen are responsible for the amount of energy that is available to the animal for production as well as microbial protein and vitamins produced through fermentation. Microorganisms in the lower gastrointestinal tract need to breakdown feedstuffs that eluded fermentation and can influence the ruminant's well-being and production (Liu *et al.*, 2020). Studies on microorganisms were limited to culture-based methods in the past, however, as many microorganisms cannot be cultured, only a small number of microorganisms within the rumen were identified and investigated (Firkins & Yu, 2015). High throughput sequencing has made it feasible to recognize and study any interaction between the microbes in the rumen. In addition, various factors that influence the rumen microbiome can be considered with regard to their role in modifying the rumen microbiome composition towards energy efficient pathways and optimal production (Terry *et al.*, 2021).

The rumen microbes metabolise and convert nutritional components that the host is unable to degrade to microbial proteins and volatile fatty acids which can be utilized by a ruminant as an energy source. Volatile fatty acids (VFA) contribute approximately 70% of the energy the animal can utilize (Perea *et al.*, 2017) and the production and proportion thereof can influence the efficiency of the animal. A higher production of VFAs, particularly butyrate, has been indicated to be associated with higher microbial fermentation and efficiency

(Guan *et al.*, 2008). The rate of fermentation can be influenced by various factors, including rumen anatomy and physiology, resulting in an effect on the rumen microbiome composition. A study with sheep showed that the rumen shape and size can influence the pH, the anaerobic environment and flow through rate of the rumen (Kamke *et al.*, 2016), ultimately affecting the rate of digestion and the microbiome composition, which is linked to overall efficiency.

The rumen microbiome composition consists of prokaryotes (bacteria and archaea), eukaryotes (protozoa and fungi) and viruses. These microbes can be classified into categories, such as cellulolytic, hemicellulolytic, amylolytic, proteolytic and lipolytic, depending on the substrate they degrade. The high rumen microbial diversity is due to the variation in substrates as well as the removal of fermentation products such as VFAs (Morgavi *et al.*, 2013).

The most predominant rumen microbes, consisting 95% of the total microbial population, are bacteria (Firkins & Yu, 2015). Firmicutes, Bacteroidetes and Proteobacteria are the main phyla identified in the rumen (Henderson *et al.*, 2015). Approximately half of the biomass of the rumen content consists of protozoa, while fungi can range from 8 to 20%. Studies have reported a core rumen microbiome that is relatively stable across individuals (Petri *et al.*, 2013; Henderson *et al.*, 2015). Rumen adapted microbes that have synergistic interactions with the host for maximum production form part of the core microbiome. A variety of factors, such as nutrition, can impact the abundance and proportion of these core microbes (Henderson *et al.*, 2015).

Propionate-producing organisms, such as *Selenomonas ruminantium*, result in more energy being available to the animal, while methanogens, such as *Methanobrevibacter*, utilize energy to produce methane, decreasing the energy available to the ruminant (Hristov *et al.*, 2012). Less methane is emitted in efficient animals compared to inefficient animals. Compared to carbon dioxide (CO_2), methane has a 28 times greater global warming potential and is therefore a key contributor to greenhouse gas emissions (Mizrahi *et al.*, 2021). Ruminants account for 37% of all anthropogenic methane emissions as part of their normal digestive fermentation (Parmar *et al.*, 2017). Methane production is an important part of rumen fermentation as it removes excess hydrogen (H) in the rumen. However, a decrease in the amount of methane produced per animal may result in more efficient animals and more environmentally friendly production. Through the modification of rumen fermentation, beneficial processes can be enhanced while inefficient processes, such as methanogenesis, can be minimized or eliminated (Nagaraja, 2016).

Various factors influence the rumen microbiome including genotype, physiological phase of the animal and the feed quality and quantity (Jami *et al.*, 2013; Shabat *et al.*, 2016), of which nutrition has the largest influence. The dietary composition offered to the animals determine the substrate amount and variety available to the rumen microorganisms for fermentation and can therefore result in a shift in the rumen microbiome composition (Petri *et al.*, 2013). A diet consisting mainly of roughage will increase cellulolytic bacteria, whereas a diet consisting mainly of maize will increase amylolytic bacteria (McDonald *et al.*, 2011). This results in the adaptation of the microbes to the rumen environment causing many species and subspecies that

perform the same function to become abundant. This redundancy and the resilience that originates from it, is one of the characteristics of the rumen microbiome (Weimer, 2015).

There are various studies that link the microbiome composition and individual microbes to efficiency traits including residual feed intake, feed efficiency ratio or average daily gain (Myer *et al.*, 2015; Shabat *et al.*, 2016). *Prevotella,* the predominant rumen microbial genus, has been reported to be abundant in both efficient and inefficient animals (McCann *et al.*, 2014; Myer *et al.*, 2015). Shabat *et al.* (2016) reported that rumen microbiome species and genes could predict the difference in feed efficiency traits and that a less diverse microbiome composition in the rumen can be linked to an increased efficiency in animals. However, a more diverse microbiome has been associated with a healthy animal (Yeoman & White, 2014; Du *et al.*, 2018). It has become a new challenge to modify the rumen microbiome towards a more energy efficient composition resulting in a decrease in methane emissions, without adverse effects on the production, quality, and health of the ruminant (Söllinger *et al.*, 2018). Strategies such as feed additive inclusion in diets have been reported to modify the rumen microbiome composition.

Feed additives are commonly used to increase the health and production of the animal. In South African feedlots, the ionophore monensin is commonly used as an AGP to increase the efficiency of the animal by decreasing feed intake as well as to act as a buffer in the rumen. A challenge that exists in the feedlot industry in present times is the banning of the use of AGPs by the European Union (EU) due to the danger of the development of antibiotic-resistant bacteria that can be transmitted to humans (Casewell *et al.*, 2003). Due to the ban, no meat can be exported to the EU from countries that use monensin. South Africa's neighbouring countries, such as Namibia and Botswana, do not use ionophores as most of the meat is exported to European countries. Alternatives, such as essential oils or probiotics, therefore, need to be investigated to replace antibiotics as performance enhancing feed additives.

Essential oils (EO) have a similar mode of action compared to monensin as it inhibits more permeable microbes, such as Gram-positive bacteria that do not have a cell membrane, by causing an ion leakage (Calsamiglia *et al.*, 2007). The influence of EOs on the production and VFA concentration have been variable, having no effect, increasing or decreasing (Kholif & Olafadehan, 2021). The active compound of the essential oil, its dose, the part of the plant it was harvested from and the diet composition in which it is included can influence its efficacy. Another potential alternative is probiotics which are live microbes that when supplemented at a sufficient dose can result in a health benefit to the animal (Markowiak & Śliżewska, 2018). Probiotics have been reported to increase the rumen microbial diversity resulting in a more resilient and healthier microbiome. The probiotic mode of action depends on the selected strains and can include the production of antimicrobial enzymes against pathogenic species (Song *et al.*, 2014) or changes in ruminal fermentation (Philippeau *et al.*, 2017). Natural feed additives, including EOs and probiotics, are potential alternatives and their mode of action and effect on the rumen and gastrointestinal tract microbiome is of interest to determine their suitability as an alternative.

There are two categories of factors that affect the rumen microbiome composition, factors that affect the rumen microbiome, such as nutrition, and that affect the sample's microbial composition, such as the sample collection methodology (Weimer, 2015). A standardization of the methodology used to investigate the rumen microbiome is needed to be able to compare studies and findings across the world (Henderson *et al.*, 2013). There are three methods commonly used to sample whole rumen content for microbiome studies. The use of cannulated animals is expensive and may limit the sample size of the study. Collecting rumen samples through oral intubation using a stomach tube or collecting the samples after the animals are slaughtered are two non-invasive alternative methods. Both these methods have been shown to result in similar microbial composition compared to cannulated animals (Terré *et al.*, 2013; Wallace *et al.*, 2014). Due to the cost of cannulation and the animal welfare implications, alternative non-invasive techniques need to be investigated.

1.2 Aim of study

As the world is constantly changing and consumers gain more insight into agricultural practices, strategies need to be developed that is conscious of the consumer's perception. The perception exists that the application of AGPs can lead to the development of an antimicrobial-resistant microorganism. Understanding the impact of different feed additives on the microbial composition of the rumen and jejunum can lead to feeding strategies that decrease the usage of AGPs. Therefore, alternatives need to be investigated that has the same or superior effect on the animal compared to ionophores, such as essential oils and probiotics (Benchaar *et al.*, 2006; Peterson *et al.*, 2007). These feed additives are of natural origin and countries that export to the EU might be able to make use of them.

Probiotics consisting of *Bacillus* strains have been used and studied in the United States of America and in Australia and is newly launched in South Africa for ruminants. The *Bacillus* strains are resistant to harsh circumstances as they are fed in spore form (Bernardeau *et al.*, 2017). Once the spore reaches a favourable environment such as the rumen it will germinate to a vegetative cell that can produce antimicrobial enzymes (Song *et al.*, 2014) as well as enzymes that can aid in the degradation of feedstuff (Pan *et al.*, 2022) resulting in a healthier and more productive animal (Kowalski *et al.*, 2009). The EO product (17% eugenol, 11% cinnamaldehyde and 7% capsicum) used in this study has been used in feedlots (De Souza *et al.*, 2018; Latack *et al.*, 2021) and is available in South Africa for commercial use. Essential oils derived from plant extracts such as eugenol, cinnamaldehyde and capsicum, have been reported to improve rumen fermentation in cattle fed high concentrate diets (Cardozo *et al.*, 2006). Studies have reported that the EO increased feed efficiency (Latack *et al.*, 2021) or had a similar feed efficiency to animals supplemented with monensin (Geraci *et al.*, 2012). However, the effect of the EO and probiotic on the rumen microbiome has not been investigated.

The microorganisms in the rumen and jejunum affect the health and production of the animal. The rumen microbiome aids in the degradation of feedstuff the animal itself cannot degrade and provides energy to the

animal (Perea *et al.*, 2017). The microorganism in the jejunum have been reported to be correlated to the immune system of the animal and would therefore influence the health of the animal (Ye *et al.*, 2022).

The aim of this study was to investigate the effect of feed additives on the rumen and jejunum microbiome of Bonsmara cattle raised under intensive feedlot conditions using amplicon sequencing techniques. The objectives are as follow:

1. To determine the shift in the rumen microbial composition from the backgrounding phase through to the starter, grower and finisher phases of a growth period based on microbial sequencing.
2. To compare the effect of monensin to essential oils or a probiotic on the rumen microbiome composition of animals raised under feedlot conditions through amplicon sequencing.
3. To determine the effect of monensin, essential oils and a Bacillus-probiotic on the jejunum microbiome of South African cattle.
4. To investigate non-invasive approaches to rumen content collection for microbiome studies.

1.3 Thesis outline

This dissertation is comprised of seven chapters, which includes a comprehensive literature review and subsequent chapters according to the objectives of the study. The literature review covers the anatomy and physiology of the rumen as it pertains to the microbiome it houses. Factors that influence the rumen microbiome are explained such as the diet the animal is fed and components within the diet. Sequencing methods commonly used to study the microbiome and the bioinformatic process needed to analyse the data is mentioned. Chapter three, which entails investigating the rumen microbiome composition of animals raised under feedlot conditions, from backgrounding through to the finisher phase, was published in the South African Journal of Animal Science. Chapter four compares the findings of the effect of monensin to essential oils or a probiotic on the rumen microbiome of feedlot animals while chapter five investigates the influence of the different feed additives on the microbial composition found within the jejunum. Chapter six compares the microbiome composition of rumen samples taken using a stomach tube or collected after the animals were slaughtered. A general discussion and critical review of the research findings, as well as recommendations for future studies are presented in chapter seven.

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Chapter 2
Literature Review

2.1 Introduction

Several factors influence the growth and efficiency of cattle such as nutrition, genetics, and the gastrointestinal tract microbiome. Increased host access to nutrients extracted from feed leading to improved production efficiency has been identified as the result of rumen microbes effectively making use of feed, ultimately contributing to food security (Huws *et al.*, 2018). Ruminants have the unique ability to convert low quality feed to high quality protein in meat and milk for human consumption. Due to the growing human population, factors that can improve feed efficiency such as the rumen microbiome need to be investigated. The implementation of next generation sequencing (NGS), including amplicon as well as shotgun metagenomic sequencing, can be used to identify microbes and their functions, leading to a better understanding of the gastrointestinal tract ecosystem (Franzosa *et al.*, 2015; Jovel *et al.*, 2016).

This chapter aims to impart a comprehensive literature review of the rumen and jejunum microbiome with specific reference to increasing efficiency in feedlot cattle using feed additives, including essential oils, ionophores, and probiotics. The sequencing techniques for studying the microbiome will be reviewed with reference to the appropriate bioinformatics required for analysis.

2.2 Rumen anatomy, physiology, and microbiome

2.2.1. Brief overview of anatomy and digestive processes

Cattle are classified as ruminants which have a modified stomach - the oesophageal region is expanded into three different segments known as the rumen, reticulum and omasum (Frandson *et al.*, 2003) (Figure 2.1). The rumen and reticulum are similar in function and anatomy and is collectively known as the ruminoreticulum.

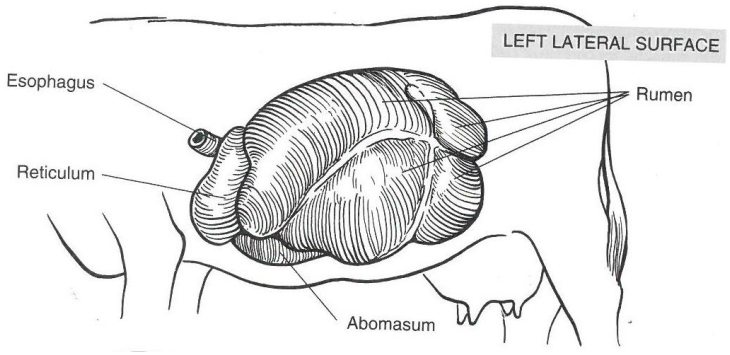

Figure 2.1 The structure of the rumen (Frandson *et al.*, 2003)

The rumen is subdivided into four compartments by muscular pillars, the right and left ventral and dorsal sacs. The ventral sacs contain papillae up to 1 cm long, however, in the dorsal sacs the papillae are almost absent (Membrive, 2016). Papillae enlarge the effective surface area and improve nutrient uptake.

After the nutrients pass through the rumen, undegraded and indigestible nutrients continue to the abomasum, small intestine and the large intestine before being excreted (Czerkowski, 1986). The contents of the rumen are heterogenous and include a large proportion of semisolid digesta, particularly in the longitudinal pillar region, which forms a mat of solid particles known as the rumen mat (Membrive, 2016). Rumen movement and rumination controls the flow of the rumen.

Rumination is a process where the animal regurgitates a portion of digesta, the bolus is mixed with saliva and chewed by the animal (Czerkowski, 1986). During rumination, the bolus mixed with saliva is squeezed into liquid which is swallowed first, a portion of the bolus is swept from the reticulum to the dorsal rumen and a portion is passed into the omasum for further digestion (Frandson *et al.*, 2003). The swallowed saliva also buffers the rumen and prevents the pH from falling too low. The major stimulation for the animals to commence rumination is stimulation from the rumen epithelium. Diets with a low roughage proportion can result in insufficient stimulation for the animal to start ruminating (McDonald *et al.*, 2011).

Rumen microbes digest cellulose or hemicellulose (McDonald *et al.*, 2011) through fermentation and convert these substrates into utilizable products for the animal (Figure 2.2). The process of fermentation produces volatile fatty acids (VFAs), microbial protein and gasses (H_2 and CH_4). The main VFAs produced are butyric, acetic, and propionic acids. Swift transformation of these VFAs to their ionized form occur, resulting in the formation of butyrate, acetate and propionate (Membrive, 2016).

The rumen provides a viable environment for microbial fermentation, due to the presence of a moist environment and saliva acting as a buffer to maintain the rumen pH between 5.5 and 6.5 (McDonald *et al.*, 2011). Fermentation products are predominantly acidic that can decrease the ruminal pH, while saliva contains phosphate and bicarbonate that regulate the pH of the rumen. Saliva acting as a buffer and the rapid absorption of the acids, maintain homeostasis. The anaerobic environment in the rumen is maintained by the rabid absorption of oxygen that enters with the food or through aerobic microbes that use oxygen as a substrate. Furthermore, the continuous supply of nutritional components from feedstuffs act as substrates for the microbes (Millen *et al.*, 2009).

Rapid fermentation, which usually occurs with high concentrate diets, can result in a decrease in pH which leads to the inhibition of cellulose fermenting organisms and a decrease in cellulose fermentation (Czerkowski, 1986). If the rumen pH decreases below 5.5, the animal could suffer from subacute rumen acidosis resulting in impaired absorption capacity from the damaged rumen walls or rumenitis (Membrive, 2016). With a high concentrate diet, predominantly consisting of grain, little mastication and rumination occurs, and this leads to insufficient salivary secretion needed as a buffer. In the circumstance that VFAs accumulate in the rumen with a low pH, a notable impact on the rumen microbial activity and function due to

lactic acid production will be observed. This can result in elevated levels of acid-tolerant bacteria, which would include *Lactobacilli*, in the rumen microbiome (Petri *et al.*, 2013).

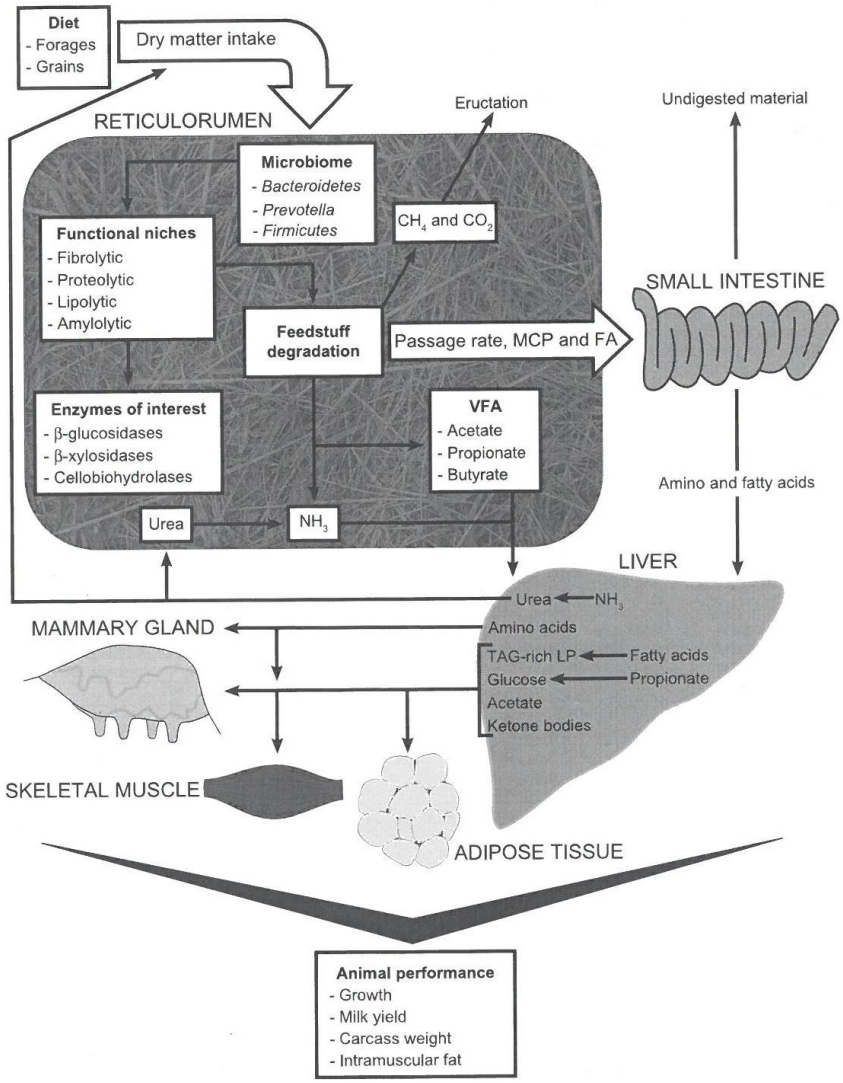

Figure 2.2 The association between the microbes in the rumen, diet and metabolism (McCann *et al.*, 2014a)

The fermentation process can also be influenced by the passage rate of the feed (Janssen, 2010). A slow passage rate results in increased fermentation and methane production. A fast passage rate results in lower fermentation, a decrease in methane as well as an increased formation of propionate and acetate (Waghorn & Hegarty, 2011). Various factors can influence the passage rate, such as the type and the structural characteristics of the feed. Forages are characterized by a large amount of structural carbohydrates, such as hemicellulose and cellulose, that are not rapidly digested in the rumen compared to starch (Sutton, 1971). In contrast, grain contains large amounts of rapidly degradable starch, which results in a faster passage rate compared to forages. It has been shown that smaller feed particles result in a faster passage rate compared to forages such as silage. Increasing the degradation rate of feed leads to a faster passage rate through the rumen

as well as a lessening in methane emissions per unit of degraded feed (Janssen, 2010). Microbial proliferation and dry matter intake has a strong positive correlation (Wadhwa *et al.*, 2016) with a faster passage rate and increased feed intake resulting in an increase in the organic matter availability for the rumen microbes.

Carbohydrate fermentation in the rumen require two steps, firstly complex carbohydrates are digested to simple sugars by microbial enzymes and secondly, simple sugars are fermented to VFAs (Figure 2.3) which serve as energy to the host (Owens & Basalan, 2016). The VFAs contribute roughly 70% of the energy that can be utilized by the ruminant (Perea *et al.*, 2017).

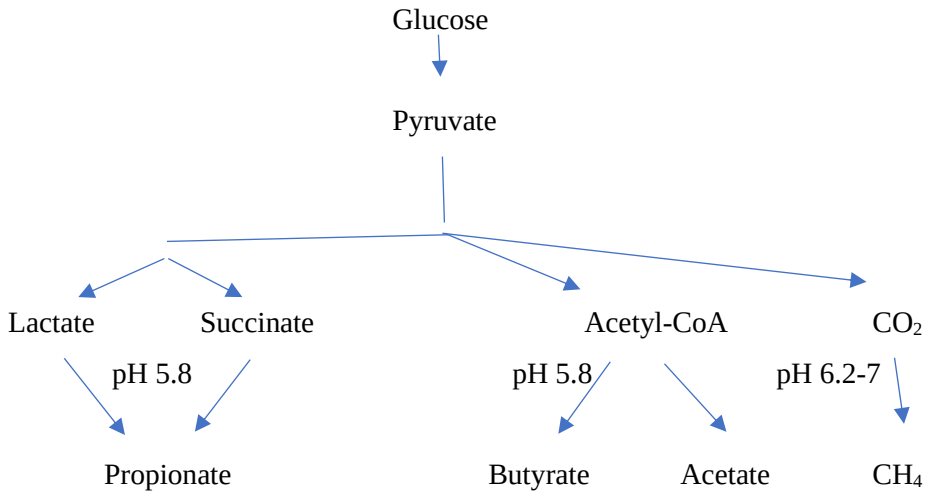

Figure 2.3 A simplified diagram depicting the end products of microbial fermentation at favourable pH (Czerkowski, 1986; Shriver *et al.*, 1986).

There are two ways in which propionate can be produced, either through lactate or succinate (Söllinger *et al.*, 2018). In the liver, propionate is converted to glucose and is available to the nervous system, muscular tissue and other organs as energy (Membrive, 2016). During propionate production, no additional H_2 is produced and therefore no methane is released.

Butyrate is produced using butyrate kinase enzymes produced by *Clostridiales* or with *Negativicutes* through the butyryl-CoA-acetate CoA transferase pathway. The VFAs, especially butyrate, increase the papillae growth on the rumen wall. Inside the rumen, the ruminal epithelial cells utilize approximately 95% of the butyrate produced for growth and maintenance (Membrive, 2016). The remaining 5% is absorbed by the rumen wall and transported, for conversion to fatty acids, acetyl-CoA, and ketones, to the liver. Both the body and lipogenesis utilize ketones as an energy source. Increased rumen butyrate concentration has been linked to animals that utilize feed more efficiently (Guan *et al.*, 2008; McGovern *et al.*, 2018). Higher butyrate concentration results in an increase in the rumen surface area by the thickening of the epithelium and growth of the papillae leading to a heightened absorption of VFAs across the rumen wall to the liver (Keogh *et al.*, 2017).

The primary source of energy for lipogenesis as well as oxidative metabolism in the ruminant is acetate (Membrive, 2016). In adipocytes, acetate is converted to triglycerides, stored as fat, and utilized by the liver in small amounts, mostly to generate ATP through oxidation for energy as well as the formation of acetyl-CoA for lipogenesis.

Volatile fatty acids are integral to the metabolic pathways of organs other than the rumen, therefore, the amount and proportion of the VFAs in the rumen are of interest (Bell *et al.*, 2017). The VFA concentration in the rumen ranges from 70 to 150 mmol/l (5-10 g/l). Propionate serves as both a primary source of glucose and as a H^+ sink in the rumen while butyrate and acetate production results in the release of H (Bell *et al.*, 2017). Other fatty acids can also be formed through fermentation, such as isobutyric and valeric acid (McDonald *et al.*, 2011). The VFA proportions within the rumen are affected by the diet composition, the feed intake (passage rate) as well as the species of microbes or activity within the rumen (Owens & Basalan, 2016).

Dietary protein and non-protein sources, including urea, are first available to the microbes in the rumen, which they use to produce microbial protein to promote microbial growth or in fermentation to produce VFAs (Rodríguez *et al.*, 2007). Microbial degradation of protein are influenced by the type of protein, the interactions with other nutrients and the microbiome population composition, which depend on the passage rate and the pH in the rumen (Bach *et al.*, 2005). The predominant microbes responsible for the breakdown of proteins are *Prevotella ruminicola*, *Peptostreptococci* species and protozoa. The microbes, microbial proteins, small feed particles and unabsorbed particles within the rumen fluid can exit the rumen and be utilised by the animal in the lower digestive tract (Janssen, 2010; Myer *et al.*, 2015b).

By-products of the fermentation process include ammonia, hydrogen ions and CO_2 (Janssen, 2010). Approximately 500 to 1000 L of gasses are eructed daily by an adult bovine (Membrive, 2016) which consists of 40% CO_2, 30% CH_4, 7% N and small and varying proportions of O_2 and H_2. During ruminal fermentation, hydrogen (H) is produced from the process of converting pyruvate to acetyl-CoA and glycolysis (Lu *et al.*, 2019). The produced H_2 needs to be removed for fermentation to continue (McAllister & Newbold, 2008) as a H_2 accumulation will be toxic to the rumen microbes and the animal. The origin of methane production lies in oxidation-reduction reactions essential to efficient microbial fermentation (Bodas *et al.*, 2012). This occurs to the detriment of co-enzymes (including electron carriers) in a synergistic process.

In anaerobic circumstances, carbon is one of the acceptors of H^+, resulting in methane (Lan & Yang, 2019). Methane production in the rumen has been indicated to be the main H-sink (Kumar *et al.*, 2014). Specialized microbes known as methanogens use H_2 and CO_2 to produce methane. There are other important H-sinks in the rumen such as propionate and acetate production, as well as sulphate and nitrate reduction (McDonald *et al.*, 2011). If the rumen pH decreases, an increase in the production of propionate takes place. As propionate production is also a H-sink, lower methane production per unit of fermented organic matter occurs (Monteny *et al.*, 2006).

The quantity of methane produced is dependent on the amount of H^+ released by the fermentation process of other microbes and is influenced by the passage rate and rumen retention times (Kittelmann *et al.*,

2014). The concentration of H_2 plays a key role in the fermentation pathway (Janssen, 2010). The fermentation patterns of rumen microorganisms are altered in response to slight changes in free energy by shifting to more favourable processes if the pathways become thermodynamically less favourable (Chin & Janssen, 2002). At low H_2 concentration, pathways producing H_2 is favoured, such as butyrate production, while at high H_2 concentration, pathways that utilize H_2 is favoured, such as propionate production. Directly after feeding, the H_2 concentration will be high as the readily degradable feed is rapidly digested. Methanogens also need a high H_2 concentration for the growth which usually occurs with high passage rates and low pH in the rumen (Janssen, 2010).

Understanding the anatomy and the physiology of the rumen is essential knowledge in microbiome studies as the environment in the rumen influences the composition and abundance of the microbiome.

2.2.2 Rumen microbiome

The rumen microbiome is unique in that it has a low diversity of phyla and classes (high taxonomic levels) and a high diversity of genera, species, and strains (low taxonomic levels) (Firkins & Yu, 2015). This unique feature combined with the influx of substrates into the rumen results in a high abundance of microbes and a high functional redundancy in which many microbes perform the same function. Components of the rumen microbiome include archaea (10^6 cells/ml), bacteria (10^{11} cells/ml), fungi (10^3-10^6 zoospores/ml), protozoa (10^4 – 10^6 cells/ml), as well as viruses (10^7-10^{10} particles/ml) (Morgavi *et al.*, 2013). Several microbes, such as *Butyrivibrio*, *Ruminococcus*, unclassified *Lachnospiraceae*, *Bacteroidales* and *Clostridiales*, have been reported to belong to the core microbiome in the rumen (Henderson *et al.*, 2015), which are always identified regardless of diet. Microbes have different characteristics with some microbes having a higher abundance in the solid phase, such as *Coriobacteriaceae*, and others in the liquid phase, such as *Prevotella* (Bowen *et al.*, 2018). The increased difficulty of forming biofilms on the liquid phase particles results in a higher abundance of methanogens in the solid fraction. It has been observed that the concentration of microorganisms is greater within the solid phase compared to the fluid portion of the rumen as the microorganisms attach to the feed particles (Bowen *et al.*, 2018). Other studies reported that the rumen microbial communities of the solid and liquid phases in animals consuming high concentrate diets did not differ (Schären *et al.*, 2017; McGovern *et al.*, 2018).

Microbes need substrates to be able to grow and therefore cannot deplete the substrates' concentration to such a level that the growth rate of the microbes fall below the dilution rate (Janssen, 2010). A decrease in the proliferation of the microbes would result in a decrease in the use of nutrients and in metabolism. The concentration of substrate needs to be constant or as close to constant as possible so that the growth rate will match the dilution rate. There are many interactions among the microorganisms, some reactions are sequential, where the end product of one microbial reaction is the substrate for other microorganisms.

Bacteria, the most abundant microorganisms in the rumen, are considered the primary fermenters, with over 7 000 species estimated and approximately 30% still unidentified (Lan & Yang, 2019). Approximately

80% of the ruminal bacteria population is Gram-negative (Nagaraja, 2016) with a Gram-positive bacteria increase of 20 to 30% in grain-fed animals. In Table 2.1, several important species are listed according to McDonald *et al.* (2011) and their associated fermentation products. These species were determined to be important *in vitro* and is not entirely accurate *in vivo* as only 15% of bacterial species have been cultured *in vitro* (Creevey *et al.*, 2014).

Table 2.1 Bacterial species reported to be abundant in the rumen through *in vitro* studies and their fermentation products (McDonald *et al.*, 2011).

Species	Typical energy source	Typical fermentation products (excluding gases)					
		Acetate	Propionate	Butyrate	Lactate	Succinate	Formate
Fibrobacter succinogenes	Cellulose	*				*	*
Streptococcus bovis	Starch				*		
Ruminococcus albus	Cellobiose	*					*
Ruminococcus flavefaciens	Cellulose	*			*	*	*
Megasphaera elsdenii	Lactate	*	*	*			
Prevotella ruminicola	Glucose	*				*	*
Lachnospira multipara	Pectin	*				*	

The rumen microorganisms are often classified considering the particular feed component that they degrade or product they utilize. Cellulolytic bacteria, including *Fibrobacter succinogenes*, *Ruminococcus albus*, *R. flavefaciens* and *Butyrivibrio fibrisolvens*, degrade fibre which consists of cellulose (Morgavi *et al.*, 2013). In ruminants, fibre degrading groups such as *Fibrobacter*, *Ruminococcus*, *Butyvibrio* and *Bacteroidetes* are predominant, but other bacteria such as *Prevotella*, *Selenomonas*, *Streptococcus*, and *Lactobacillus* are also commonly found (Gaggìa *et al.*, 2010). A large number of methanogens, anaerobic fungi and ciliate protozoa also form part of the microbiome composition (Deusch *et al.*, 2015).

The most abundant bacterial phyla are Firmicutes, Bacteroidetes and Proteobacteria (Henderson *et al.*, 2015). One of the predominant rumen microorganisms from the Bacteroidetes phylum; *Prevotella* degrade starch, fibre and protein (Carberry *et al.*, 2012) to produce a range of end products, which include VFAs. The *Prevotellacea* family is genetically diverse and occupies liquid and solid phases in the rumen (Jami & Mizrahi, 2012). Research has indicated that four species from the *Prevotellaceae* family, *P. ruminocola*, *P. bryantii*, *P.*

albensis and *P. brevis*, make up 70% of the rumen bacteria (Stevenson & Weimer, 2007; Jami & Mizrahi, 2012). In animals fed concentrate-based diets, *Succinivibrionaceae* and *Prevotella* are vital succinate and propionate producers in the rumen (Chen *et al.*, 2017). Succinate is a pre-cursor of propionate and is swiftly converted (Suen *et al.*, 2011), with propionate being absorbed through the rumen wall and converted to glucose.

The protozoa in the rumen are classified into two categories, either holotrichs or entodiniomorphs. Holotrichs use simple carbohydrates and store excess carbohydrates as microbial starch. Entodiniomorphs are more specific in their nutritional requirements and morphologically more complex. Protozoa do not match bacteria in numbers, but are physically larger compared to bacteria; they can equal or exceed them in mass (Lan & Yang, 2019). *Entodinium*, from the family *Ophryoscolecidae*, has been noted as a predominant protozoal genus (Lan & Yang, 2019). Some protozoal genera have an affinity for high grain conditions such as *Polyplastron* and *Ophryoscolex* (Tymensen *et al.*, 2012). Protozoa have been associated with methanogenesis as they provide archaea with substrates (mostly H_2) that is used for methane production (Huws *et al.*, 2018).

The rumen fungi are the least studied of the rumen organisms, but it has been indicated that they have a higher abundance in the rumen of animals offered diets consisting predominantly of roughage (Gruninger *et al.*, 2014). Fungi invade and colonize plant tissues for degradation, while the other microbes make use of the products and degradation caused by the invading fungi. The phylum Neocallimastigomycota is the most predominant anaerobic fungi in the rumen (Gruninger *et al.*, 2014), while aerobic fungi, such the Ascomycota and Basidiomycota phyla, has been observed (Zhang *et al.*, 2020). Their function is mostly to scavenge for free O_2 to ensure that the environment in the rumen stays anaerobic.

Archaea produce methane from H_2 formed as a by-product through microbial fermentation. Theoretically, there should be a correlation between archaea and methane emissions, however limited evidence have been reported (Zhou *et al.*, 2009). The ratio between archaea and bacteria might play a part in methane production (Roehe *et al.*, 2016) and has been shown to be a possible marker for methane emissions (Wallace *et al.*, 2014; Auffret *et al.*, 2018). Within the rumen, methanogenic archaea are limited in abundance and diversity, with three major methanogenic genera, *Methanomicrobium* (14.9%), *Methanobrevibacter* (61.6%), and a group of ruminal archaea (15.8%) related to *Thermoplasmatales* known as rumen cluster C (Janssen & Kirs, 2008; Pitta *et al.*, 2018). The proportion of these three groups differ widely between studies (Wright *et al.*, 2007; Lan & Yang, 2019), which may be due to host genetics, methodological differences, nutrition or animal management differences. *Methanobrevibacter ruminantium* and *M. gottschalkii* were indicated to be the most abundant methanogens (Henderson *et al.*, 2015; Gruninger *et al.*, 2019). These two species combined with *Methanomassilicoccaceae* and *Methanosphaera* species comprise 89.2% of the community.

There are three pathways that can produce methane as shown in Figure 2.4. The hydrogenotrophic methanogens produce up to 82% of the rumen methane and include *Methanobrevibacter*, *Methanosphaera*,

Methanimicrococcus and *Methanobacterium*. The methylotroph communities, such as *Methanosarcinales*, *Methanomassillicoccaceae*, and *Methanosphaera* are found at lower levels.

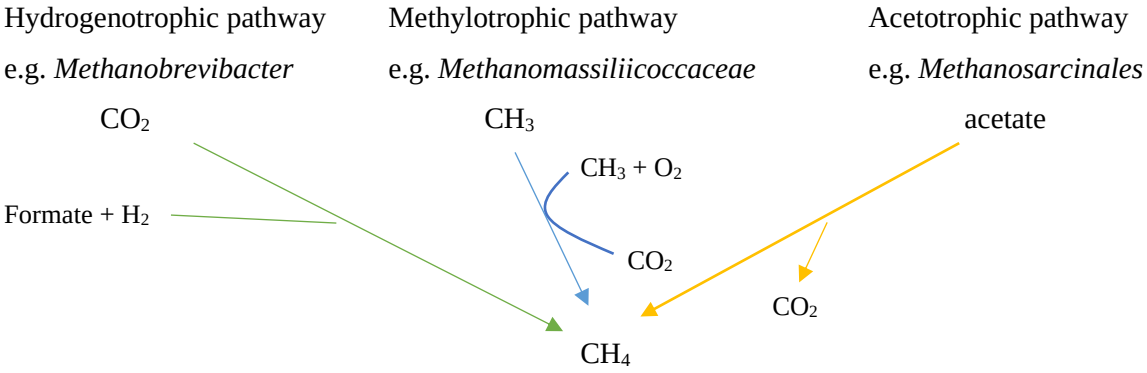

Figure 2.4 The various pathways in which methane is produced (Russell & Rychlik, 2001; Poulsen *et al.*, 2013).

Methane production and emission waste between 2 to 15% of the energy that could be used by the ruminant (Kumar *et al.*, 2014). Various mitigation strategies are being investigated to decrease methane emissions to result in more energy being available to the host for production.

The identification of microbes found in the rumen and gastrointestinal tract and factors that can influence their composition, such as diets, can lead to strategies to increase the efficiency of the animal.

2.3 Jejunum microbiome

Different segments of the gastrointestinal tract house different microbial populations and the diversity among these populations play a critical part in the breakdown and absorption of nutrients from feed, ultimately determining the efficiency of the animal. Ruminants are mostly pre-gastric fermenters and much research has been done on the link between the rumen microbiome and feed efficiency (Hernandez-Sanabria *et al.*, 2012; Myer *et al.*, 2015a) and little on the small intestine microbiome (Mao *et al.*, 2015; Han *et al.*, 2021). Variation exist in the microbiome community composition between segments of gastrointestinal tract due to metabolic and morphological differences (Donaldson *et al.*, 2016) with the same or adjacent regions (rumen, small intestine and large intestine) of the gastrointestinal tract housing a more similar microbial composition to each other compared to those from other regions. The jejunum plays a key part in the enzymatic breakdown of nutrients and their absorption, and the microorganisms found within this part of the gastrointestinal tract might also affect the production efficiency of the animal.

The absorption of nutrients, including glucose, glycerol, fatty and amino acids, primarily occur in the small intestine after the breakdown of nutrients by bile acids and enzymes (Donaldson *et al.*, 2016). The abundance and diversity of microbes are low within the small intestine, but bacteria found within the small

intestine play a prominent part in digestion, degradation of bile acids and inhibition of pathogens (Kamada *et al.*, 2013; Deusch *et al.*, 2015). The health of the animal can also be impacted by the small intestine microbiome (Zhang *et al.*, 2021b) as it prevents the proliferation of pathogenic bacteria through the decrease of binding sites and the competition for substrates. The epithelium of the small intestine is protected by a mucin layer that is maintained by the microorganisms and prevents pathogens from colonizing. Xenobiotic(s) degradation, biodegradation and metabolism pathways were noted to be enriched in the small intestine of crossbred cattle indicating that the microbes in the small intestine play a crucial part in the health (Wang *et al.*, 2022) and can influence the immune system of the host (Hooper & MacPherson, 2010).

Microbial diversity within the jejunum is lower compared to the rumen and large intestine due to bile acids, as well as the fast passage rate of the digesta through the small intestine leaving little time for bacteria to proliferate (De Oliveira *et al.*, 2013). Due to the lower diversity, there is little redundancy which is a characteristic of the rumen microbiome. The jejunum microbiome, in contrast to the rumen microbiome, has a high functional efficiency where a small number of microorganisms perform various functions (De Oliveira *et al.*, 2013; Mao *et al.*, 2015). The small intestine microbiome has a bigger variation between individual animals compared to the rumen and large intestine microbiome (Perea *et al.*, 2017).

The small intestine microbiome composition consists predominantly of facultative anaerobes and acid-tolerant microorganisms (Donaldson *et al.*, 2016). The most notable phylum within the small intestine microbiome is Firmicutes (Liu *et al.*, 2020; Zhong *et al.*, 2022). However, it was observed that feed efficient animals had a higher Proteobacteria abundance and a lower Firmicutes abundance in the jejunum compared to less efficient animals (Myer *et al.*, 2016). A high abundance of Proteobacteria in the gastrointestinal tract may indicate dysbiosis in less efficient animals (Perea *et al.*, 2017). Differences exist between the mucosa and the digesta microbial compositions. Bacteria that aid in digestion, such as *Ruminoccoccus*, *Butyrivibrio*, and *Eubacterium*, are abundant within the digesta while bacteria that can be associated with the immune system, such as *Faecalibacterium*, *Clostridium*, and *Bifidobacterium*, can be found in the mucosa microbiome (Zhang *et al.*, 2021b).

The small intestine microbiome is influenced by various factors related to diet. In the small intestine microbiome of ruminants fed concentrate-based diets, an increase in lactate producers and utilizers and a reduction in Firmicutes abundance were reported (Liu *et al.*, 2020; Zhong *et al.*, 2022). An elevation in the number of possible pathogenic bacteria, including *Moraxella* and *Veillonella*, within the jejunum has also been reported in animals fed high grain-diets (Lai *et al.*, 2022). A study with Hu sheep reported that the small intestine microbiome differed significantly between sheep fed pelleted and non-pelleted feed (Zhong *et al.*, 2022). Animals fed the pelleted diet had a higher abundance of acid-producing bacteria, including *Olsenella* and *Pseudoramibacter*, and a lower abundance of pro-inflammatory bacteria, such as *Marvinbryantii* and *Mogibacterium*. Changes in the bacterial community with specific taxa in the jejunum could have important health implication for ruminant production (Liu *et al.*, 2020). Investigating all factors that can influence the

efficiency of an animal is important as through these investigations, strategies can be developed that lead to more efficient and sustainable ruminant production.

2.4 Factors that influence the rumen microbiome

A number of factors influence the rumen microbiome composition which include the host's genetics, age, and physiological condition as well as the feed available and the geographical location (Henderson *et al.*, 2015). Variation in the rumen microbiome can be observed from animals in different geographical regions, which is likely to be caused by differences in temperature, humidity, soil types. The first microorganisms are introduced to the new-born animal during birth and via the dam's milk (Malmuthuge *et al.*, 2015). The colonization of the gut in new-born animals is crucial for developing the gastrointestinal tract, especially the rumen, and the immune system of the animal (Jami *et al.*, 2013). This early colonization has been shown to influence the microbiome and performance of the adult animal.

2.4.1 Host genetics

Quantitative trait loci have been reported to be correlated with microbial taxa (McKnite *et al.*, 2012), thus proving that host genotype can influence the core rumen microbiome and the microbial community composition (Paz *et al.*, 2018). The animal's genetics and its physiological traits have an effect on its rumen microbial composition and most likely on the efficiency of the animal to convert feed into energy (Lima *et al.*, 2019). The influence of the animal on the microbiome was confirmed by Roehe *et al.* (2016) where the difference between sire progeny groups in terms of methane production was larger compared to the variation found between diets varying in proportion of roughage. A study focussing on sire progeny groups in dairy cows reported that 22 bacterial operational taxonomic units (OTU), which is equivalent to a microorganism in bioinformatic terms, exhibited a heritability of 0.7 (Sasson *et al.*, 2017). These microbes were correlated with dry matter intake and feed efficiency measured as residual feed intake (RFI).

A difference in the individual taxa in the rumen microbiome was observed in genetically divergent individuals (Henderson *et al.*, 2015), as well as between breeds (Parmar *et al.*, 2017). The differences in the rumen microbial composition because of the breed may be as a result of physiological and anatomical differences. Rumen size, as determined by the frame size of the animal, as well as feed intake has an influence on the rumen passage rate (Shriver *et al.*, 1986). Studies have reported that sheep with a smaller rumen have lower methane production (Goopy *et al.*, 2014; Kamke *et al.*, 2016) and a lower retention time impacting production and efficiency of the animals. These differences between animals may be due to the quantity and species of methanogens found in the microbiome.

There is an association between genes from rumen microbes and feed intake, growth rate and feed efficiency (Lima *et al.*, 2019). In a study by Roehe *et al.* (2016), 88.3% of the difference observed in the feed conversion ratio (FCR) was explained by 49 microbial genes. These microbial genes could be used as potential

markers in selection programmes. Selecting animals for breeding based on their feed efficiency, combined with nutritional strategies, holds potential as an effective strategy to breed more efficient animals with a faster growth rate (Gerber *et al.*, 2013). Including information from rumen metagenomic studies into the models can improve this strategy (Ross *et al.*, 2013).

2.4.2 Diet

The microbiome is affected by the roughage to concentrate ratio of a diet. As discussed earlier in this literature review, microbes are specialized in the substrate they degrade. Cellulolytic bacteria degrade mostly cellulose-type feedstuff, therefore, high roughage diets will have a higher number of bacteria that can degrade structural carbohydrates while diets consisting predominantly of concentrates have microbes specializing in degrading soluble starch (Cammack *et al.*, 2018). Microbes that were reported to be abundant in grain- or roughage-based diets were recorded in Table 2.2.

Table 2.2 Bacteria observed to be more abundant within grain- or roughage based diets (Deusch *et al.*, 2015; Henderson *et al.*, 2015; Li & Guan, 2017; Gruninger *et al.*, 2019)

Grain-based diet	Roughage-based diet
Butyrivibrio fibrisolvens	*Bacteroidales*
Prevotella species	*Ruminococcaceae*
Streptococcus bovis	*Clostridiales*
Clostridium species	*Fibrobacter succinogens*
Selenomonas ruminantium	*Ruminococcus flavefaciens*
Fibrobacter succinogenes	*Ruminococcus albus*
Succinimonas amylolytica	
Succinivibrio species	
Ruminobacter amylophilus	

The *Ruminococcaceae* family from the Firmicutes phyla was reported to be highly abundant in animals fed a concentrate-based diet (Li & Guan, 2017). This was unexpected as this family consists mainly of cellulolytic organisms. However, some of the members of this family are involved in starch hydrolysis (Klieve *et al.*, 2012). In ruminants fed mainly forage diets, hemicellulose, cellulose, and pectin are the predominant substrates, and the main bacteria to degrade cellulose include *Ruminococcus albus*, *R. flavefaciens* and *Fibrobacter succinogenes*. The species from *Ruminococcaceae* both also ferment hemicellulose, along with *Butyrivibrio fibrisolvens* and *Prevotella ruminocola* (Puniya *et al.*, 2015). Pectin is degraded by *B. fibrisolvens*, *F. succinogenes*, *Succinivibrio dextrinosolvens*, and *P. ruminocola*. Microbial populations from ruminants fed forage-based diets have a higher diversity of microbes belonging to the Fibrobacteres and a lower diversity from the Bacteroidetes and Firmicutes phyla.

Shifts in the microbial population occur when the diets are changed from a predominantly forage diet (60:40) to a predominantly grain-based diet (40:60; 20:80) (Fernando *et al.*, 2010). The rumen microbiome needs to adjust to changes in the composition of the diet for efficient nutrient digestibility and fermentation (Loor *et al.*, 2016). Increasing highly fermentable components in the diet results in a reduction in the diversity of the rumen microbiome composition as microbes that utilize these components more efficiently will dominate the population (Fernando *et al.*, 2010; Tapio *et al.*, 2017). The microbial richness (or the number of species) remains similar, but the composition (abundance of the species) changes significantly to adapt to the new rumen environment. An increase in the concentrate portion will also decrease the Firmicutes to Bacteroidetes ratio (Mao *et al.*, 2013) and increase Proteobacteria abundance (Auffret *et al.*, 2018). An increased abundance of the Proteobacteria phylum has been associated with dysbiosis in the microbial community (Shin *et al.*, 2015) as many pathogenic species originate from the Proteobacteria phylum. Firmicutes, Bacteriodetes and Proteobacteria are phyla that have been observed to be present in cattle regardless of the diet composition (Petri *et al.*, 2013; Myer *et al.*, 2015a; Paz *et al.*, 2018). Cellulolytic bacteria, including *Fibrobacter succinogenes* and *Butyrivibrio fibrisolvens*, reduce with increasing levels of concentrates in the diet. In research conducted by Pitta *et al.* (2010) in which 16S rRNA sequencing was performed, the abundance of *Prevotella* increased while *Rikenella* decreased when the cattle were changed from a bermudagrass hay-based diet to grazing winter wheat pastures. The abundance of *Succiniclasticum* was higher on the pasture and wheat diet resulting in an increase in the propionate production and more energy provided to the animal (Bell *et al.*, 2017).

A rapid shift from a predominantly roughage diet to a high starch diet may lead to metabolic diseases, such as acidosis, and dysbiosis in the rumen microbiome (Zebeli & Metzler-Zebeli, 2012). Volatile fatty acids can accumulate in the rumen if the fermentation rate of the feed is increased, and they are produced at a faster rate than they can be transported out of the rumen. This results in a decrease in the rumen pH which inhibits cellulolytic bacteria and encourages the growth of lactate producing acid tolerant species, such as *Lactobacillus* (Khafipour *et al.*, 2009). This increase in lactate producing bacteria can be countered by the increase in abundance of lactic acid utilizing bacteria, including *Megasphaera elsdenii*, resulting in the stabilization of the rumen microbiome.

2.4.3 Feed additives

Feed additives are used to modify the rumen environment to improve digestive efficiency and improve the production of the animal. The ideal feed additive should increase propionate concentration to improve the energy balance while decreasing methane emissions, improve nitrogen efficiency by reducing nitrogen excretion, stabilize the rumen pH to decrease the risk of acidosis and increase fibre degradation (Jouany & Morgavi, 2007). It should increase fermentation without diminishing feed intake and digestibility (Cobellis *et al.*, 2016). There is growing pressure to find strategies that can enhance the animals' immune system naturally, decrease the use of antibiotics (Michalak *et al.*, 2021), and increase the growth and productivity of the animal.

Natural feed additives, including essential oils, probiotics and fibrolytic enzymes, may be viable alternatives to antibiotic growth promoters.

Ionophores

Antibiotic growth promoters (AGPs), especially ionophores, remain the most predominant feed additive used in a sub-therapeutic practises in feedlots, as studies have shown an increase in the animal's production efficiency (Duffield *et al.*, 2012; Marques & Cooke, 2021). Ionophores are especially effective in diets low in roughage and high in concentrate, typically fed in the feedlot (Millen *et al.*, 2009; Samuelson *et al.*, 2016). Ionophores, especially monensin, can influence the production of fermentation products and thus potentially affecting ATP production (Russell & Strobel, 1989). The use of ionophores result in the decrease of methane emissions as a change in the VFA production occur favouring propionate above acetate production (Bell *et al.*, 2017). The mode of action of ionophores are as a consequence of their lipophilic characteristics causing an energy wastage cycle by changing the ion exchange gradient across the bacterial membrane (Russell & Strobel, 1989). Ionophores interfere with the electrolyte balance, especially with the sodium-potassium pump in the membrane of the microorganisms' cell. It overloads the cell with potassium (K) and the cell struggles to pump it out as it requires additional energy (Marques & Cooke, 2021). This results in an inefficient ion pump and K accumulation inside the bacterial cell. The K concentration is diluted by allowing water to enter by osmosis, resulting in the rupture of the bacterial cell.

Monensin is one of the most predominantly used ionophores in beef production systems and it's product is recommended at 20 to 30 mg/kg dosage. Monensin increases propionate production in the rumen by influencing the rumen microbiota leading to increased formation of glucose in the liver and more energy available to the ruminant (Russell & Strobel, 1989). Monensin significantly alters the rumen microbiome composition and decreases its diversity by decreasing the abundance of specific microbes from Bacteroidetes and Firmicutes phyla and increasing microbes from the Actinobacteria, Firmicutes, Cyanobacteria and Bacteroidetes phyla (Creevey *et al.*, 2014; Schären *et al.*, 2016). Monensin has been noted to inhibit Gram-positive bacteria due to cell wall permeability. Ionophores might not be able to inhibit Gram-negative bacteria because of the impenetrability of the outer membrane resulting in a protective barrier. The result is the inhibition of Gram-positive bacteria in the rumen and a reduction in the acetate to propionate ratio (Russell & Strobel, 1989; Thomas *et al.*, 2017). Gram-negative bacteria are predominantly succinate and propionate producers and Gram-positive bacteria mostly lactate producers (Nagaraja, 2016). However, studies have shown that ionophores might not have a preference for Gram-positive or Gram-negative bacteria but have varying effects that depend on the cell wall permeability (Weimer *et al.*, 2008), inhibiting more permeable bacteria.

As ionophores decrease the number of lactic acid-producing bacteria, it also decreases the occurrence of metabolic disorders, including ketosis (Drong *et al.*, 2016) or lactic acidosis (McGuffey *et al.*, 2001). The main cause of acidosis prevention when feeding monensin has been suggested to be the inhibition of lactate-

producing bacteria (Coe *et al.*, 1999; Ogunade *et al.*, 2018), however the manipulation of feed intake by ionophores has the greatest benefit of preventing acidosis, rather than alterations in fermentation patterns and pH (Coe *et al.*, 1999). Monensin has been indicated to decrease the feed intake of the animal while maintaining its production. In addition, the supplementation of ionophores can result in more efficient utilization of dietary protein and energy, as ammonia and methane production is decreased (Marques & Cooke, 2021). This reduction in methane emissions can be due to a decrease in the protozoa abundance, that are colonized by methanogens as well as playing an important role in hydrogen production (Russell & Strobel, 1989) or due to the competition in substrates between ionophores and methanogens (Schären *et al.*, 2016).

Probiotics

Probiotics, or direct-fed microbials, are live microbial feed additives that are beneficial to the animal by encouraging the growth of beneficial microbes in the rumen at the expense of less desirable bacteria when supplemented at a sufficient dose (Markowiak & Śliżewska, 2018). The mode of action in which probiotics can influence the rumen microbiome is not completely understood (Markowiak & Śliżewska, 2018). However, it may include: the stabilization of the gastrointestinal barrier (Salminen, 1996), the decrease of potential binding sites for pathogens, supporting the immune system of the host, the proliferation of beneficial as well as commensal bacteria, and the production of antimicrobial substances (Song *et al.*, 2014). Enzymes are also produced by probiotics that increase nutrient digestibility and absorption (Latorre *et al.*, 2016). Probiotics attach to the rumen lining, thus surviving difficult conditions and resulting in the beneficial effect of stabilizing and protecting the rumen environment. Advantages of probiotics include an increase in production due to increased feed absorption and utilisation (Qiao *et al.*, 2010; Sun *et al.*, 2011), and an improved immunity (Michalak *et al.*, 2021).

Probiotics used in formulas and products must adhere to strict criteria such as a sufficient count of viable cells, as well as an advantageous effect on the host animal's health, production, and digestive tract (Markowiak & Śliżewska, 2018). The primary concern for a probiotic is the selection of a suitable strain for high efficiency. Probiotic products vary from containing only one strain to containing multiple strains (Musa *et al.*, 2009). Multi-strain products act in broad spectrum and is expected to be active in different host animal species against microbial infections (Timmerman *et al.*, 2004). Combinations of probiotic strains result in more beneficial effects compared with individual strains due to synergistic behaviour (Collado *et al.*, 2007). Probiotics should be manufactured to be able to withstand the environment within the rumen, as well as the heat and the pressure that forms part of the process of feed pelleting.

The characteristics and functions of probiotics commonly used in ruminant production is summarized in Table 2.3. Some probiotic bacteria, including *Enterococcus* and *Lactobacillus*, naturally occur in the rumen microbiome, however others, such as *Bacillus*, do not. Most of these bacteria are safe to use in probiotics, however a risk still exists that the use of probiotics can result in antibiotic resistance, such as with *Enterococcus* strains, or in the production of endotoxins, such as with *Bacillus cereus* (Gaggìa *et al.*, 2010). *Lactobacilli* and

Streptococci are the most commonly used genera in production of probiotics (Bayatkouhsar *et al.*, 2013). Yeast and fungi strains, such as *Saccharomyces cerevisiae*, can also be used as probiotics (Markowiak & Śliżewska, 2018).

Table 2.3 The characteristics and functions of commonly used probiotics for ruminant production.

Microorganism	Characteristic	Function	Reference
Bacillus	Spore-forming	Produce antimicrobial enzymes	Seo *et al.*, 2010
		Produce enzymes that aid in nutrient digestibility	Latorre *et al.*, 2016
Enterococcus	Lactate producing	Decrease risk of acidosis	Nocek and Kautz, 2006
		Maintain lactate concentration	
Lactobacillus	Lactate producing	Decrease *E. coli* O157	Peterson *et al.*, 2007
		Prevent acidosis	
Megasphaera elsdenii	Lactate utilizing	Decrease lactate concentration	Beauchemin *et al.*, 2003
		Maintain rumen pH	
Propionibacterium	Lactate utilizing	Increase propionate concentration	Philippeau *et al.*, 2017
		Decrease H_2	

Genera, species, or strain can influence the probiotic activity. The *Lactobacillus* genera, known to be lactate producers, comprise of more than a 100 different species (Gaggìa *et al.*, 2010). *Lactobacillus casei* and *L. acidophilus* were the first two bacteria used as probiotics (Musa *et al.*, 2009). Some of the bacteria in this genus are associated with human diseases whereas others are commonly used as human and animal probiotics. The use of *Enterococcus* as a probiotic is also a controversial issue as some of its species and strains are associated with human diseases, and the occurrence of multiple strains with antimicrobial resistance have raised consumers concern (Gaggìa *et al.*, 2010). However, it is commonly used as starter cultures for food products, such as cheese, and as silage additives in animal nutrition (Foulquie Moreno *et al.*, 2006).

The effect of probiotics on acidosis is not completely understood, however in part it may be due to the adaptation of the rumen to a high lactate concentration, either by feeding lactate-producing or lactate-utilizing bacteria (Beauchemin *et al.*, 2003). Bacteria that produce lactate, such as *Lactobacillus* and *Enterococcus*, might prevent acidosis as these bacteria facilitate the growth of microorganisms which are adapted to the presence of lactate and stimulating lactate-utilizing bacteria. Bacteria that use lactate, including *Propionibacterium* and *Megasphaera elsdenii*, decrease the concentration of lactate and maintain the pH in the rumen. Applying *M. elsdenii* intra-ruminally has been reported to increase the ruminal pH and decrease lactate concentration during transition from a high roughage to a grain-based diet (Henning *et al.*, 2010). Variable effects of probiotics on acidosis in feedlot cattle have been reported (Beauchemin *et al.*, 2003).

Bacillus probiotics are fed in spore form and can therefore withstand harsh circumstances such as extreme heat during the pelleting process. Probiotics with *Bacillus* species mostly focus on improving or

maintaining the health of the animal, such as with *Bacillus licheniformis* and *Bacillus subtilis* which can inhibit pathogens (Song *et al.*, 2014). The administration of *Bacillus* probiotics resulted in higher fermentation production and feed efficiency as well as improved milk production and quality in dairy cows (Peng *et al.*, 2012; Sun *et al.*, 2013).

Benefits of probiotics involve an increase in the diversity and richness of the rumen microbial population, cellulolytic microorganisms as well as fibre digestibility (Marden *et al.*, 2008). A higher growth rate (Krehbiel *et al.*, 2002) and average daily gain (Bayatkouhsar *et al.*, 2013) were observed when probiotics were fed to feedlot cattle, however the effect of probiotics on growth has been inconsistent (Peterson *et al.*, 2007; Vasconcelos *et al.*, 2014). Elam *et al.* (2003) reported that the supplementation of *Lactobacillus acidophilus* and *Propionibacterium freudenreichii* did not significantly affect the growth of feedlot cattle. Conversely, Vasconcelos *et al.* (2014) reported an increase in feed efficiency when supplementing the same probiotic and strains as used in Elam *et al.* (2003) to feedlot cattle. This difference between the two studies might be due to the difference in management or feeding strategies.

Essential oils

Plant secondary metabolites have been shown to affect metabolic processes in animals and the growth rate of microorganisms (Bodas *et al.*, 2012). Saponins, tannins, organosulphur compounds, and essential oils are mostly used as feed additives. Essential oils (EO) are usually derived from the aromatic component of secondary metabolites, including terpenoids, alcohols, hydrocarbons, lactones, aldehydes, and acyclic ester (Bodas *et al.*, 2012). These secondary metabolites have antimicrobial characteristics and decrease the abundance of food-borne pathogens and are therefore commonly used.

As EOs consist of different compounds, there are various modes of action on the microbial cells (Burt, 2004). The most common mode of action of EOs is by interacting and disrupting bacterial cell membrane processes, such as protein translocation, ion gradients, electron transport and other enzymatic reactions (Calsamiglia *et al.*, 2007). The mode of action mirrors some AGPs in that they selectively inhibit Gram-positive bacteria by altering the ion transport across the membrane through the interaction of EOs with the bacterial membrane (Calsamiglia *et al.*, 2007). However, Gram-negative bacteria can be affected by losing the lipopolysaccharides of their outer membrane (Burt, 2004). Due to their hydrophobic nature, EOs are attracted to the lipids of the bacterial cell membrane, and this determines their antibacterial properties. Essential oils suppresses the attachment of bacteria to and degradation of fermentable carbohydrates by proteolytic and amylolytic bacteria without inhibiting cellulolytic bacteria (Wallace *et al.*, 2002). Saponins, EOs and tannins decrease the ammonia nitrogen concentration, and this improves the absorption and utilization of feed in the rumen.

The chemical components of EOs in the form of monoterpene alcohols, aldehydes and hydrocarbons can inhibit or stimulate the growth and metabolism of rumen microbes (Benchaar *et al.*, 2008; Bodas *et al.*, 2012). Essential oils decrease the abundance of protozoa and increase the abundance of bacteria as there is no

predation and competition from protozoa (Bodas *et al.*, 2012). Some essential oils, their active components and functions within the rumen is shown in Table 2.4.

Table 2.4 The active component and function of essential oils

Essential oil	Active component	Function	Reference
Cinnamon oil	Cinnamaldehyde	Disrupt cytoplasmic membrane, bind to proteins, deactivate microbial enzymes, and deplete cellular ATP	Benchaar and Greathead, 2011
Clove oil	Eugenol	Antimicrobial characteristics Inhibit both Gram-positive and Gram-negative bacteria	Benchaar and Greathead, 2011
Oregano oil	Carvacrol	Transmembrane carriers of cations, decrease ATP synthesis, cell death Inhibit *E. coli* O157:H7	Busquet *et al.*, 2006 Elgayyar *et al.*, 2001
Thyme oil	Thymol	Transmembrane carriers of cations, decrease ATP synthesis, cell death Antimicrobial characteristics	Busquet *et al.*, 2006 Benchaar and Greathead, 2011

The effects of the antimicrobial properties of EOs depend on a variety of factors. The part of the plant, for example the stem, leaves, roots, seeds, flowers, or bark, used for the EO can affect the concentration of the main compound which is responsible for the antimicrobial activity. Combinations of EOs seem to be more effective compared to using the individual compounds. Lambert *et al.* (2001) found that thymol and carvacrol, two components of oregano EO, combined had a better antimicrobial effect against *Staphylococcus aureus* and *Pseudomonas aeruginosa* than used individually. However, the effects between the individual compounds have been found to work antagonistic, additive or synergistic (Burt, 2004).

The interest of EOs in ruminant nutrition stemmed at first from their effect on the palatability of the feed (Calsamiglia *et al.*, 2007; Wallace *et al.*, 2002). The first to investigate the effect of EOs on the rumen microbiome was Oh *et al.* (1967) and Nagy & Tengerdy (1968). Both studies reported that EOs extracted from plants (Sage bush and Douglas fir needles) inhibited the rumen bacteria *in vitro*. The chemical component of the EO influenced the degree of inhibition. Some rumen bacteria seem to be more susceptible to the effect of EO, these include *Prevotella ruminicola*, *P. bryantii*, *Clostridium sticklandii*, *C. aminophilum*, *Butyrivibrio fibrisolvens* and *Peptostreptococcus anaerobius* compared to others including *Streptococcus bovis* (McIntosh *et al.*, 2003; Patra & Yu, 2012). Phenolic compounds, such as oregano EO, had a stronger antimicrobial effect by decreasing the abundance of *Clostridia*, *Firmicutes* and *Butyrivibrio* compared to other EO.

Current research on EO has shown the potential to improve nitrogen and energy utilization (Benchaar *et al.*, 2008; Zhang *et al.*, 2021a). Assorted studies have indicated a positive effect (Benchaar *et al.*, 2006;

Yang *et al.*, 2007) or no effect (Meyer *et al.*, 2009; Schären *et al.*, 2016) of EOs on the feed degradability in the rumen as well as the VFA production (Table 2.5). The effect of EOs on feed intake is also not consistent and dependent on the dose and type of EO.

Table 2.5 The effect and dosage of essential oils used in various *in vivo* studies in cattle

Essential oil blend	Dosage	Effect	Reference
Thymol, eugenol, vanillin & limonene	2-4 g/animal/day	Increased FE No effect VFA	Benchaar *et al.*, 2006
Thymol, eugenol, vanillin & guaiacol, limonene	1 g/animal/day	Increased FE No effect VFA	Meyer et al., 2009
Cinnameldehyde & eugenol	0.27 g/animal/day	Increased rumen fermentation Decrease feed intake Decreased acetate portion	Cardozo *et al.*, 2006
Anise, capsicum, eugenol & cinnameldehyde	3.9 g/animal/day	Increased rumen fermentation Decreased feed intake	
Eugenol, thymol & vanillin	4 g/animal/day	Increased feed intake	De Souza *et al.*, 2018
+ clove	4 g/animal/day	Increased feed efficiency Increased gain	
+ clove & rosemary	4 g/animal/day	Increased feed intake Increased feed efficiency Increased gain	
Cinnameldehyde, eugenol & capsicum oleoresin	400 mg/animal/day	Increased gain	Geraci *et al.*, 2012
Carvacrol, allicin, limonene, pinene, thymol, citronellal & citronellol	500-1000 mg/kg	No effect	Rivaroli *et al.*, 2017
Cashew & castrol EO	550	Increased feed efficiency Increased gain	Valero *et al.*, 2016
Clove & cinnamon EO	3.5-7 g/animal/day	Increased feed intake	Ornaghi *et al.*, 2017

In certain studies, supplementation with EOs did decrease the ammonia concentration in the rumen as EOs can inhibit the degradation of amino acids to ammonia (McIntosh *et al.*, 2003). The use of a cinnamon EO *in vitro* resulted in the largest decrease in ammonia concentration (Macheboeuf *et al.*, 2008) whereas a combination of cinnamaldehyde and eugenol resulted in a decrease in protein digestibility and the bacterial nitrogen flow (Tager & Krause, 2010). In contrast to this, other studies (Calsamiglia *et al.*, 2007) observed no

effect of EOs on NH_3-N concentration. Castillejos *et al.* (2007) and Spanghero *et al.* (2008) observed no change in ammonia concentration when using a mix of EOs in short term *in vitro* trials, suggesting that rumen bacteria are slower in adapting to these compounds (more than 4 weeks) to reduce NH_3-N. The effect of EOs on NH_3-N in the rumen seems to be dose dependent. Eugenol given in high doses resulted in a significant decrease of NH_3-N in *in vitro* cultures, low doses increased the ammonia concentration whereas intermediate doses had no effect (Cardozo *et al.*, 2006).

One of the mode of actions of EOs is to inhibit microbes resulting in a change in the fermentation pattern of the animal. Studies have shown contrasting results with total VFA concentration unaffected (Benchaar *et al.*, 2006; Meyer *et al.*, 2009), decreased (Spanghero *et al.*, 2008) or increased (Newbold *et al.*, 2004). Castillejos *et al.* (2007) observed an increase, a decrease, and no effect in VFAs depending on the EO and its dose used in the feed. The acetate to propionate ratio increased (Busquet *et al.*, 2005; Cardozo *et al.*, 2006) or decreased (Busquet *et al.*, 2006; Cardozo *et al.*, 2006) after supplementation with EOs. A reduction in the acetate and an increase in butyrate concentration was also noted (Castillejos *et al.*, 2007). A reduction in the concentration of VFAs due to the antimicrobial characteristics of EOs might be dose dependent; at low doses the VFA concentration was unchanged, but at higher doses a decrease in VFAs were observed in the above-mentioned studies.

Essential oils seem to influence VFAs in a similar manner compared to monensin by increasing the propionate and decreasing the acetate concentration (McGuffey *et al.*, 2001) resulting in more energy being available to the animal. Together with the increase in propionate concentration, the concentration of butyrate also increases, suggesting that the mode of action of EO is slightly divergent from that of monensin. Meyer *et al.* (2009) reported an increase in the feed efficiency of feedlot steers fed a blend of EOs, containing eugenol, guaiacol, limonene, thymol, and vanillin, with the antibiotic tylosin. In contrast, no effect was found in the group of animals that received the mix of EOs but was not supplemented with tylosin. However, no treatment influenced the fermentation characteristics. A limitation to the use of EOs is that the rumen microorganisms adapt to EOs resulting in a decrease in effectivity over time (Benchaar *et al.*, 2008).

Further interest in EOs is the effect these compounds have on methane emissions. The challenge in using EO is to identify a product that will decrease methane emissions without decreasing the feed degradation (Benchaar *et al.*, 2008). Methanol, from garlic, was reported to be the most effective mitigator of methane emissions (Busquet *et al.*, 2005). Patra & Yu (2012) also found that garlic and oregano EOs had the highest mitigating effect on methane production in comparison with EOs from eucalyptus, clove, and peppermint. However, *in vitro* studies have conflicting results due to variation in dose and EOs used (Cobellis *et al.*, 2016), as a decrease in methane by EOs depends on its dose (Macheboeuf *et al.*, 2008). The effect of EOs on mitigating methane seems to be due to the suppression of rumen methanogenic archaea and/or changing the methanogenesis pathway.

2.5 The effect of rumen microorganisms on efficiency

Feed efficiency is a crucial trait used in animal production as an efficient animal has a lower feed intake and higher production. Various studies have investigated the microbes found in efficient and inefficient ruminants (Guan *et al.*, 2008; Myer *et al.*, 2015a; Tapio *et al.*, 2017). Feed efficiency can be measured by various traits. Residual feed intake (RFI) can be described as the variation in the predicted and actual feed intake (Koch *et al.*, 1963). This measurement is phenotypically independent of the production level and the weight of the animal whereas feed conversion ratio (FCR) is a ratio between feed intake and growth. An estimated heritability of 0.06 to 0.62 for feed efficiency measurements have been reported (Lu *et al.*, 2020), the wide range indicates a strong environmental influence on feed efficiency. This environmental influence can be the result of nutrition as well as the rumen microbes among others (Shabat *et al.*, 2016; Li *et al.*, 2019). Rumen microbes influence the feed efficiency of the animal by delivering energy to the host through fermentation (Paz *et al.*, 2018).

One of the aims of feed efficiency studies is to utilize omics-based techniques, including metagenomics, to analyse the rumen microbiome to identify indicators that show variation in average daily gain (ADG), daily feed intake (DFI), feed efficiency (RFI, FCR), and VFA production to select for efficient animals in breeding programs (Guan *et al.*, 2008; Myer *et al.*, 2017). Differences in feed efficiency might be due to more active microbial fermentation in the form of VFA production and difference in energy metabolism (Khiaosa-ard & Zebeli, 2014). Specific microbes and their abundance can influence the efficiency of the animal. The efficiency of the animal may increase if the microbes convert a broader range of dietary components more efficiently.

The diversity of the microbes, as determined by alpha diversity indices, found in the rumen influences the efficiency of the animal as differences in diversity have been reported between more and less efficient ruminants (Guan *et al.*, 2008). A lower diversity in the rumen microbial composition has been reported in more efficient animals (Shabat *et al.*, 2016; Li & Guan, 2017) and inefficient cattle have more variation in metabolic pathways and fermentation end products. More variation in metabolic pathways increase the chance that carbon will be directed to pathways that use energy, such as methanogenesis. However, a higher diversity has been associated with a more stable and healthy rumen (Yeoman & White, 2014). Table 2.6 shows bacterial species that were reported to be abundant in more efficient and less efficient animals in various studies.

Studies show contrasting results with regard to feed intake, growth and efficiency, and associated rumen microbes. Species from the *Veillonellaceae* family, the *Clostridiales* order (Myer *et al.*, 2015a) as well as the *Bifidobacteriaceae* and *Prevotellaceae* families (Paz *et al.*, 2018) have been positively associated with feed intake while the *Lachnospiraceae*, *Prevotellaceae*, *Veillonellaceae* and *Victivallaceae* families have been positively associated with ADG in cattle (Myer *et al.*, 2015a). It has been indicated that the abundance of *Lachnospiraceae* differed between efficient and inefficient cattle through a rumen metatranscriptomic study with a higher abundance of *Lachnospiraceae* observed in inefficient cattle (Li & Guan, 2017). Some members of this family are butyrate producers, which have been observed to have a higher concentration in more feed

efficient ruminants (Guan *et al.*, 2008). Myer *et al.* (2015a) found that the genera *Succiniclasticum* was more abundant in the feed efficient group with low ADG. *Succiniclasticum* specializes in converting succinate to propionate (Van Gylswyk, 1995). Propionate-producing bacteria divert H_2 away from methanogenesis (De Menezes *et al.*, 2011), resulting in a reduction in methane emissions. Diets consisting predominantly of concentrates, such as grain, produce lower methane emissions compared to diets consisting predominantly of roughage (Auffret *et al.*, 2018).

Table 2.6 Literature reporting bacterial groups or taxa found to be more abundant within efficient and inefficient animals.

Reference	Efficient animals	Inefficient animals
Carberry *et al.*, 2012		*Prevotella*
Hernandez-Sanabria *et al.*, 2012	*Succinivibrio sp*	*Eubacterium rectale*
	Eubacterium rectale	*Robinsoniella sp*
Li *et al.*, 2019	*Firmicutes*	
	Chloroflexi	
	Blautia	
McCann *et al.*, 2014b	*Bacteroidales*	
Myer *et al.*, 2015a	*Firmicutes sp*	
	Butyvibrio	
	Leucobacter	
Shabat *et al.*, 2016	*Megasphaera elsdenii*	*Lachnospiraceae*
	Lachnospiracea	*Methanobrevibacter ruminantium*
	Coprococcus Catus	
Zhou *et al.*, 2009		*Methanobrevibacter* sp
		Methanosphaera stadtmanae

A change in the Firmicutes to Bacteroidetes ratio has been reported to influence energy harvesting and has been associated with increased fat deposition (Jami *et al.*, 2014) and ADG (Myer *et al.*, 2015a). A higher abundance of Firmicutes was reported in the rumen of feed efficient animals showing that the ratio is linked to higher gain and less feed intake. Microbes of the *Bacteroidales* order has been associated with more efficient steers in grazing conditions (McCann *et al.*, 2014b) whereas Shabat *et al.* (2016) observed an abundance of *Bacteroidales* in inefficient dairy cows. Inefficient cattle on a high energy diet seem to have an abundance of *Eubacterium rectale* (Hernandez-Sanabria *et al.*, 2012).

The majority of taxa and microbes identified by Myer *et al.* (2015a) that was associated with feed efficiency was found to be related to fibre-degrading, fermentative, and metabolic activities in the rumen. The abundance of *Succiniclasticum*, *Lactobacillus*, *Ruminococcus* and *Prevotella* have been reported to affect the feed efficiency and methane production of the animal (Myer *et al.*, 2015a). Moreover, Myer *et al.* (2015a) also

reported a lack of difference in the microbes between four different feed efficiency groups and concluded that the variation in the microbial population between feed efficiency groups might lie at a finer resolution. Positive (Hernandez-Sanabria *et al.*, 2012; Paz *et al.*, 2018), negative (Carberry *et al.*, 2012; McCann *et al.*, 2014b; Paz *et al.*, 2018) and no (Myer *et al.*, 2015a) associations have been reported between *Prevotella* and feed efficiency which indicates that the strain of the microbes also influences the efficiency of the rumen microbiome population. *Prevotella* is the predominant bacterial genus in the rumen microbial composition (Stevenson & Weimer, 2007; Pitta *et al.*, 2010; Henderson *et al.*, 2015). Manipulating the rumen microbiome to shift towards microbial species that can increase the efficiency of the animals, such as *Succiniclasticum*, can result in increased animal production.

Methanogenic species in the bovine rumen include *Methanobrevibacter ruminantium*, *M. smithii*, *Methanosphaera stadtmanae*, and *Methanosarcina barkeri* (Zhou *et al.*, 2009). Methanogenic bacteria use energy to produce methane causing a loss in energy that could have been used by the host (Tapio *et al.*, 2017). Feed efficient cattle produce 20 to 26% less methane in comparison with inefficient cattle (Hegarty *et al.*, 2007). However, Zhou *et al.* (2009) observed that the total methanogenic population did not differ significantly between efficient and inefficient animals. It has been indicated that the diversity of the methanogenic population, instead of the abundance of the methanogens, can influence feed efficiency (Lan & Yang, 2019). *Methanosphaera stadtmanae* and a strain from *Methanobrevibacter* species, were present in abundant amounts and proportions in inefficient animals (Zhou *et al.*, 2009). Bacteria not directly involved in the methanogenesis process can also have an influence on methane emissions due to the H_2 concentration and the pH in the rumen (Russell, 1998). A high abundance of *Succinivibrionaceae*, *Megasphaera* and *Dialister* were found in cattle with low methane emissions and could possibly offer a mitigation strategy (Wallace *et al.*, 2015) while the abundance of *Desulfovibrio*, *Mogibacterium* and *Pyramidobacter* were observed to be higher in cattle emitting more methane.

Various mitigation strategies have been suggested ranging from using rumen protected feed components to the selection of more efficient cattle using RFI (Gerber *et al.*, 2013). Despite the low heritability of feed efficiency traits, such as RFI, it holds potential to select more efficient animals together with microbial markers. Methane emissions can also be predicted with a phenotypic correlation of 0.49 from the methanogen to bacteria ratio in the rumen microbiome (Wallace *et al.*, 2014; Auffret *et al.*, 2018). As a host effect on the core rumen microbiome exists, microbes associated with efficiency holds potential as markers to select animals for increased efficiency. It may be less expensive and labour intensive as well as faster to use microbial genes as markers for hard to measure traits, such as RFI, compared to recording the traits (Ross *et al.*, 2013; Roehe *et al.*, 2016). This can be used to test sire progeny groups to determine the breeding value for feed efficiency and methane emissions.

2.6 Techniques to study the microbiome

Historically, *in vitro* cultivation methods, such as culture-based techniques, were the primary method for identifying rumen microbes (Deusch *et al.*, 2015). Despite the major contribution of culture-based methods, the variation between the microbes in the environment and the cultivation of bacteria *in vitro* led to limited information as certain cultures and strains were difficult to cultivate (Deusch *et al.*, 2015). As a consequence of the culture conditions, protocol design and difficult-to-cultivate microbes, studies using culture based methods are unable to represent the full scope of the rumen microbiome (Hiergeist *et al.*, 2015). However, these studies are still important in characterizing and understanding the function and biochemistry of the microbes as they are used to build reference databases used in microbiome sequencing analysis. The reference databases that are used to identify the taxa can influence the accuracy of taxonomic identification (Smith *et al.*, 2022).

The rumen microbiome was also studied using classic molecular techniques (Gruninger *et al.*, 2019), such as quantitative polymerase chain reaction (qPCR), restriction fragment length polymorphism (RFLP), as well as denaturing gradient gel electrophoresis and these have become largely obsolete as the intricacy of the rumen microbiome makes omics-based techniques and amplicon sequencing more suited. Quantitative polymerase chain reaction (qPCR) amplifies the DNA of a specific gene and can be used to identify specific species that are also culturable. The qPCR technique is limited to species that can be queried by species-specific probes (Franzosa *et al.*, 2015). However, it is a sensitive tool that can be used to track specific microbes and to study the shift in the microbial population and is therefore used for validation.

The development of next generation sequencing (NGS) provided new ways to study microbial diversity, the functions and interactions of the microbiome community, as well as the influence of the microorganisms on the host (Deusch *et al.*, 2015). Next generation sequencing platforms are continuously being upgraded and developed to lower the cost as well as increase the quality and throughput of sequencing. Various sequencing platforms commonly used today, their advantages and limitations were summarized in Table 2.7.

Metagenomics can be used to study the microbiome structure, the phylogenetic composition, microbial and functional diversity, metabolic capacity, and microbial genes (Shah *et al.*, 2011; Roehe *et al.*, 2016; Auffret *et al.*, 2018). It can also be used to predict the functional capacity of microbiota through 16S and 18S rRNA gene sequencing (McCann *et al.*, 2014a) or shotgun metagenomic sequencing. The abundance of microbial genes determined with metagenomics, can be used to understand the interaction between the microbiome and the host leading towards predictive models for methane emissions and feed efficiency (Roehe *et al.*, 2016).

There are predominantly three methods to study the microbiome, amplicon or shotgun metagenome sequencing and metatranscriptomics. Shotgun sequencing investigates all the genes and their functions in a community, while amplicon techniques focus on the taxonomic identification of the microbes in the community (Caporaso *et al.*, 2011). Unrestricted sequencing of all the microorganisms in the sample can be done by whole-metagenome shotgun analyses and metatranscriptomics, while PCR amplicons from the 16S

rRNA, 18S rRNA or ITS genes can only sequence one group at a time, bacteria and archaea or fungi (Janda & Abbott, 2007). Both amplicon and shotgun sequencing techniques can be accompanied by functional profiling. Amplicon sequencing relies on previously build databases to determine the functional potential of the microbes while shotgun sequencing provides real time data of the microbes' functional and metabolic properties (Jovel *et al.*, 2016). The most accurate way of describing the functional activity of the microbes are to make use of transcriptomics, proteomics and metabolomics in combination with metagenomics (Franzosa *et al.*, 2015). There are advantages and disadvantages to each sequencing method (Porath-Krause *et al.*, 2022; Schloss, 2018; Wensel *et al.*, 2022) and the selection of the sequencing method for the study depends on the research question asked. The various methods, advantageous and limitations will be discussed further below.

Table 2.7 Various platforms that can be used for sequencing (Caporaso *et al.*, 2011; Di Bella *et al.*, 2013; Deusch *et al.*, 2015; Quince *et al.*, 2017)

Sequencing platforms	Chemistry	Manufacturer	Advantages	Limitation
454 GS FLX+	Pyrosequencing	Roche	Long read lengths (600-800 bp), high speed	Low throughput, high error rate
HiSeq 2000/2500	Sequencing by synthesis	Illumina	Wide availability High output (15 Tb/run) High accuracy (0.1-1.1%)	Short read length
5500 xl W SOLiD	Ligation	Applied Biosystems/ Life Technologies	High accuracy	Long run time, short read length
Nanopore MinION	Read through nanoscale-sized pore	Oxford	Read length 25 kbp per 1 bp/s speed	Low throughput, and lack of nucleotide specificity
Single-Molecule Real-Time sequencing (SMRT)	Fluorescent dye modified nucleotides	Pacific Biosciences	Long reads Low reagent cost	High error rate (> 10%) Low throughput Difficult set-up
IonTorrent	Proton detection	Life Sciences	High throughput, short run time	Short read length High error rate

Samples taken from the same environment can result in different microbial compositions. This presents a challenge of detecting statistically significant differences among a small sample size (Quince *et al.*, 2017). As a possible solution to this problem, Quince *et al.* (2017) recommended that samples be taken over time or replicates must be taken. Given the intricacy of metagenomic data and the tremendous number of possible comparisons, multiple-test correction is necessary. Sample collection, such as time or location where the

sample was taken, and extraction of the DNA or RNA can have a greater effect on the microbial composition compared to the biological variable being investigated (Henderson *et al.*, 2013). Another method to increase the accuracy of sequencing and discover low abundance microbes is through the sequencing depth or coverage, which is the amount of reads that can be produced during sequencing (Di Bella *et al.*, 2013), with a deeper coverage uncovering rare species or detecting small differences between samples. A high depth coverage in amplicon sequencing results in the detection of more microbes while in shotgun metagenomic analysis more genes are detected (Di Bella *et al.*, 2013).

2.6.1 Amplicon sequencing

The first study to categorise prokaryotic cells into bacteria and archaea based on 16S rRNA genes was performed by Woese and Fox (1977). The 16S rRNA gene encodes small ribosomal subunits ideal to identify bacterial species and is present in all species. The application of 16S rRNA profiling in research was increased due to the developments in DNA sequencing technology and the application of barcoded pyrosequencing (Hamady *et al.*, 2008). The 16S rRNA sequence of prokaryotes is approximately 1 550 base pairs and contain various conserved and hypervariable regions (V1-V9) which are targeted by 16S rRNA amplification primers (Chakravorty *et al.*, 2007). These hypervariable regions are unique to different microbial species and can therefore be used for identification. The conserved regions are used to create universal PCR primers suitable for closely related microbial species containing the same hypervariable regions (Wensel *et al.*, 2022). Variation in these genes indicate evolutionary distance and relatedness of organisms; it does not indicate rate of change in the gene sequence. Hypervariable region V2 and V4 have the lowest error rate of the nine regions (Wang *et al.*, 2007). The hypervariable regions V2, V3 and V6 contain the maximum heterogeneity and would therefore provide the most discriminating power to analyse the bacterial groups (Chakravorty *et al.*, 2007). The V1-V3 region of the 16S rRNA gene is commonly used to study the microbiome as it is highly conserved and has variable regions with a substantial reference sequences database (McCann *et al.*, 2014a). The use of the hypervariable regions,V4, V5-V6 and V6-7, has been reported to result in the best indication of richness (Youssef *et al.*, 2009). Usage of the V3-V4 hypervariable region can result in simultaneous amplification of both archaea and bacteria. Although this may not be as accurate as using different hypervariable regions for separate analysis (Fischer *et al.*, 2016), it is appropriate for rumen microbiome diversity studies as the interaction between archaea and bacteria can affect the production of the animals (Roehe *et al.*, 2016). The hypervariable region used can influence the accuracy of taxonomic identification and the estimation of the microbial diversity (Youssef *et al.*, 2009).

Advantages of using 16S rRNA sequencing includes the routine identification of bacteria, discovery of new pathogens and identification of bacteria not yet cultured (Jovel *et al.*, 2016). In a study comparing 16S rRNA amplicon and shotgun sequencing, greater detail of the bacterial communities was generated by 16S rRNA amplicon sequencing with improved identification of low abundance species compared to shotgun sequencing (Shah *et al.*, 2011). Zhou *et al.* (2021) reported that the impact of diet on the microbiome in the

rumen became more evident through amplicon sequencing compared to shotgun sequencing. One of its biggest advantageous is the lower cost associated with amplicon sequencing compared to shotgun and metatranscriptomic sequencing.

Sequencing 16S rRNA is best used with a large sample size , however it has limited taxonomical and functional power only identifying to genus level and the primers used may result in inaccurate results (Jovel *et al.*, 2016). Biases can also be introduced through sample dilution and lane-to-lane variation through the sequencing methodology (Porath-Krause *et al.*, 2022). Other challenges in using 16S rRNA sequencing is the generation of chimera sequences caused by PCR amplification and sequencing errors. Chimera sequences are produced when there is an incomplete PCR amplification in the region of interest and the resulting partial sequences are seen as primers that can merge with the molecules (Jovel *et al.*, 2016). Chimera sequences can be removed via bioinformatic means with programs such as USEARCH (Edgar, 2010) or DADA2 (Callahan *et al.*, 2016). A limitation of 16S rRNA sequencing is that some bacteria have more than one section that contain the 16S RNA gene (Di Bella *et al.*, 2013), which may lead to false positives. A further limitation is that the use of amplicon sequencing is restricted to the analysis of taxa that are already identified, can be amplified and reported in a database (Sharpton, 2014).

2.6.2 Metagenomic and alternative sequencing techniques

Shotgun sequencing is the sequencing of all microbial genes present (Quince *et al.*, 2017). This technique sequences the whole genome of the microbes in the microbiome (Quince *et al.*, 2017) and can profile the composition and functional potential of microbiome community. The quantification of the microbiome's taxonomic diversity involves the determination of the microbes present in the community, and the abundance of these microbes (Sharpton, 2014). The abundance of the taxa can be determined through analysing marker genes, by arranging sequences into defined taxonomic groups through binning and by constructing the sequences into specific genomes.

Binning is the grouping of metagenomic DNA sequences into groups that are closely related (Thomas *et al.*, 2012). This can be done through the classification of the sequence into genomic groups, such as OTUs or amplicon sequence variants (ASVs), or by clustering the sequences into representative groups based on similar properties, such as the GC content of the sequence (Sharpton, 2014). Binning reduces the complexity of data and therefore increases the ease of post-processing analysis. Further simplification is through the assembly of the sequences into a single contiguous sequence (Sharpton, 2014) and this longer sequence length results in more accurate information (Thomas *et al.*, 2012).

The potential functions of microbial communities can be determined by comparing the reads identified through sequencing to databases containing the functions of the various genes, proteins or metabolic pathways (Sharpton, 2014). A comparison between metagenomic and metatranscriptomic analysis indicated that there was little difference between communities at a transcriptional and genomic level, metagenomics may be a good representation for functional activity (Mason *et al.*, 2012).

An advantage to shotgun metagenomics is that with a high quality metagenome assembly, microbial communities can be identified using gene databases (Quince *et al.*, 2017). Shotgun sequencing is expensive and computationally intensive (Sharpton, 2014); however, it has a higher resolution and coverage that leads to more specific taxonomic and functional classification (Jovel *et al.*, 2016). It might also result in the detection of new microbial species, genes, and genomes. The greatest advantage lies in the simultaneous study of all microbes in the environment and less biases are introduced through the sequencing method. The main limitation in the use of shotgun sequencing to identify the metabolic potential of the microbial composition is the absence of annotation for genes in most species. Shotgun metagenomics cannot be used to quantify the genes expression or determine the function of the microbes but it offers a more reliable analysis, provided that there is sufficient coverage, compared to 16S rRNA metagenomics (Franzosa *et al.*, 2015).

Shotgun metagenomic sequencing provide the most detailed data to characterise the microbiome, however the cost, labour and time associated with the process of library preparation, sequencing and bioinformatic and statistical analyses pose a limitation when analysing many samples. Recently techniques such as the Axiom microarray (Thissen *et al.*, 2019) were developed. The Axiom microbiome array was designed as a less expensive version of the Lawrence Livermore Microbial Detection Array (Thissen *et al.*, 2019) and contains 1.38 million DNA probes to identify microbial species. Although it does not detect microorganisms that have not been sequenced, the advantage of the Axiom microarray is its detection of viruses, fungi, archaea, protozoa, and bacteria. The disadvantage of this technique is that it is only designed for taxonomic identification of the microbes and does not produce any functional description of genes and other sequences. This technique has been used for the detection of single nucleotide polymorphisms in humans (Hoffmann *et al.*, 2011), and animals (Kranis *et al.*, 2013).

A limitation of metagenomics is the inability to directly measure functional activity of the community under a given set of conditions. To face this limitation, other ‘omics methods can be implemented; defining the microbial community in terms of the RNA (transcriptomics), protein (proteomics) and metabolite (metabolomics) abundances (Franzosa *et al.*, 2015). Metatranscriptomics determine the microbial genes that are expressed in a given moment in a specific environment (Loor *et al.*, 2016). This technique can elucidate accurately which of the genes annotated through metagenomics are expressed and to what extent. From this data, active metabolic pathways can be investigated in the microbial community and associated to specific treatment conditions. The functional potential and capabilities of the rumen microbiome can then be studied (Li *et al.*, 2019).

The community function can be analysed based on metabolites and expressed proteins. Metaproteomic studies focus on the expressed proteins in a particular sample at a specific point in time (Franzosa *et al.*, 2015). The functionality of a community of microbes can be validated by assessing the protein produced (Seifert *et al.*, 2013). Active microbes and their expressed metabolic pathways can be identified using this technique. Metabolomics is a technique that detects metabolites, such as VFAs, and other small molecules within

microbiome communities (Franzosa *et al.*, 2015). This is a direct, experimental measurement of metabolite abundances.

2.6.3 Bioinformatic and statistical analyses

Post-sequencing analysis uses statistical tools in bioinformatic programs to interpret the data received from sequencing to investigate how the results correlate with the quantitative data (metadata) collected. Figure 2.5 depicts the workflow necessary for post-sequencing analysis for 16S rRNA amplicon and shotgun sequencing compiled from various studies (Liu *et al.*, 2008; Di Bella *et al.*, 2013; Sharpton, 2014; Deusch *et al.*, 2015; Jovel *et al.*, 2016; Quince *et al.*, 2017).

Post-processing analysis of amplicon sequencing is commonly performed by clustering the sequences into OTUs or ASVs. Operational taxonomic units (OTUs) are assembled by grouping reads based on their similarity with the similarity threshold set at 97% (Di Bella *et al.*, 2013). Amplicon sequence variants (ASVs) do not have a similarity threshold and are often distinguished by a single nucleotide (Callahan *et al.*, 2017). Methods using ASVs have been shown to be as or more sensitive and specific compare to OTUs (Callahan *et al.*, 2016). The most widely used pipelines (Di Bella *et al.*, 2013; Jovel *et al.*, 2016) for amplicon sequencing includes mothur (Schloss *et al.*, 2009) and Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso *et al.*, 2010). Further packages that can be used include R packages such as DADA2 with ASVs (Callahan *et al.*, 2016) for quality control, vegan (Oksanen *et al.*, 2020) for assessing diversity, ape (Paradis & Schliep, 2019) for generating a phylogenetic tree and phyloseq (McMurdie & Holmes, 2013) for analysing abundance data.

Specific challenges to post-processing analysis of shotgun metagenomic data include the proportional nature of the microbes and the distribution of abundances (Quince *et al.*, 2017). The common post-processing analysis for shotgun metagenomic reads involve binning, assembly and mapping (Di Bella *et al.*, 2013). Grouping the reads can be performed before or after assembly and is useful as it simplifies the post-processing analysis. The reads should not be assembled if gene abundances between samples are going to be compared as abundance information may be lost during the assembly process (Di Bella *et al.*, 2013). Software programs that assemble metagenomes differ from those that assemble genomes as they are programmed for data containing multiple species using algorithms to separate the species where possible, thereby reducing the number of chimeric reads. The analysis result is a metabolic pathway reconstruction, which is usually performed with the KEGG (Kyoto encyclopaedia of genes and genomes) database (Kanehisa *et al.*, 2014).

Metagenomic sequences generated from shotgun sequencing can also be used to study the genes in the sample (Di Bella *et al.*, 2013). The KEGG database is then used to annotate the genes (Kanehisa *et al.*, 2014). Pipelines such as MEGAN4 (Huson *et al.*, 2011) or MG-RAST (Meyer *et al.*, 2008) uses the assembled or raw reads for gene annotation and prediction. The diversity of the genes in the sample is analysed to identify closely related or significantly different samples (Di Bella *et al.*, 2013).

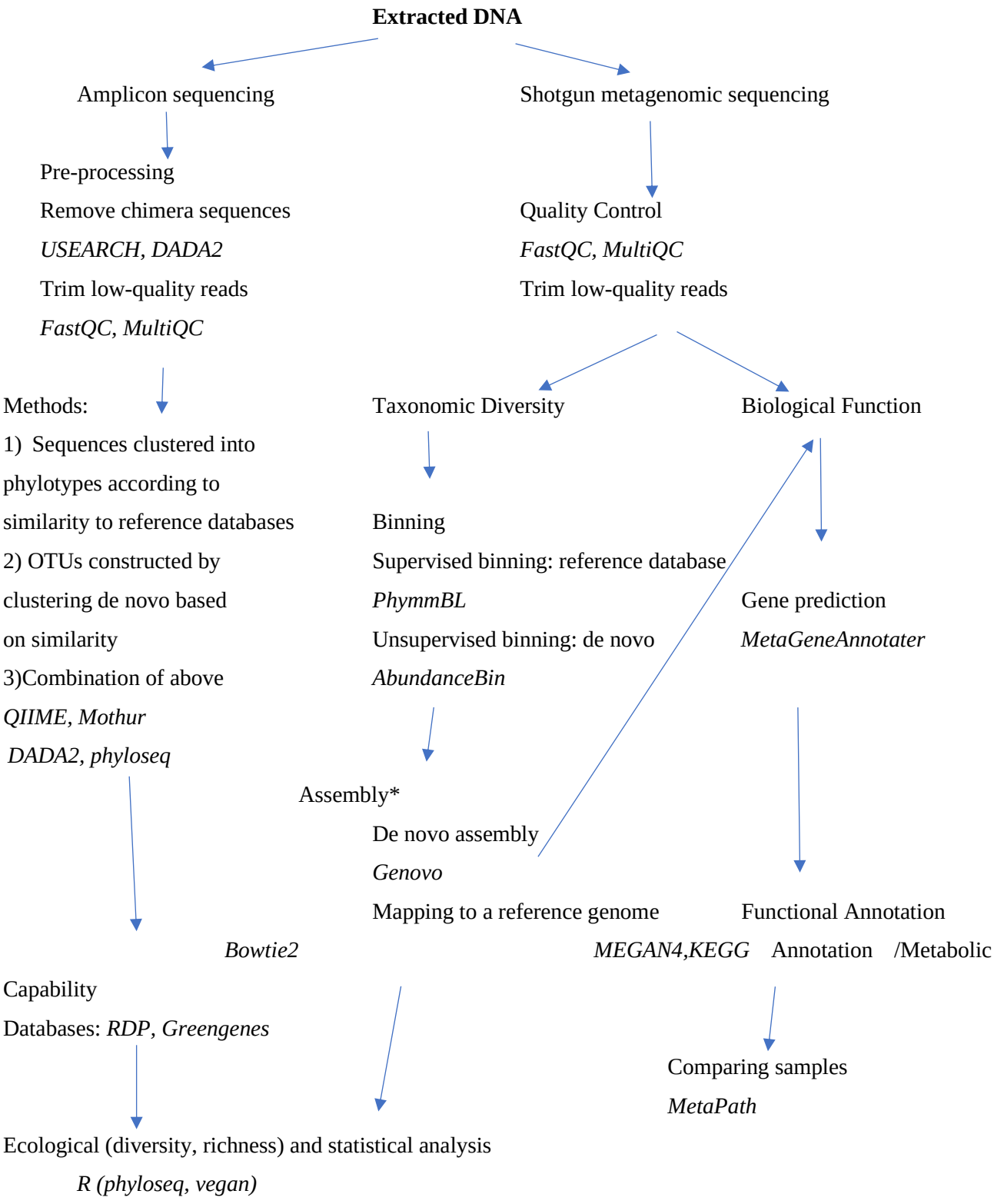

Figure 2.5 Post-processing workflow for two sequencing techniques to study the rumen microbiome featuring various steps to reach ecological and statistical analyses. Compiled from Liu *et al*. (2008), Di Bella *et al.* (2013), Sharpton (2014), Deusch *et al.* (2015), Jovel *et al.* (2016), Quince *et al.* (2017).

Bioinformatic programs are shown in italic. USEARCH (Edgar, 2010), Mothur (Schloss *et al.*, 2009), QIIME (Caporaso *et al.*, 2010), phyloseq (McMurdie & Holmes, 2013), PhymmBL (Brady & Salzberg, 2011), AbundanceBin (Wu & Ye,

2011), Genovo (Laserson *et al.*, 2011), Bowtie2 (Langmead & Salzberg, 2013), MetaGeneAnnotater (Noguchi *et al.*, 2008), MEGAN4 (Huson *et al.*, 2011), KEGG (Kanehisa *et al.*, 2014), MetaPath (Liu & Pop, 2011).
Databases: Greengenes (DeSantis *et al.*, 2006), Ribosomal Database Project (RDP) (Cole *et al.*, 2014).
*Assembly can be performed before or after binning.

Software programs and packages, such as MetaPath (Liu & Pop, 2011), uses metagenome data combined with KEGG pathway information to determine different abundances for genes, functions or pathways between treatments. The metabolic capability of the microbiomes can be determined and the interactions between the microbes and the environment can be identified by comparing the metagenomic data between different treatments.

Microbial ecology measurements are used in microbiome studies to characterize and explain the microbial population. There are two types of diversity measures that can be used to characterize a microbial population, alpha diversity (Lozupone & Knight, 2008) and beta diversity (Whittaker, 1972) (Table 2.8). The alpha diversity of the samples are determined through the richness, evenness and diversity of the sample (Lozupone & Knight, 2008). Richness is described as the number of different microbes in the sample while evenness and diversity is the abundance of the microbes as determined by Chao1 index and the number of OTUs/ASVs (Kim *et al.*, 2017). The Shannon index combines richness (total number) and the evenness (abundance) and is the overall indicator of the diversity within a sample.

Table 2.8 The different diversity measures used for microbiome studies.

Diversity measure	Definition	Indices
Alpha diversity	Microbial composition within samples as determined by richness and evenness of the sample	Simpson and Shannon Indices Observed number of ASVs/OTUs, Chao1 Index
Beta diversity	Difference in microbial profile between samples	Weighted and unweighted UniFrac distances

There are primarily two approaches for analysing beta diversity: the phylogenetic approach (Lozupone & Knight, 2008) which considers the evolutionary differences between microbial communities, and the taxon-based approach (Kuczynski *et al.*, 2010). Differences in the abundances of closely related microbes are given less weight with phylogenetic beta diversity, on the presumption that they have corresponding genetic capabilities. The Bray Curtis dissimilarity (Bray & Curtis, 1967) is the most popular taxon-based approach to quantify beta diversity (Jovel *et al.*, 2016). Weighted and unweighted UniFrac distances are also often used to determine the beta diversity (Lozupone & Knight, 2008). Weighted UniFrac distances consider the abundance of the taxa in the sample and is more suited to study the compositional differences that occur due to factors that influence the relative abundance of the taxa such as changes in diet whereas unweighted UniFrac distances

are more suited to identify factors that restrict microbial growth. A principal coordinate analysis (PCoA) illustrates beta diversity measurements.

There are various methods to analyse the population of the rumen microbiome, some of which are mentioned above. Amplicon sequencing has been suggested to be more sensitive when studying the impact of diet on the rumen microbial composition (Zhou *et al.*, 2021). However, studies that investigate the metabolic pathways in the rumen microbiome perform shotgun sequencing. The disadvantage to shotgun metagenomic sequencing is the complex bioinformatic analysis that is needed to analyse the data.

2.7 Conclusion

Climate change and the growing world population as well as consumer preferences are necessitating the research for methods to increase the production and efficiency of livestock. There is a direct correlation between the rumen microbiome and the efficiency of the animal, modifying the rumen microbiome towards energy favourable pathways can lead to an increase in the efficiency of the ruminant. This can be done by manipulating the rumen microbiome towards more energy efficient pathways through feed additives. The addition of feed additives to a ruminant diet might decrease the amount of non-productive or pathogenic microbes while increasing beneficial microbes.

Due to next generation sequencing, the influence of different feed additives and nutritional components on the rumen microbiome can be investigated. The rumen microbiome is a complex ecosystem that is influenced by various factors. The knowledge gained from these techniques can result in strategies to mitigate greenhouse gas emissions and simultaneously maintain or even increase the efficiency of the animal. Although many aspects of the microbes in the gastrointestinal tract have been studied, many influences on the microbiome composition are yet to be fully understood as shown by the many contrasting studies.

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Chapter 3

Rumen microbial diversity of Bonsmara cattle using amplicon sequencing during a 120-day growth trial

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Abstract

An improved understanding of the microbial populations during intensive feeding of feedlot cattle holds potential for optimizing production efficiency. Ionophores are used to increase the production and efficiency of ruminants and are commonly used in South African feedlots. Bonsmara bull calves (n=24) were subjected to a four-phase feedlot diet in a growth trial commencing with backgrounding, followed by a starter, grower and finisher diet. Animals were randomly divided into two groups: a control and a group with monensin inclusion. Four animals from each group were randomly selected for rumen content collection using an oesophageal tube during the phases within the growth trial. Samples were analysed using 16S rRNA and internal transcribed spacers amplicon sequencing. A total of 42 008 and 35 442 amplicon sequence variants were identified from 16S rRNA and internal transcribed spacers amplicon sequencing, respectively. The rumen microbiome composition and alpha diversity differed significantly between the phases while no significant difference was observed between the control and monensin groups. Backgrounding had the highest bacterial richness while the grower phase had the highest fungal richness. Bacteroidetes, Firmicutes and Proteobacteria were the most abundant phyla with Bacteroidetes being most abundant in the backgrounding and starter phases, while Proteobacteria was the most abundant in the grower and finisher phases. Ascomycota, Basidiomycota and Neocallistigomycota were the most abundant fungal phyla. Improved knowledge of the shift in microbiome population during the growth period is a tool that can assist to adapt feeding strategies to improve the efficiency of beef production.

Key words: Bacteria, fungi, microbial shift, rumen microbiome, ruminant

3.1 Introduction

Beef producers are presented with the challenge of increasing the supply of high-quality beef, while maintaining an economic and environmentally sustainable enterprise. A potential solution is to increase the feed efficiency of the animal (Capper, 2011). South African beef production is characterized by medium to large extensive commercial cow-calf operations with up to 65 to 70% of all cattle slaughtered originating from feedlot systems (DAFF, 2019). Several factors play a role in economic beef production, such as the feeding regime, price of weaners, and general health and management of the animals. Overall, the efficiency of the animals is the determining factor for sustainable feedlot production (Koenig *et al.*, 2020).

The efficiency of an animal is determined by various factors including the rumen microbiome (Guan *et al.*, 2008; Myer *et al.*, 2015), where the microbes (Firkins & Yu, 2015) are responsible for fermentation and degradation of the feed components into nutrients such as volatile fatty acids (VFAs). These VFAs are responsible for approximately 70% of the energy available to the animal for maintenance and production (Perea *et al.*, 2017). The composition and balance of the rumen microbiome determines the concentration of the VFAs thus greatly influences the metabolic energy efficiency of the animal.

In South Africa, the efficiency and production of feedlot animals are further increased by the addition of monensin, an in-feed ionophore, to the diet. Ionophores change the rumen microbiome by inhibiting Gram-positive bacteria and methanogens resulting in a shift in the VFA production in favour of propionate (Samuelson *et al.*, 2016). Propionate is glucogenic and can provide more energy to the host resulting in an increase in the efficiency of the animal.

Transitioning from a roughage- to a concentrate-based diet has been reported to modify the rumen microbiome (Fernando *et al.*, 2010; Stanton *et al.*, 2020) with a sudden transition resulting in digestive disorders (Klieve *et al.*, 2003). A stepwise adaptation, from a roughage to a high energy diet, is known to stabilize the rumen microbiome (Klieve *et al.*, 2003; Bevans *et al.*, 2005). These feeding regimes are characteristic of feedlot feeding and therefore necessitates an improved understanding of the dynamics of the rumen microbiome (Mackie *et al.*, 1978; Tajima *et al.*, 2001). Improving feed efficiency in the livestock sector is crucial for sustainable animal production as it has the potential to improve nutrient utilization from feed, increase profitability and reduce greenhouse gas emissions (Huws *et al.*, 2018).

Modern sequencing-based methods, such as 16S rRNA and internal transcribed spacer (ITS) amplicon sequencing, have improved the detection and quantification of microbes in the rumen as previous methods such as culturing could not capture the full diversity of the rumen microbiome (Huws *et al.*, 2018; Gruninger *et al.*, 2019). These sequencing techniques can be used to conduct research on the total microbial diversity and microbial population function (Myer, 2019) and can lead to an improved understanding of the interaction between the diet, the rumen microbiome and the efficiency of production (Pitta *et al.*, 2018).

In this study, next generation sequencing of the 16S rRNA gene and the ITS region was used to perform the first investigation of the rumen microbial diversity in Bonsmara cattle during a 120-day growth trial in Southern Africa. It is hypothesized that the bacterial, archaeal and fungal populations in the rumen of Bonsmara cattle will differ across the phases within the feedlot period.

3.2 Materials and methods

The Animal Ethics committee of the University of Pretoria granted approval for the project (NAS445/2019). The trial was conducted at a commercial feedlot, Edenvale, Free State, South Africa. Twenty-four Bonsmara bull calves (10-12 months old, 228 ± 22 kg) originating from a single Bonsmara breeder was backgrounded for 40 d on veldt grazing with lick supplementation before the onset of the growth period. The animals were divided into two treatment groups: a group receiving a standard feedlot diet including monensin with an inclusion of 30 mg/animal/day (n = 12) and a control group with no feed additive (n = 12). The diets were mixed at the Sernick feed mill, marked, and bagged for the trial.

The animals were allocated in a randomized block design according to weight, three to a pen with eight pens in total for the growth study that consisted of a starter, grower, and finisher phase. The composition of the diets used during the feedlot period is presented in Table 3.1. All animals received a Revalor S (Intervet GesmbH, Austria) hormone implant at the beginning of the starter phase as per standard feedlot operation in South Africa.

Table 3.1 Composition of the diets (as fed in kg/day and % of diet) as calculated with predicted daily feed intake.

Ingredients	Starter		Grower		Finisher	
	kg/d	% of diet	kg/d	% of diet	kg/d	% of diet
Wheat straw 5% Crude Protein	1.10	17.2	1.40	13.9	1.60	11.9
Yellow maize	1.80	28.1	3.12	31.0	4.36	32.3
Hominy chop	1.80	28.1	3.12	31.0	4.36	32.3
Salt	0.02	0.3	0.03	0.3	0.04	0.3
Urea	0.06	0.9	0.10	1.0	0.15	1.1
Limestone	0.10	1.6	0.11	1.1	0.14	1.0
Sunflower Oilcake	0.26	4.1	0.15	1.5	0.00	0.0
Molasses	0.51	8.0	0.81	8.1	1.08	8.0
Wheat bran	0.70	11.0	1.01	10.0	1.35	10.0
Feedlot vitamin/mineral Pre-Mix	0.01	0.2	0.01	0.1	0.01	0.1
Megalac (rumen bypass fat)	0.03	0.5	0.20	2.0	0.41	3.0
Predicted feed intake (kg/d)	6.39		10.06		13.5	

Animals received the starter diet for 21 days, followed by the grower (fed for 80 days) and the finisher (fed for 14 days) diet. Three days was used for the adaptation between phases (starter to grower to finisher diet) by decreasing the percentage of the diet fed and increasing the percentage of the new diet until the animals only received the diet of the new phase. Animals were given *ad libitum* access to feed and water.

Eight animals, one from each pen, were randomly selected at the start of the trial for collection of rumen content in the different phases namely backgrounding, starter, grower, and finisher (32 samples). The same animals were used for rumen content collection throughout the trial. A trained veterinarian inserted a flexible plastic oesophageal tube into the rumen through the mouth to the ventral sac of the rumen for collection of rumen content. The microbial community composition of samples collected via oesophageal tube with both fluid and solid particles is comparable with samples collected via rumen fistula (Paz *et al.*, 2016). Care was taken to ensure both fluid and solid particles were present in the samples. The first 50 ml of rumen content was discarded due to potential saliva contamination. A further 50 ml was collected in a sterilized 50 ml plastic container. Immediately after collection the pH was measured using a portable pH meter (EcoSense pH100A, YSI Environmental, USA) and the sample was frozen in liquid nitrogen and stored at -80º C until DNA extraction. Due to technical problems with the portable pH meter, the pH readings of the backgrounding phase had to be discarded.

After thawing, the rumen content samples (300 mg) were first homogenized using a BeadBug microtube homogenizer (Benchmark Scientific, USA) for approximately 12 mins at maximum speed (400 x 10 rpm) followed by DNA extraction using a QIAamp PowerFecal Pro DNA extraction kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. After extraction, DNA concentration and purity (A260/A280) were quantified using a Qubit Fluorometer v2 (Invitrogen, USA) and a Nanodrop ND-1000 Spectrophotometer (Thermo Scientific, USA), respectively.

Thirty-two DNA samples were shipped to Novogene (NovogeneAIT, Singapore) for pair-ended (250 x 250 bp) sequencing with an Illumina NovaSeq 250 (Illumina, USA). Sequencing targeted the V3-V4 hypervariable region of the 16S rRNA gene and the 5F region of ITS1. Data was received from Novogene Singapore with primers removed with an average number of reads per sample of 198 775 and 194 585 for 16S rRNA and ITS sequencing, respectively. Sequence data was deposited into the NCBI Sequence Read Archive under accession number PRJNA721531.

Microbiome analysis was performed with various packages in R software v4.0.2 (R Core Team, 2013). Processing and analysis of reads were performed using DADA2 v1.16.0 (Callahan *et al.*, 2016) including read filtering, dereplications, sample inference, chimera removal, merging of paired-end reads, and taxonomic classification. Reads were trimmed at 220 base pairs for both the forward and reverse reads of the 16S rRNA generated reads resulting in a total of 186 933 reads remaining after trimming. Taxonomy was assigned to genus level by using RDP database (Cole *et al.*, 2014) for 16S rRNA and UNITE database (Nilsson *et al.*, 2019) for ITS. The ape package v5.4.1 (Paradis & Schliep, 2019) was used to construct a phylogenetic tree. The amplicon sequence variant (ASV) table, the taxonomy table, phylogenetic tree and sample data were combined to construct a phyloseq object using phyloseq v1.32.0 (McMurdie & Holmes, 2013). The microbiome package v1.10.0 (Lahti *et al.*, 2017) was used to generate figures.

Low abundance ASVs (detected at least 10 times in 5% of the samples) were removed for downstream analysis. Reads were rarefied to minimum sampling depth for normalization. The remaining number of reads

are reported as average and standard deviation. Weighed and unweighted UniFrac distances were used to perform a principal coordinate analysis (PCoA) for ordination analysis to visualize differences between the phases. Beta diversity was determined using the Adonis, betadisper and permutest functions in vegan v2.5.6 (Oksanen *et al.*, 2020). The following linear model was used to test for beta diversity with the above mentioned functions: unifrac.dist ~ phase + animal + group. These same fixed effects (phase, animal, group) were tested for significant influence on alpha diversity and the relative abundance of the microbes with an analysis of variance. Animal and group were found to not have a significant effect and were not included in any further tests. Three alpha diversity indices were calculated using phyloseq: the observed number of ASVs, Chao1 richness estimator and Shannon diversity index. The Kruskal-Wallis and Wilcoxon rank sum tests were used to determine statistical significance for the relative abundance of the taxa as well as alpha diversity. Analysis was corrected for multiple testing with Bonferroni correction. For all statistical tests, results were considered significant at $p < 0.05$ and trends were recognized at $p < 0.1$.

3.3 Results

Following quality control, chimera detection and removal, the samples had an average read count of 116 943 ± 19 832 for the 16S rRNA and 149 447 ± 15 014 for the ITS sequencing. From the sequences, 42 008 and 35 442 amplicon sequence variants (ASVs) were detected for 16S rRNA and ITS sequencing, respectively. There was no difference in the rumen microbiome composition between the control and monensin groups (P >0.05) while a difference (P = 0.001) was observed between the four phases for both the 16S rRNA and ITS rumen populations.

In Table 3.2, alpha diversity is shown based on the observed number of ASVs, Shannon and Chao1 indices. The diversity within the samples were higher for the backgrounding phase with a consequent decrease up to the finisher phase for the bacterial and archaea population. The most ASVs for the fungi population was observed in the grower period with the least in the finisher phase. All alpha diversity indices were significantly different across the four phases with no significant difference between the groups or animals.

Table 3.2 The average and standard deviation of the alpha diversity indices (observed number of ASVs, Shannon diversity index and Chao1 richness index) of rumen bacterial, archaeal, and fungal communities across the various phases in the feedlot period.

Phase	Bacterial/Archaeal			Fungal		
	Observed	Shannon	Chao1	Observed	Shannon	Chao1
Backgrounding	1547±59^{a}	6.51±0.11^{a}	1554±58^{a}	214±37^{a}	3.76±0.33^{a}	215±37^{a}
Starter	1322±141^{b}	5.89±0.30^{b}	1331±142^{b}	271±28^{b}	3.58±0.27^{b}	273±27^{b}
Grower	923±125^{c}	4.15±0.39^{c}	935±126^{c}	301±29^{c}	3.93±0.51^{c}	302±29^{c}
Finisher	662±37^{d}	3.34±0.47^{d}	667±38^{d}	162±18^{d}	3.23±0.16^{d}	162±18^{d}

^{a,b,c,d} different superscripts within a column indicates significant difference at $p < 0.05$.

There was a difference in the beta diversity between the phases (p = 0.001, R^2 = 0.601) but not between the animals or groups (p = 0.395, R^2 = 0.301). The backgrounding and starter phases clustered separately (Figure 3.1), while the grower and the finisher phases formed a larger dispersed cluster.

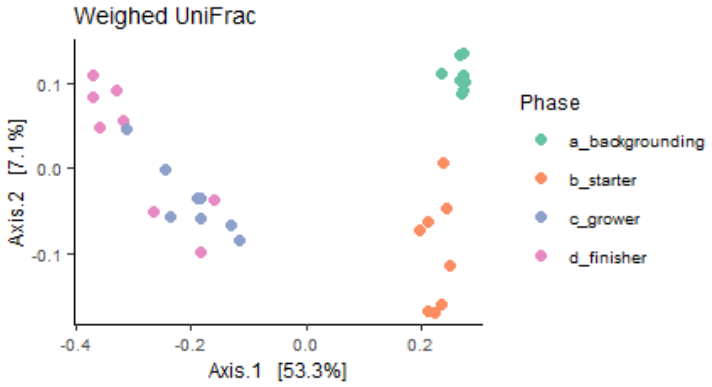

Figure 3.1 A principal coordinate analysis (PCoA) using weighted UniFrac distances to indicate beta diversity of the various phases within the feedlot period for the bacteria and archaea (backgrounding = green, starter = orange, grower = purple, finisher = pink).

In Figure 3.2, a PCoA using weighted UniFrac distance of the fungal population of the phases (depicted as different colours) can be observed to cluster separately. The groups (monensin and control) did not cluster separately within the phases.

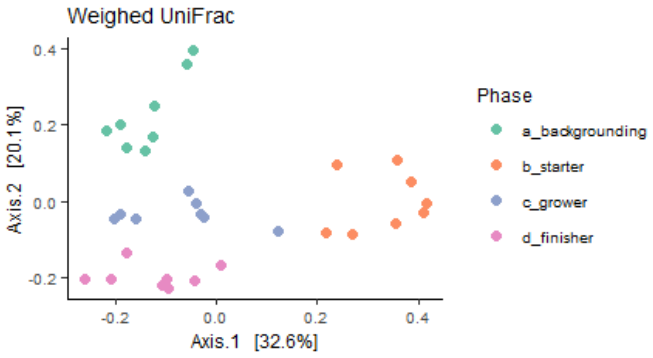

Figure 3.2 A PCoA plot using weighted UniFrac distances to depict the beta diversity between the various phases within the feedlot period for the rumen fungi (backgrounding = green, starter = orange, grower = purple, finisher = pink).

There was a difference in microbial composition between the phases (p = 0.001, R^2 = 0.568) but not between the animals or groups (p = 0.221, R^2 = 0.123) in the fungal population. There was also a difference in

the dispersion between the phases (p = 0.002). The significance test based on weighted UniFrac distances for the homogeneity of dispersions is reported in Table 3.3.

The results of the permutation test for homogeneity of multivariate dispersions are shown in table 3. There was a significant difference in the microbial composition between backgrounding and starter in the 16S rRNA and between starter and grower as shown in the ordination plot (Figure 3.1) and confirmed by the permutation test. As seen in Figure 3.2, the rumen fungi population of backgrounding clustered separately from the starter phase showing a significance difference between the communities of these two phases (Table 3.3).

Table 3.3 Weighted UniFrac distance-based test for homogeneity of multivariate dispersions for rumen microbial communities of the animals in the phases.

Phases	Bacterial/Archaeal	Fungal
Backgrounding – Starter	0.003*	0.001*
Starter – Grower	0.046*	0.946
Grower – Finisher	0.902	0.763

* indicates statistical significance at $p < 0.05$

Overall, the most abundant phylum in the rumen microbiome was Bacteroidetes (56%), followed by Firmicutes (29%) and Proteobacteria (5%) as shown in Figure 3.3 and Table 3.4. At phylum level, 2% of the microbes were not characterized. Of the ASVs identified in the rumen, 0.7% were archaea from the Euryarchaeota phylum.

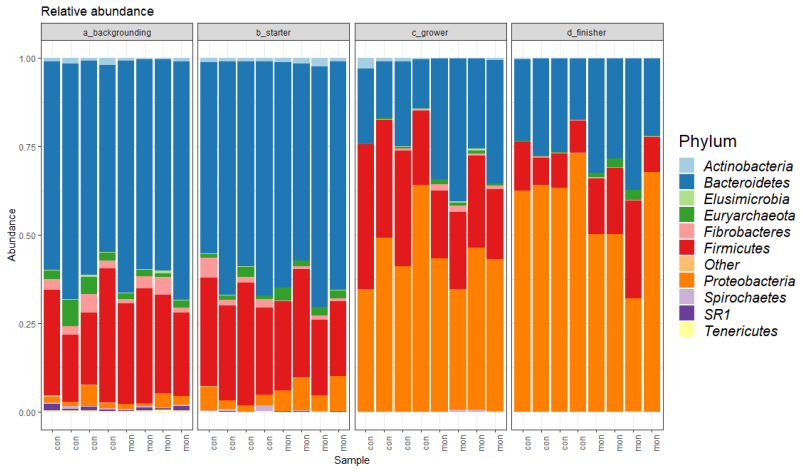

Figure 3.3 The relative abundance of the various bacterial phyla during the four phases of the feedlot period in the control (CON) and the monensin (MON) groups. The x-axis depicts the different rumen samples per phase and the y-axis the relative abundance. Each colour represents a specific phylum as indicated by the legends on the right side of each plot.

At family level, *Prevotellaceae* from the Bacteroidetes phylum as well as *Ruminococcaceae* from the Firmicutes phylum were the most abundant overall with *Prevotellaceae* more abundant in the starter phase while *Ruminococcaceae* was more abundant in the grower phase (Figure 3.4). Other families also present were *Porphyromonadaceae*, *Lachnospiraceae*, *Sphingobacteriaceae*, *Fibrobacteraceae* and *Methanobacteriaceae*.

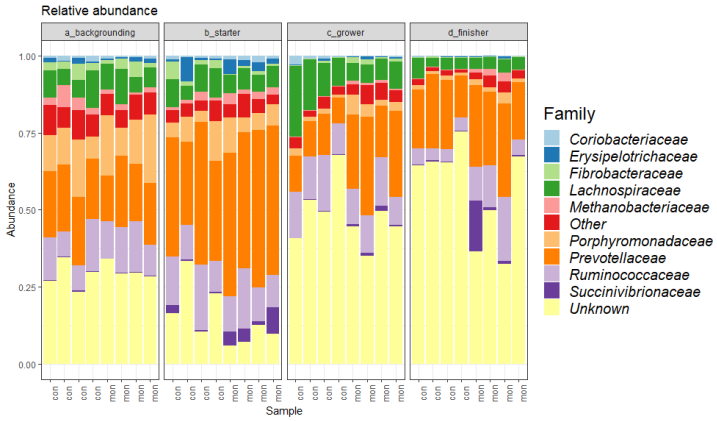

Figure 3.4 The relative abundance of the various bacterial families during the four phases of the feedlot period in the control (CON) and the monensin (MON) groups. The x-axis depicts the different rumen samples per phase and the y-axis the relative abundance. Each colour represents a specific phylum as indicated by the legends on the right side of each plot.

For the fungi population, the phyla Ascomycota, Neocallistigomycota and Basidiomycota were the most abundant (Figure 3.5, Table 3.4). Ascomycota was more abundant in the grower period whereas Neocallimastigomycota was more abundant in the finisher phase.

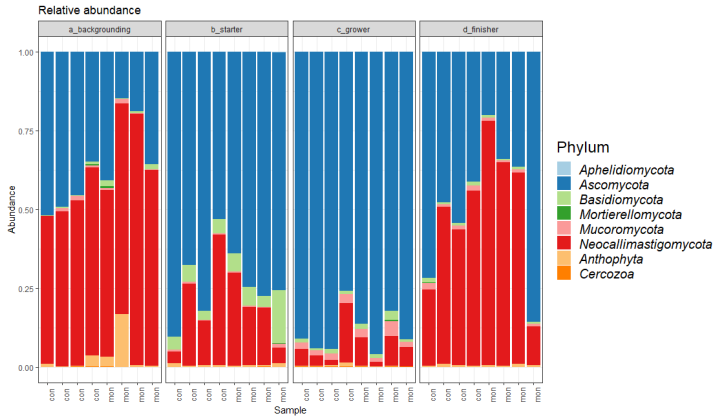

Figure 3.5 The relative abundance of the various fungal phyla in the feedlot phases of the control (CON) and monensin (MON) groups. The x-axis depicts the different rumen samples per phase and the y-axis the relative abundance. Each colour represents a specific phylum as indicated by the legends on the right side of each plot.

Table 3.4 The relative abundance of the bacterial, archaeal, and fungal phyla (in percentage) and the significant difference depicted as p-value between phase, backgrounding and starter (bvs), starter and grower (svg) and grower and finisher (gvf).

Phylum	Relative Abundance (%)				p-value			
16S rRNA (Bacterial & Archaeal)	Backgrounding	Starter	Grower	Finisher	phase	bvs*	svg[#]	gvf[†]
Euryarchaeota	2.9	2	0.6	0.9	*0.006*	0.721	*0.003*	0.959
Actinobacteria	2.2	1.4	0.8	0.2	*0.0004*	0.105	*0.028*	*0.010*
Bacteroidetes	62.4	62	26.2	26.6	*0.00002*	*0.015*	*0.0002*	0.721
Elusimicrobia	0.3	0.1	0	0.1	*0.0007*	*0.003*	0.052	*0.001*
Fibrobacteres	2.9	1.8	0.7	0.2	*0.0006*	0.161	0.156	0.128
Firmicutes	27.3	26.8	26.7	14	*0.007*	0.505	1	*0.003*
Proteobacteria	2.3	5.3	0.44	57.8	*0.00001*	*0.010*	*0.0002*	*0.049*
SR1	0.8	0	0	0	*0.000003*	*0.0009*	*0.0008*	0.076
Tenericutes	0.4	0.1	0	0	*0.000007*	*0.002*	*0.016*	*0.002*
ITS (Fungal)								
Ascomycota	37.2	74.5	89.4	49.8	*0.006*	*0.015*	0.721	*0.021*
Basidiomycota	0.6	5.7	1.3	0.9	*0.0002*	*0.0002*	*0.0002*	0.195
Mucoromycota	0.7	0.4	2.2	1.2	0.407	0.798	*0.0002*	*0.038*
Neocallimastigomycota	58.3	18.6	6.5	47.6	*0.00004*	*0.0002*	*0.010*	*0.0003*
Anthophyta	3.1	0.6	0.3	0.5	0.059	*0.013*	*0.015*	0.059

p-values in *italics* are significantly different ($p < 0.05$)

*backgrounding versus starter

[#]starter versus grower

[†]grower versus finisher

At the phylum level, 45% of the ASVs were non-characterized. *Neocallimastigaceae* from the Neocallimastigomycota phylum and *Aspergillaceae* from the Ascomycota phylum were the most abundant (Figure 3.6). The abundance of the phyla (in percentage) and significance of the various factors are depicted in Table 3.4. Most phyla did not differ ($p > 0.05$) between the groups or between the animals.

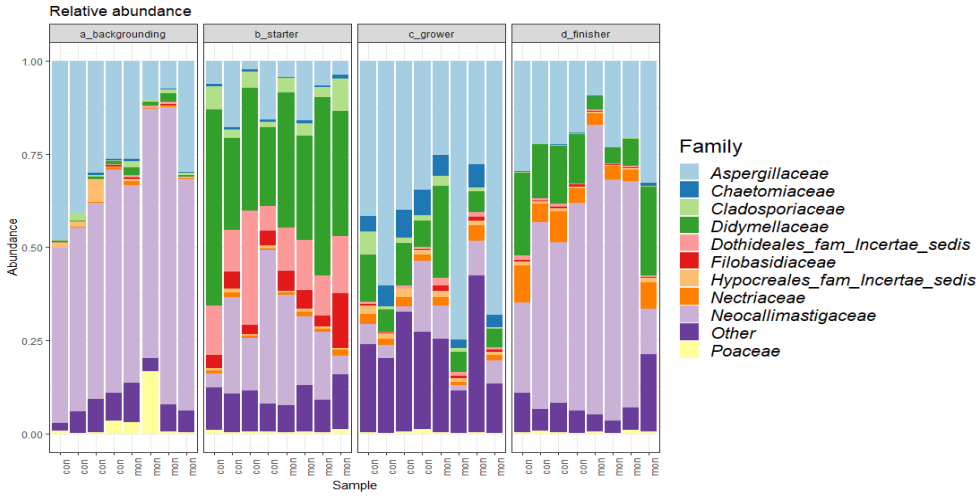

Figure 3.6 The relative abundance of the various fungal families in the feedlot phases of the control (CON) and monensin (MON) groups. The x-axis depicts the different rumen samples per phase and the y-axis the relative abundance. Each colour represents a specific phylum as indicated by the legends on the right side of each plot.

There was no difference ($p < 0.5$) in the pH measurement between the phases as well as between the control and monensin groups (Table 3.5).

Table 3.5 The average, standard deviation (SD) and range of the pH measurements of the monensin and control groups in the starter, grower and finisher phases.

Group	Starter		Grower		Finisher	
	Average ± SD	Range	Average ± SD	Range	Average ± SD	Range
Control	6.55 ± 0.37	6.10 - 7.03	6.48 ± 0.27	6.09 - 6.72	6.02 ± 0.20	5.77 - 6.27
Monensin	6.09 ± 0.27	5.76 - 6.43	6.29 ± 0.25	6.06 - 6.58	6.24 ± 0.48	5.52 - 6.56

3.4 Discussion

The microbial diversity within the rumen microbiome determines the amount of energy, in the form of volatile fatty acids and other end products, available to the animal for production (Guan *et al.*, 2008; Shabat *et al.*, 2016) with a higher propionate to acetate ratio resulting in more energy available to the animal (Wolin, 1960). In this study, the shift in the diversity and abundance of microbes in the

rumen through a growth trial was investigated in Bonsmara cattle using microbial sequencing of 16S rRNA and ITS regions.

The weighted UniFrac distances were able to differentiate bacterial and fungal communities between the phases depicting a significant difference. This was expected as the composition of the diets fed differed from backgrounding to finisher and diet is known to be one of the most influential factors on microbial composition (Belanche *et al.*, 2012; Gruninger *et al.*, 2019; Stanton *et al.*, 2020).

To further increase the production of animals and decrease the occurrence of digestive disorders, feed additives such as monensin are commonly used in feedlots in South Africa. Monensin is known to decrease the abundance of Gram-positive bacteria and methanogens by limiting the nutrient supply available, resulting in a decrease in the acetate to propionate ratio as well as methane emissions (Boadi *et al.*, 2004; Thomas *et al.*, 2017). This decrease could result in a more energy efficient process. Feed additives are known to have an effect on the rumen microbiome composition (Schären *et al.*, 2017). In this study no significant difference was found between the group receiving monensin and the control group for both bacterial, archaeal, and fungal populations. This finding warrants further investigation as it may hold positive outcomes for countries or situations where feed additives are not allowed in feedlot diets.

The most notable change in the rumen microbiome composition occurs during the transition from a forage-based to a concentrate-based diet as fermentation substrates switch from cellulolytic to amylolytic (Carberry *et al.*, 2012). This was also observed in this study where the bacterial population of the backgrounding phase differed significantly from the starter phase with alpha diversity indexes indicating a higher richness in the backgrounding phase. Roughage-based diets such as fed during backgrounding have a wider range of carbohydrate substrates such as cellulose and heteropolysaccharides that is fermented by microbes resulting in a more diverse rumen microbiome (Belanche *et al.*, 2012). These diets also have a less acidic rumen environment, which also play a role in rumen diversity as many microbes are sensitive to acidic conditions (Russell & Wilson, 1996). The pH measurements taken in this study (pH 6.0-7.0) indicate a less acidic environment for the starter phase.

The Bacteroidetes phylum was the most abundant during backgrounding as expected for animals on a roughage diet (Li *et al.*, 2012). *Prevotella* from the Bacteroidetes phylum was the most abundant genus in backgrounding compared to the starter phase. Several studies reported *Prevotella* as the most abundant bacterial genus in the rumen microbiome regardless of diet (Stevenson & Weimer, 2007; Jami & Mizrahi, 2012) as it is involved in the degradation of multiple substrates (Rosewarne *et al.*, 2014) and production of various acetate, succinate and propionate (Carberry *et al.*, 2012; Chen *et al.*, 2017). *Ruminococcus*, *Clostridium* and *Pseudobutyrivibrio* from the Firmicutes phylum are plant fibre degraders (Danielsson *et al.*, 2017) and expected to be abundant in the backgrounding phase. *Pseudobutyrivibrio* have been observed to have a significant effect on average daily feed intake (Paz *et al.*, 2018), as well as with average daily gain (Myer *et al.*, 2015a). Both *Methanobrevibacter* and

Pseudobutyrivibrio were more abundant in the backgrounding phase compared to the starter phase. These microbes have been shown to be correlated as *Pseudobutyrivibrio* was previously identified as a potential biomarker for methane emissions (Auffret *et al.*, 2018) and *Methanobrevibacter* is a methanogen (Tapio *et al.*, 2017). Their lower abundance in the starter phase can be due to the higher energy content of the diet as propionate is favoured above acetate production. This results in more energy being available to the animal for production (Jeyanathan *et al.*, 2019).

Fungi play a role in the degrading of fibrous materials in the rumen (Gruninger *et al.*, 2014) and is therefore observed to be more abundant in roughage-based diets, such as during backgrounding. However, in this study the number of observed ASVs showed a higher abundance of fungi in the starter phase. Further research is needed to elucidate this observation. Most studies (Gruninger *et al.*, 2014; Zhang *et al.*, 2017, 2020; Belanche *et al.*, 2019) report that Neocallimastigomycota is the most abundant fungal phylum in the rumen microbiome. In contrast to this, the Ascomycota phylum was found to be the most abundant fungus in the current study followed by Basidiomycota and Neocallimastigomycota phyla. Limited studies are available regarding Ascomycota or Basidiomycota as these phyla are aerobic fungi (Zhang *et al.*, 2020) and rarely found in animals (Zhang *et al.*, 2017). Zhang *et al.* (2017) reported an increase in their abundance as the proportion of concentrates increased in dairy cattle, which was also observed in this study. It is unclear how these aerobic microbes survived in the anaerobic environment of the rumen; however, they might play a role in the scavenging of oxygen entering the rumen and might have a beneficial effect on the anaerobic fermentation in the rumen (Zhang *et al.*, 2020).

Aspergillaceae family from the Ascomycota phylum have been used as feed additives in animal nutrition (Adegbeye *et al.*, 2020) as they decrease methane emissions by reducing the growth and activity of methanogenic bacteria (Wolin & Miller, 2006). The abundance of *Aspergillus* in the starter phase might therefore indicate a decrease in methane emissions. This is in line with the earlier observation regarding *Methanobrevibacter* and *Pseudobutyrivibrio*.

There is a beneficial symbiotic relationship between anaerobic fungi from the Neocallimastigomycota phylum and methanogens, such as *Methanobrevibacter* (Cheng *et al.*, 2009). Both *Methanobrevibacter* and the *Neocallistigaceae* family were more abundant in the backgrounding phase. Roughage-based diets have a higher methane production per unit of feed compared to diets high in concentrates (Beauchemin & McGinn, 2006).

In this study, the most prominent shift in the rumen microbiome composition was found between the starter and the grower phases. The increase in the proportion of carbohydrates in the diet, as from the starter to the grower phase in this study, is known to shift the rumen microbial composition from predominantly Firmicutes to Proteobacteria (Petri *et al.*, 2018). The proportion of carbohydrates in the diet has a significant effect on the rumen microbiome population (Raabis *et al.*, 2019) as an increase in easily digested carbohydrates results in more propionate producing bacteria, lower fibre-degrading organisms, lower protein breakdown and higher feed efficiency (Fernando *et al.*, 2010; Belanche *et al.*,

2012). Bacteroidetes was the most abundant phylum in the starter phase whereas Proteobacteria was more abundant in the grower phase. Microbes from the Proteobacteria phylum have diverse metabolic functions and indicate an increase in the number of bacteria that are metabolically capable of handling the easily fermentable carbohydrates (Fernando *et al.*, 2010). *Succinivibrio* was more abundant in the starter phase whereas *Vampirovibrio* and *Ruminobacter* were more abundant in the grower phase. A higher abundance of propionate-producing bacteria such as *Succinivibrio* (Suen *et al.*, 2011) may divert hydrogen away from methanogenesis, reducing enteric methane emissions, and increasing the energy available to the animal (De Menezes *et al.*, 2011) resulting in a more efficient animal.

Eubacterium as well as *Ruminococcus* showed significantly higher abundance in the grower phase. *Eubacterium* has been observed to be abundant in efficient steers and has a tolerance of low pH while *Ruminococcus* has been associated with residual feed intake (Hernandez-Sanabria *et al.*, 2012). As the grower diet had a higher proportion of easily fermentable carbohydrates, a lower pH can be expected in comparison to the diet fed during the starter phase. The pH measurements taken in this study did not show the expected decrease in pH from starter to grower. This might be explained by possible saliva contamination in the samples due to the method of rumen content collection or due to selective feeding by the cattle before collection. In highly efficient steers the acetate utilization characteristics of *Eubacterium* may interact with the acetate-producing capacity of *Succinivibrio* to utilize excessive hydrogen, which otherwise would be directed to methanogenesis (Chassard & Bernalier-Donadille, 2006).

Based on the alpha diversity, more fungi were present in the grower compared to the starter phase. This was unexpected, as fungi are known to decrease in abundance as the proportion of carbohydrates in the diet increase. A low pH, which would be found in high concentrate diets such as the grower diet, can inhibit the growth of anaerobic fungi (Han *et al.*, 2019) leading to a decrease in their abundance. *Cyllamyces* and *Orpinomyces* were the genera more abundant in the starter phase. These genera from the Neocallistigomycota phylum are known to be present in the rumen (Gruninger *et al.*, 2014; Zhang *et al.*, 2017) and degrades cellulose and xylose (Kittelmann *et al.*, 2012). Genera from Ascomycota (*Neoascochyta*, *Selenophoma* and *Cecomyces*) were found to be more abundant within the grower compared to the starter, however little literature could be found to elucidate their abundance within the starter. Belanche *et al.* (2019) did report that fungi detected could be ingested with the feed materials such as plant-pathogens, saprotrophs, yeast, and other species of unclassified fungi. The abundance of these genera might be due to external factors and their role within the rumen require further research to confirm their origin and functions.

There was no significant difference in the rumen microbe population between the grower and finisher groups for either the bacteria and archaea or the fungi population. This might be due to the fact that Proteobacteria had a high abundance in both the grower and finisher phases. Even though the high abundance of Proteobacteria could indicate possible dysbiosis in the rumen of the cattle (Auffret *et al.*, 2017), no physical effects of acidosis or metabolic disorders were observed in the animals during the

grower and finisher phases. Many pathogenic bacteria belong to the Proteobacteria phylum and these pathogens' abundance are sensitive to dietary change (Baümler & Sperandio, 2016). Metabolic diseases in cattle such as bloat or acidosis have been associated with an unbalanced rumen microbiome (Khafipour *et al.*, 2009) and are known to occur in finishing diets. High concentrate diets, such as the finisher diet in this study, increase production of lactate and are associated with acid tolerant microbes such as Proteobacteria (Fernando *et al.*, 2010). As the finisher period has the highest abundance of Proteobacteria, strategies could be formulated to decrease the abundance of pathogenic microbes while maintaining beneficial microbes.

The finisher phase had the lowest alpha diversity compared to the other phases. A lower alpha diversity has been associated with more efficient animals (Zhou *et al.*, 2009; Shabat *et al.*, 2016). It is therefore more desirable to have low diversity within the rumen microbial population to focus on promoting energy yield from the feed (Shabat *et al.*, 2016). However, a too low alpha diversity has been observed to be associated with an unbalanced and unhealthy rumen microbiome composition. It is therefore imperative to balance the microbiome composition to prevent dysbiosis.

The finisher phase exhibited the least abundance of fungi compared to the other phases based on the alpha diversity. A similar observation was reported by Kumar *et al.* (2015), but is in contrast to Zhang *et al.* (2017). This decrease in the richness and diversity of the fungi in the finisher might be due to the higher proportion of concentrates as fungi are mostly fibre degrading microbes. *Neocallismastix* from the Neocallistogomycota phylum was the most abundant in the finisher phase. This genus is able to utilize a wide range of substrates such as cellulose, xylose, glucose, starch, grass, and straw (Edwards *et al.*, 2017). However, its abundance has been reported to decrease with increasing concentrates (Han *et al.*, 2019), which is in contrast with this study. Further studies on the function and prevalence of fungi in the rumen microbiome is needed.

There are other factors that must also be mentioned when discussing the rumen microbiome. Although most of the differences discussed above can be explained by the influence of diet, the age of the animals (Jami *et al.*, 2013), host genetics can also influence the rumen microbiome. Studies have reported that animals fed the same diet can exhibit substantial differences in microbiome composition (Welkie *et al.*, 2010; Firkins & Yu, 2015) due to host genetics or the interaction between the host and the rumen microbiome (Hernandez-Sanabria *et al.*, 2013).

3.5 Conclusion

This is the first study to investigate the rumen microbiome of South African Bonsmara cattle under intensive feedlot conditions. Improvement of feed efficiency in feedlot cattle hold several advantages including cost, a decrease in the environmental impact and food safety. There was no significant difference in the overall rumen microbiome population between the monensin and the control groups, but differences within the phases require further investigation. This study allowed for

an improved understanding of microbial shift in the feedlot period. This could provide integrative information about rumen function and lead to improvements in ruminant production, such as increased digestion and feed efficiency.

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Authors' contributions

All authors participated in the planning of the project. DAL conducted the trial, performed the laboratory work as well as the bioinformatic and statistical analysis and wrote the original paper. EvMK supervised the project, wrote, and edited the paper. CJLdT assisted in the nutritional aspects of the project. DS assisted in the bioinformatic and statistical analysis. MMS revised the paper. All authors have read and agreed to the published version of the manuscript.

Conflict of interest declaration

The authors declare no conflict of interest.

3.6 References

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Chapter 4

A comparison of the effect of a probiotic and essential oils to an ionophore on the rumen microbiome composition of Bonsmara cattle raised under feedlot conditions using 16S rRNA and ITS amplicon sequencing

Abstract

The rising concern of antibiotic use in subtherapeutic practices in livestock and the potential development of antibiotic-resistant bacteria has necessitated the investigation into alternative feed additives. The effect of a probiotic (*Bacillus*) and essential oils (eugenol, cinnamaldehyde and capsicum) to an ionophore on the rumen microbiome composition of Bonsmara bulls raised under intensive feeding conditions was compared in this study. Forty-eight Bonsmara bull calves were allocated to four groups: the control group with basal diet (CON) and three groups with monensin (MON), probiotic (PRO) and essential oils (EO) included in the basal diet. The animals were finished over a period of 120 days under feedlot conditions following standard starter, grower, and finisher phases. Rumen content was collected from four animals per group within each phase via a stomach tube for 16S rRNA and internal transcribed spacer (ITS) sequencing as well as volatile fatty acid analysis. In the starter phase the MON group had a significantly lower acetate to propionate ratio compared to the other treatment groups. The amplicon sequence variants (ASVs) detected in total were 41 300 and 35 442 for 16S rRNA and ITS sequencing, respectively. A significant difference in the Chao1 richness index of the 16S rRNA population in the grower phase was observed, with the lowest number of ASVs being observed the probiotic group. In the finisher phase, the probiotic group had a significantly higher bacterial diversity (Shannon index). A higher abundance of Euryarcheaota and Fibrobacteres was observed in MON compared to CON. *Lachnospiraceae* was higher in abundance in the essential oils as well as the probiotic-supplemented groups in comparison with the monensin group. The alpha diversity did not differ between the fungal populations of the treatment groups. Little variation was observed between the rumen microbiome composition of monensin compared to the other treatment groups. Alternatives, such as probiotics or essential oils might therefore be considered to replace monensin.

Keywords: Archaea, bacteria, Bonsmara, feed additives, fungi

4.1 Introduction

The modification of the rumen microbiome composition through feed additives has a beneficial effect on the animal's production and health by reducing excess nitrogen (N) from protein degradation, controlling rumen pH, increasing fibre digestion and decreasing methane emissions (Jouany & Morgavi,

2007). Monensin is commonly used in feedlot diets as it alters ruminal fermentation and increases feed efficiency (Duffield *et al.*, 2012). However, due to the danger of the development of an antibiotic-resistant bacterium as well as the ban on the usage of antibiotics in subtherapeutic practices by the European Union (Markowiak & Śliżewska, 2018), alternatives need to be investigated that can replace the use of ionophores. Probiotics and essential oils (EOs) are being studied as viable options with contrasting effects on the production of animals (Benchaar *et al.*, 2006).

Probiotics are live microbes that are advantageous to the animal's health when supplemented in adequate doses (Markowiak & Śliżewska, 2018). *Bacillus* strains have been used as probiotics and beneficial effects include increased milk quality and growth (Sun *et al.*, 2013; Du *et al.*, 2018). The inclusion of *Bacillus* in the diet *in vitro* resulted in the growth of beneficial microorganisms including *Bifidobacterium* and *Lactobacillus* (Hosoi *et al.*, 2000). *Bacillus* bacteria produce a number of antimicrobial compounds that inhibit Gram-positive bacteria and pathogens, however some also display activity against Gram-negative bacteria (Khochamit *et al.*, 2015).

Essential oils (EO) favourably modify rumen fermentation by inhibiting methanogens, and other undesirable microbes resulting in decreased methane emissions and higher VFA production (Patra & Yu, 2012). The mode of action of EOs are similar to ionophores in that they interact with the cell membrane, targeting more permeable microorganisms and changing the VFA proportions (Calsamiglia *et al.*, 2007). The interaction with the cell membrane is influenced by fermentation conditions such as rumen pH and the fermentation substrate (Cardozo *et al.*, 2006). Various EOs can be used, however synergistic effects have been reported when fed in combinations or blends (Calsamiglia *et al.*, 2007). Due to EOs having a similar or superior effect on the animals' performance compared to monensin (Benchaar *et al.*, 2006; Meschiatti *et al.*, 2019), EOs might be an alternative to the use of monensin in feedlot diets.

Although there have been studies that showed that EOs can potentially replace monensin in feedlots, these results have been inconsistent and limited evidence has been reported on the potential of probiotics. This study compared the effect of a probiotic (*Bacillus*) and essential oils (capsicum, cinnamaldehyde and eugenol) to an ionophore on the rumen bacterial, archaeal, and fungal populations in South African Bonsmara bulls raised under intensive feedlot conditions.

4.2 Materials & methods

4.2.1 Animals and diet

Ethical approval was received from the University of Pretoria's Animal Ethical Committee (NAS445/2019). The trial was completed at the facilities of a commercial feedlot in Edenville, Free State, South Africa (-27.6096553, 27.7221717). Forty-eight Bonsmara calves (228 ± 22 kg; 10-14 months old) were sourced from the same farm. Natural grazing was used to background the animals for 40 days where after they were randomly allocated to four groups: basal diet (CON), the basal diet

supplemented with either monensin (MON, 0.3 g/animal/day), probiotic (PRO, 2.75 g/animal/day) or essential oils (EO, 1 g/animal/day). The probiotic consisted out of two strains, *Bacillus subtilis* and *Bacillus licheniformis* (3.20 x 10^9 CFU/g), while the essential oils consisted of eugenol, capsicum and cinnamaldehyde (17%:7%:11%).

The animals were blocked by weight and allocated three to a pen, resulting in twelve animals per group. The animals were fed starter, grower, and finisher diets for 21, 80, and 14 days, respectively. The feed for each phase was mixed at the feed mill on farm, bagged, and marked for the trial. The composition of the diets for each phase was reported in Linde *et al.* (2022) (Chapter 3). The animals were processed and received an ear implant (Revalor® S, Intervet GesmbH, Austria) as per standard feedlot procedures. Adaptation of the animals to the starter diet was managed by decreasing the amount of hay supplied while increasing the volume of the starter diet over five days. During the grower and the finisher phases, adaptation of the animals to the new diet occurred over three days by increasing the proportion till fed only the new diet. Water and feed were supplied *ad libitum* to the animals. Feed intake per pen was calculated by subtracting the refusals of the day from the amount of feed provided the previous day. The calves were weighed once a week.

4.2.2 Rumen content collection

Rumen content was collected a week before the start of a new phase (Backgrounding: Day -16; Starter: Day 7; Grower: Day 90) and slaughter (Finisher: Day 112). From each group, four animals were selected (one per pen) at the start of the trial for collection of rumen content within each phase (total number of samples = 64). Rumen content was collected approximately two hours after morning feeding. A flexible stomach tube was inserted through the animal's mouth into the ventral sac of the rumen by a trained veterinarian. Samples of rumen microbiome composition collected via stomach tube have been reported to be similar to those collected via cannula if both particles and fluid are obtained (Paz *et al.*, 2016). Negative pressure was applied via a dosing gun to draw out rumen content (particles and fluid). To safeguard against saliva contamination, the first 50 ml was removed, the next 50 ml was instantly frozen in liquid nitrogen and placed in a -80 ºC fridge until DNA extraction could be performed.

4.2.3 Volatile fatty acid analysis

Rumen fluid from the frozen samples (Prates *et al.*, 2010) were submitted to the Department of Animal Science laboratory (University of Pretoria) for VFA analysis. For preservation of the samples, orthophosphoric acid (25% H_3PO_4) was added and the samples were stored in a -20 ºC freezer. Volatile fatty acid concentration was analysed through gas chromatography (SCION GC-456, SCION Instruments, Scotland) according to FAO (2011) with modifications. The gas chromatograph was equipped with a flame ionization detector, an auto-sampler and CP-WAX 58 (FFAP) CB column with a length of 25 m and a 0.53 mm internal diameter with a 2.0 µm acid-modified chemically bonded polyethylene glycol-film thickness. The oven temperature (100 °C) was sustained for 2 min, then

increased to 150°C where it was once again sustained for 2 min and increased to 195°C. The molar proportions of the VFAs were compared between groups (Terré *et al.*, 2013).

4.2.4 DNA extraction and sequencing

Thawed rumen content samples (300 mg) were homogenized for twelve minutes at maximum speed (400 x 10 rpm) with a BeadBug homogenizer (Benchmark Scientific, USA). DNA extraction was completed using the repeated bead beating plus column method (Yu & Morrison, 2004) with a QIAamp PowerFecalPro extraction kit (Qiagen, Germany) following manufacturer's guidelines. A Qubit Fluorometer (Invitrogen, USA) and a Nanodrop ND-1000 Spectrophotometer (Thermo Scientific, USA) were used to determine sample quality. One sample from the probiotic group, collected during the backgrounding phase, did not have sufficient quality, and was discarded. Extracted DNA was sent to Novogene (NovogeneAIT, Singapore) for 16S rRNA (V3-V4) with 341F (5'-CCTAYGGGRBGCASCAG) and 806R (5'-GGACTACNNGGGTATCTAAT) as primers and ITS1 sequencing (F-5'GGAAGTAAAAGTCGTAACAAGG and R-5'GCTGCGTTCTTCATCGATGC) using an Illumina NovaSeq 250 (Illumina, USA) to generate 250 bp pair-ended raw reads. Average reads per samples generated were 200 126 ± 11 204 for 16S rRNA sequencing and 196 787 ± 16 115 for ITS sequencing. Primers were removed in the data received from Novogene Singapore. Data was deposited in the NCBI Sequence Read Archive under accession number PRJNA721531.

4.2.5 Statistical analyses

Both the forward and reverse reads were cut at 220 base pairs using DADA2 (Callahan *et al.*, 2016) to enhance the quality of the samples. The Ribosomal Database Project (Cole *et al.*, 2014) and the UNITE database (Nilsson *et al.*, 2019) was used for 16S rRNA and ITS annotation, respectively. Taxonomy was assigned to family level. The data was rarefied, and amplicon sequence variants (ASVs) detected in 5% of the samples less than 10 times were discarded. The alpha diversity of the samples (observed number of ASVs, Shannon diversity and Chao1 richness indices) was determined using phyloseq (McMurdie & Holmes, 2013). The Shannon index determines the richness and evenness found within the samples while the Chao1 index estimates the expected amount of ASVs in the community (Kim *et al.*, 2017). Beta diversity was determined with PERMANOVA in vegan (Oksanen *et al.*, 2020) as well as with a principal coordinate analysis (PCoA) depicting weighted UniFrac distances. The Proteobacteria ratio, as an indicator for dysbiosis, was calculated by dividing the Proteobacteria abundance with the combined abundance of Bacteroidetes and Firmicutes (Auffret *et al.*, 2017). Dysbiosis is indicated by values equivalent or above 0.19. Significant differences were determined by the Kruskal-Wallis and Dunn tests as well as ANOVA between alpha diversity, relative abundance of the microbes and the performance traits using statistical packages in R (R Core Team, 2013). The Holm-Bonferroni procedure was performed for multiple test correction. Significant differences were recognized at $p < 0.05$ and trends were acknowledged at $0.05 < p < 0.1$.

4.3 Results

The treatment groups did not differ significantly in live weight (LW), feed conversion ratio (FCR), or average daily gain (ADG) except for daily feed intake as is (DFI) (Table 4.1).

Table 4.1 The average and standard deviation of the live weight (LW), average daily gain (ADG), daily feed intake (DFI), and feed conversion ratio (FCR) for the four treatment groups.

Performance traits	CON	MON	PRO	EO	p-value
LW (kg)	468 ± 26.44	471 ± 27.30	455 ± 34.95	460 ± 30.42	0.497
ADG (kg/day)	1.81 ± 0.11	1.85 ± 0.08	1.70 ± 0.71	1.68 ± 0.16	0.603
DFI (kg/day)	11.3 ± 0.38	11.6 ± 0.71	10.5 ± 0.43	11.6 ± 0.47	0.037*
FCR	6.25 ± 0.52	6.25 ± 0.46	6.24 ± 0.66	6.91 ± 0.46	0.255

* Significance at $p < 0.05$

The VFA concentrations did differ significantly across the phases ($p < 0.05$) (Table 4.2).

Table 4.2 The total volatile fatty acid (tVFA; mmol/L), acetate, propionate, and butyrate (mol/100 mol) concentrations and the acetate to propionate ratio (A:P) of the control (CON), monensin (MON), probiotic (PRO) and essential oils (EO) groups within the three phases.

	CON	MON	PRO	EO	p-value
Starter					
tVFA	70.07 ± 23.17	84.85 ± 18.87	73.03 ± 18.03	81.65 ± 13.27	0.589
Acetate	66.33 ± 2.42^{a}	57.99 ± 1.54^{b}	65.26 ± 2.58^{a}	66.97 ± 2.94^{a}	0.033*
Propionate	16.17 ± 4.86^{b}	28.87 ± 3.38^{a}	17.67 ± 2.44^{b}	16.96 ± 2.72^{b}	0.033*
Butyrate	12.70 ± 2.38	9.91 ± 1.70	12.73 ± 1.58	12.44 ± 1.96	0.277
A:P	4.52 ± 1.44^{a}	2.04 ± 0.28^{b}	3.77 ± 0.61^{a}	4.08 ± 0.81^{a}	0.034*
Grower					
tVFA	105.19 ± 26.02	110.48 ± 13.57	100.01 ± 15.74	91.20 ± 15.15	0.657
Acetate	58.63 ± 0.81	58.46 ± 2.57	57.75 ± 2.41	59.56 ± 2.43	0.724
Propionate	28.19 ± 2.30	26.84 ± 2.57	28.94 ± 3.12	22.43 ± 5.36	0.235
Butyrate	8.19 ± 1.32	9.73 ± 0.29	9.41 ± 0.31	13.16 ± 5.13	0.134
A:P	2.09 ± 0.17	2.21 ± 0.29	2.03 ± 0.31	2.84 ± 0.79	0.474
Finisher					
tVFA	110.59 ± 13.10	94.41 ± 11.23	115.10 ± 18.52	95.03 ± 18.72	0.231
Acetate	56.16 ± 1.07	59.65 ± 3.26	56.23 ± 1.53	55.96 ± 1.56	0.382
Propionate	29.59 ± 1.89	22.52 ± 6.37	29.51 ± 2.11	29.62 ± 1.91	0.531
Butyrate	8.95 ± 1.33	10.96 ± 2.29	9.29 ± 0.99	9.29 ± 1.43	0.562
A:P	1.91 ± 0.15	2.89 ± 0.86	1.92 ± 0.18	1.90 ± 0.16	0.171

* Significance at $p < 0.05$

^{a,b} superscripts indicate significant difference within rows at $p < 0.05$

The VFA concentration of the groups within the backgrounding period, before the addition of the feed additives, did not differ. The acetate and propionate concentrations in the starter phase did differ significantly between the various treatment groups with MON differing from CON, EO and PRO.

4.3.1 Rumen microbial composition

An average of 116 127 ± 19 264 and 150 668 ± 13 495 reads remained following quality control and chimera removal for the 16S rRNA and ITS sequencing, respectively. From the reads, 41 300 bacterial and archaeal and 35 442 fungal ASVs were identified. Samples taken during backgrounding, before the feed additives were added to the diets, indicated no significant differences in terms of alpha and beta diversity of the bacterial population. Bacteroidetes was the most abundant phylum during the backgrounding period in the rumen, followed by Firmicutes. *Prevotellaceae* was the most predominant family followed by *Ruminococcaceae* and *Porphyromonadaceae*.

Within the starter phase, the bacterial alpha diversity indices did not differ between the feed additive groups (Table 4.3).

Table 4.3 The alpha diversity indices average and standard deviation (observed number of ASVs, Chao1 and Shannon indices) of the bacterial and archaeal population of the control (CON), monensin (MON), essential oils (EO) and probiotic (PRO) treatment groups within the various phases of the feedlot period.

Alpha diversity indices	CON	MON	EO	PRO	p-value
Starter					
Observed number of ASVs	1398 ± 48	1213 ± 56	1277 ± 70	1186 ± 105	0.461
Chao1 Index	1402 ± 49	1222 ± 55	1281 ± 70	1196 ± 106	0.492
Shannon Index	6.03 ± 0.15	5.74 ± 0.09	5.88 ± 0.21	5.65 ± 0.21	0.576
Grower					
Observed number of ASVs	805 ± 53^{ab}	969 ± 37^{a}	808 ± 47^{ab}	701 ± 33^{b}	0.046*
Chao1 Index	816 ± 54^{ab}	980 ± 39^{a}	819 ± 48^{ab}	708 ± 32^{b}	0.046*
Shannon Index	3.92 ± 0.20	4.33 ± 0.12	4.39 ± 0.13	3.95 ± 0.19	0.306
Finisher					
Observed number of ASVs	626 ± 12^{a}	608 ± 17^{a}	641 ± 66^{ab}	737 ± 20^{b}	0.100
Chao1 Index	629 ± 13^{a}	612 ± 17^{a}	649 ± 66^{ab}	742 ± 20^{b}	0.108
Shannon Index	2.98 ± 0.13^{a}	3.63 ± 0.21^{ab}	3.43 ± 0.24^{ab}	4.13 ± 0.11^{b}	0.044*

* Significance at $p < 0.05$

^{a,b} superscripts indicate significant difference within rows at $p < 0.05$

The observed number of ASVs and the Chao1 richness index of the bacterial/archaeal population were significantly different between MON and PRO in the grower phase. The bacterial/archaeal diversity (Shannon index) between the treatment groups did differ significantly in the finisher phase with a higher diversity within PRO in comparison with CON. The PRO group also had a significantly

higher richness (Chao1 index and observed number of ASVs) compared to MON and CON in the finisher phase.

In the fungal population of the animals in the starter and finisher phases, the alpha diversity indices did not differ between the feed additive groups (Table 4.4). In the grower phase, there was a tendency to differ in the richness of the fungal population between CON and PRO.

Table 4.4 The average and standard deviation of the alpha diversity indices (observed number of ASVs, Chao1 index, Shannon index) of the fungal population in the control (CON), monensin (MON), essential oils (EO) and probiotic (PRO) treatment groups within the various phases.

Alpha diversity indices	CON	MON	EO	PRO	p-value
Starter					
Observed number of ASVs	263 ± 12	275 ± 15	275 ± 10	284 ± 12	0.814
Chao1 Index	264 ± 12	276 ± 15	276 ± 10	285 ± 12	0.782
Shannon Index	3.52 ± 0.17	3.65 ± 0.07	3.69 ± 0.18	3.86 ± 0.12	0.405
Grower					
Observed number of ASVs	304 ± 12^{a}	287 ± 14^{ab}	293 ± 10^{ab}	249 ± 5^{b}	0.055**
Chao1 Index	304 ± 12^{a}	287 ± 13^{ab}	294 ± 10^{ab}	250 ± 5^{b}	0.063**
Shannon Index	4.10 ± 0.15	3.77 ± 0.31	4.27 ± 0.02	4.17 ± 0.02	0.362
Finisher					
Observed number of ASVs	175 ± 4^{a}	151 ± 8^{ab}	149 ± 5^{b}	153 ± 10^{ab}	0.186
Chao1 Index	175 ± 4^{a}	152 ± 9^{ab}	149 ± 5^{b}	153 ± 10^{ab}	0.183
Shannon Index	3.30 ± 0.05	3.17 ± 0.09	3.21 ± 0.06	3.15 ± 0.08	0.618

** Tendency towards significance at $p < 0.10$

^{a,b} superscripts indicate significant difference within rows at $p < 0.05$

The principal coordinate analysis (PCoA) (Figure 4.1) showed that MON did cluster apart from the rest of the treatment groups in both the starter and grower phases indicating different bacterial compositions. The bacterial composition between the feed additive groups did differ significantly in the starter ($p = 0.001$) and grower phases ($p = 0.022$) as indicated by beta diversity analysis. In the finisher phase, the samples were widely spread with no groups clustering separately. Although PERMANOVA showed significant differences in terms of the bacterial beta diversity ($p = 0.006$), there was only a tendency to differ between CON and MON ($p = 0.087$) in the finisher phase. The two axes of the PCoA explained 34.3 %, 34.7% and 50.5% of the variance in the bacterial/archaeal composition of the starter, grower, and finisher phases, respectively.

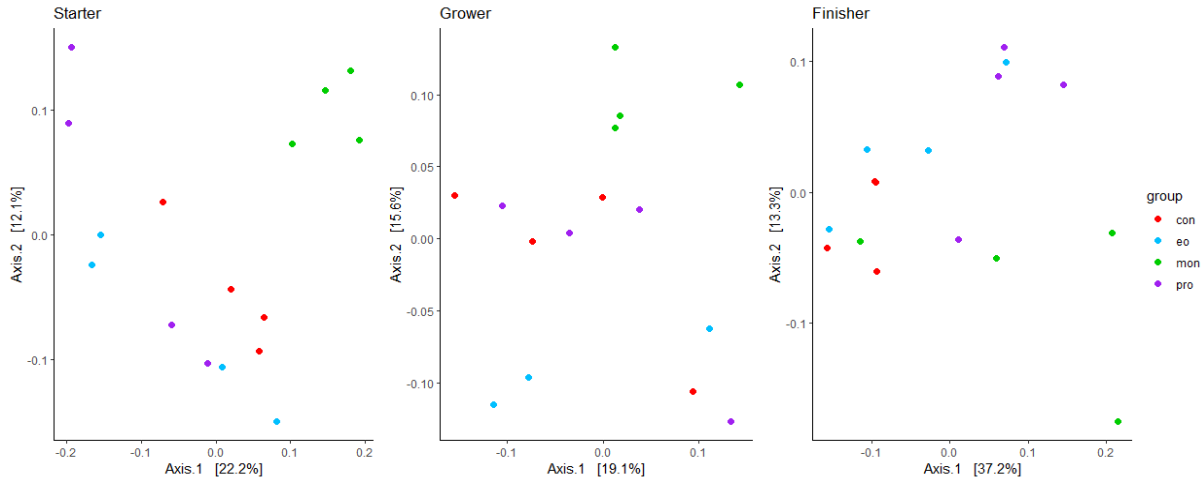

Figure 4.1 A principal coordinate analysis (PCoA) based on weighted UniFrac distances of the treatment groups in the starter, grower, and finisher phases for the 16S rRNA microbial population. Red depicts the control (CON), blue depicts the essential oils (EO), green the monensin (MON) and purple the probiotic group (PRO).

There was no clustering of the treatment groups in terms of the fungal composition within the PCoA (Figure 4.2) for the starter and grower phases. No difference was determined by PERMANOVA in the fungal composition between the treatment groups in terms of beta diversity in the starter phase ($p = 0.125$) however, a tendency to differ was found in the grower phase ($p = 0.084$). In the finisher phase, the EO group clustered separate from the other treatment groups in the PCoA plot with the beta diversity analysis showing a significant difference in the fungal composition ($p = 0.002$). The two axes in the PCoA explained approximately 41.4%, 63.8% and 61.0% of the microbial composition variation of the treatment groups in the starter, grower, and finisher phases, respectively.

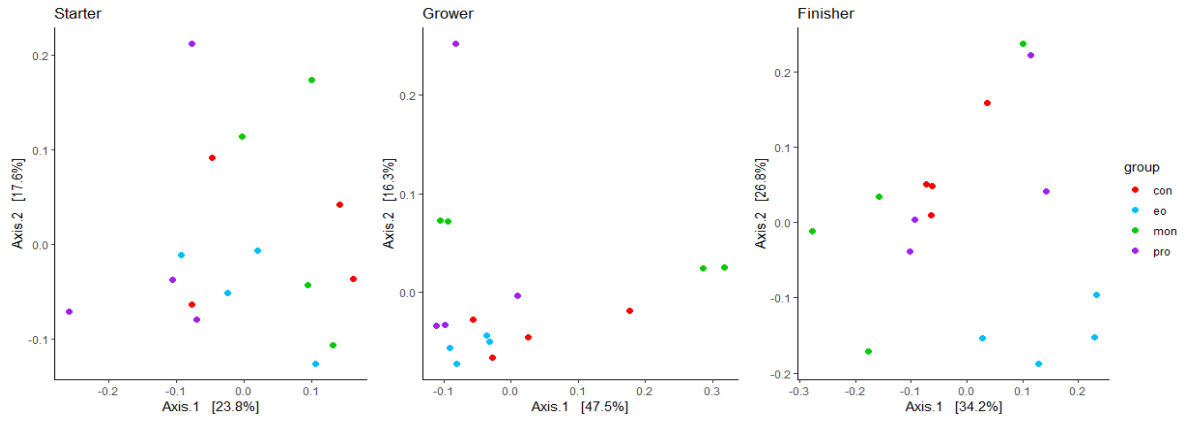

Figure 4.2 A PCoA plot based on weighted UniFrac distances of the fungal composition of the treatment groups in the starter, grower, and finisher phases. The control group (CON) is depicted in red, essential oils (EO) in blue, monensin (MON) in green and the probiotic (PRO) group in purple.

Within the starter phase across the treatment groups, the predominant phyla were Bacteroidetes and Firmicutes (Figure 4.3). The compositional relative abundance and the p-values for the bacterial and archaeal phyla and families were reported in Supplementary Table 4.1.

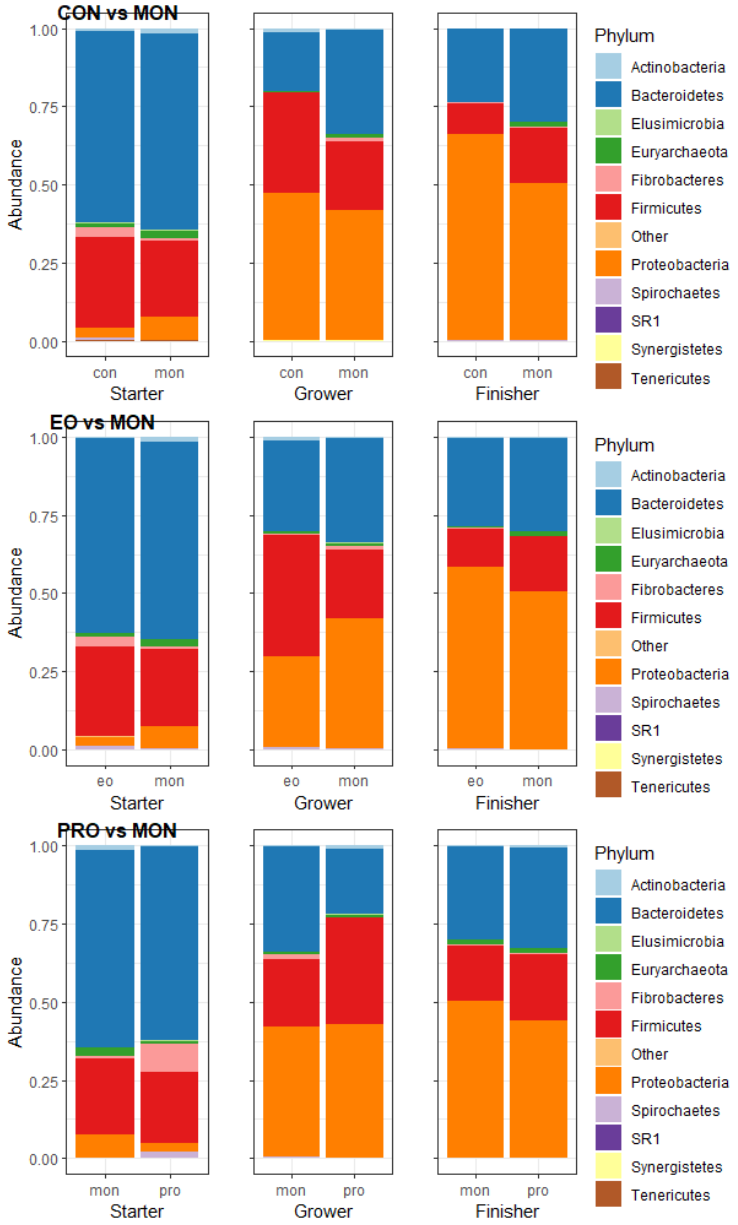

Figure 4.3 The average relative abundance of the bacterial/archaeal phyla compared between monensin (MON) and control (CON), MON and essential oils (EO) and MON and probiotic (PRO). The x-axis depicts the different samples averaged per treatment group and phase while the y-axis the compositional relative abundance. Each colour represents a specific phylum as indicated by the legend on the right side of the plot.

Fibrobacteres differed significantly ($p = 0.01$) across the treatment groups in the starter phase with a lower abundance in MON compared to CON. Within the grower phase, Bacteroidetes was

significantly (p = 0.045) higher and there was a tendency towards a higher abundance of Euryarchaeota (p = 0.069) in MON compared to CON. *Prevotellaceae* and *Succinivibrionaceae* were more abundant within MON compared to CON throughout the whole period whereas *Veillonellaceae* was more abundant within the finisher phase.

Between EO and MON in the starter phase, Actinobacteria had a higher abundance in MON (p = 0.058). A higher abundance of Firmicutes (p = 0.045) in MON in comparison with EO was observed in the grower phase. *Succinivibrionaceae* and *Veillonellaceae* were lower in abundance while *Lachnospiraceae* was higher in abundance in EO compared to MON. There was no difference between MON and EO in the finisher phase.

A difference in the abundance of Actinobacteria (p = 0.045) and Fibrobacteres (p = 0.005) were observed between MON and PRO within the starter phase with a higher and lower abundance in MON compared to PRO, respectively. Spirochaetes had a tendency towards a difference (p = 0.097) with a higher abundance in PRO compared to MON in the starter phase. *Veillonellaceae* and *Succinivibrionaceae* were higher in abundance within MON compared to PRO in the starter phase. Within the grower phase, Fibrobacteres had a significantly higher (p = 0.036) abundance in MON compared to PRO. The families *Lachnospiraceae*, *Clostridiales_XI*, *Clostridiales_XIII* and *Elusimicrobiaceae* were more abundant within PRO compared to CON in the finisher phase.

During the finisher phase, a higher abundance of Proteobacteria was observed in CON while MON and PRO had a lower abundance (p-value = 0.058). All treatment groups had a Proteobacteria ratio above 0.19, indicating dysbiosis. The Proteobacteria ratio of PRO (0.84 ± 0.14) was significantly lower compared to CON (2.06 ± 0.49) and numerically lower compared to MON (1.21 ± 0.62) and EO (1.59 ± 0.59) in the finisher phase.

Ascomycota was the fungal phylum with the highest abundance across the treatment groups (Figure 4.4) followed by Neocallimastigomycota. The compositional abundance of the fungal phyla and families can be found in Supplementary Table 4.2. In the grower phase, CON had a tendency towards a lower abundance of Basidiomycota (p = 0.056) compared to MON. The abundance of individual phyla did not differ between MON and EO in any of the phases.

A tendency towards a difference was indicated in the Neocallimastigomycota phylum (p = 0.084) between the treatment groups in the starter phase, with the highest abundance in PRO. Ascomycota had a significantly (p = 0.029) lower abundance in PRO compared to MON in the grower phase, while a tendency towards a difference in the abundance of Ascomycota (p = 0.056) and Basidiomycota (p = 0.092) between the treatment groups were observed in the finisher phase.

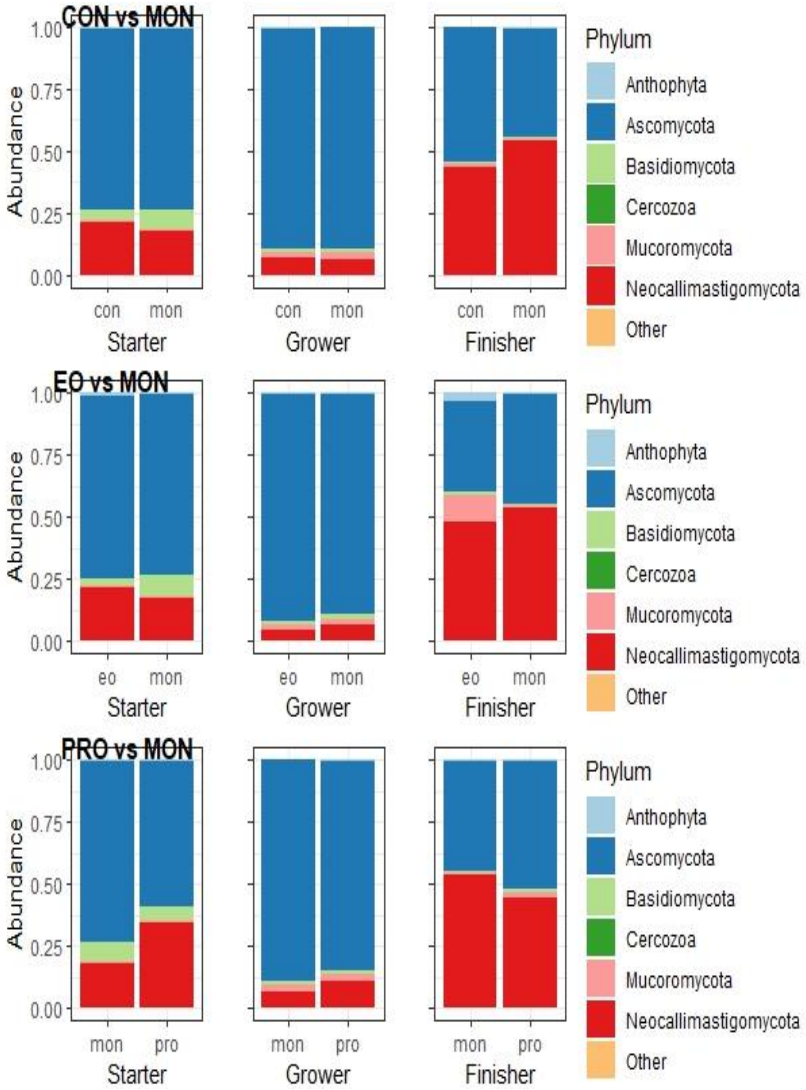

Figure 4.4 The averaged relative abundance of the fungal phyla compared between monensin (MON) and control (CON), MON and essential oils (EO) and MON and probiotic (PRO). The x-axis depicts the different samples averaged per treatment group and separated by phase while the y-axis the relative abundance. Each colour represents a specific phylum as indicated by the legend on the right side of the plot.

4.4 Discussion

Studies on natural feed additive alternatives have been inconsistent with varying results on the production of the animals; an increase, a decrease or no effect (Benchaar *et al.*, 2006). In this study, the emphasis was on the microbiome composition (Wu *et al.*, 2021) and a significant difference in production was not expected due to the small sample size. The MON group, however, had a numerically higher LW and ADG compared to the other feed additive groups. In a meta-analysis, monensin decreased feed intake by 3% and increased the feed efficiency by 1% (Duffield *et al.*, 2012). The use of monensin is a common practise in South African feedlots as it increases the production of the animals

and prevents conditions such as ruminal acidosis. Other studies have also reported no significant difference in production between MON and EO (Benchaar *et al.*, 2006; Tomkins *et al.*, 2015). In the finisher phase, EO had a numerically lower acetate to propionate ratio compared to MON. Inclusion of EOs, such as eugenol (Cardozo *et al.*, 2006), in a diet fed more than 91 days resulted in a reduction in acetate concentration (Torres *et al.*, 2020). The feed intake of PRO was lower compared to the other treatment groups which is in contrast to previous studies performed with probiotics, such as *Enterococcus faecium* and *Bacillus*, where DMI was increased (Nocek *et al.*, 2003; Du *et al.*, 2018).

This study compared the effect of essential oils and a probiotic to the effect of an ionophore on the rumen microbiome in Bonsmara cattle under intensive feeding conditions. The bacterial and archaeal microbial composition did exhibit significant differences between the treatment groups within the different phases based on beta diversity. For the fungal population, the treatment groups tended to differ in the grower phase and differed significantly in the finisher phase. Different growth rates of the various rumen microbes have been reported (Belanche *et al.*, 2012) with anaerobic fungi having a slower growth rate compared to rumen bacteria (Theodorou *et al.*, 1996). Fungal organisms might take longer to adapt or to respond to feed additives which may explain why there was no difference in the starter phase and a significant difference in the finisher phase of the fungal microbial community between the treatment groups.

Monensin vs control

Although, numerically, CON had a higher richness and diversity compared to MON, the alpha diversity did not differ significantly. Weimer *et al.* (2008) and Schären *et al.* (2016) reported that monensin supplementation decreased bacterial diversity in the rumen which has been linked with higher efficiency (Shabat *et al.*, 2016).

Succinate-producing microbes, such as *Succinivibrionaceae* and *Veillonellaceae*, were significantly higher in abundance within MON, both are associated with higher weight gain (Myer *et al.*, 2015). The supplementation of monensin is known to impact the fermentation characteristics by decreasing the acetate and butyrate concentration while increasing the propionate concentration (Duffield *et al.*, 2012) resulting in more energy being accessible to the animal. In this study, the MON group had the lowest acetate to propionate ratio within the starter phase in comparison with the other groups. However, in the finisher phase, MON had the highest acetate to propionate ratio. Over the past forty years research has indicated a decrease in the efficacy of monensin on feed efficiency which can be partially explained by an increase in dietary energy in feedlots (Duffield *et al.*, 2012) or the adaptation of the rumen microorganisms to monensin (Lima *et al.*, 2009). The reduction in the acetate to propionate ratio when using monensin (Weimer *et al.*, 2008) is due to the decrease in the Gram-positive microbes which are primarily acetate producers and the likely growth of Gram-negative bacteria, such as succinate producer *Fibrobacter succinogenes*, and *Selenomonas ruminantium* which converts succinate to propionate.

The abundance of Fibrobacteres was significantly decreased in MON in the starter phase. Fibrobacteres are Gram-negative, obligate anaerobes that are cellulolytic colonisers that produce succinate and acetate (Ransom-Jones *et al.*, 2012). It is therefore unexpected that *Fibrobacter* would have a lower abundance within MON compared to CON. Monensin is known to affect Gram-positive bacteria more compared to Gram-negative bacteria (Weimer *et al.*, 2008). However, recently, a study has observed that monensin can inhibit Gram-negative bacteria as well (Ogunade *et al.*, 2018), as observed in this study. Other factors, besides the outer membrane and its presence or absence, determines the vulnerability of bacteria to monensin (Weimer *et al.*, 2008). The abundance of Fibrobacteres increased to be significantly higher in MON within the grower period while no significant difference in abundance between MON and CON in the finisher phase was observed. This may indicate an interaction between the roughage to concentrate ratio, monensin, and Fibrobacteres.

A higher abundance of Euryarchaeota in MON compared to CON was observed throughout the feeding period with a significant difference in the grower phase. The phylum Euryarchaeota consists mainly of methanogenic archaea such as *Methanomassilicoccaceae* and *Methanobacteriaceae* which were observed to be abundant in the MON group. Monensin has been reported to decrease methane emissions by inhibiting bacteria that produce hydrogen, resulting in a decrease in the substrates needed for methanogenesis (Busquet *et al.*, 2005), instead of affecting methanogen abundance (Schären *et al.*, 2016).

Within the grower phase, the Basidiomycota abundance was significantly higher in MON compared to CON. The role of aerobic fungi, such as Basidiomycota, in the rumen is unclear, however they scavenge for free oxygen within the rumen to ensure an anaerobic environment with Ascomycota (Zhang *et al.*, 2020). Monensin has been indicated to inhibit anaerobic fungi in the rumen of sheep (Elliott *et al.*, 1987) as a consequence the abundance of aerobic fungi might increase. Basidiomycota had a higher abundance within MON in the grower phase, with a higher abundance in CON in the finisher phase. This could be attributed to the interaction between the microbes, the feed additive and the roughage to concentrate component of the diet.

Monensin vs essential oils

No significant difference between EO and MON was observed in alpha diversity, similar to results reported in dairy (Schären *et al.*, 2016) and beef (Tomkins *et al.*, 2015) cattle where EO did not alter the diversity in the rumen microbiome. In contrast, Patra & Yu (2012) indicated that various EO decreased the rumen microbiome diversity *in vitro*. Factors such as ruminant species and age, active component in EO, extraction methods, supplementation period and dose administered are possible sources of variation on the effect of EOs (Torres *et al.*, 2020).

Compared to MON, EO was characterised by a low abundance of *Succinivibrionacea* and within the grower phase, a higher abundance of *Lachnospiraceae*. *Lachnospiraceae* is a family of Gram-positive bacteria that might be inhibited by both monensin and EO as they affect more permeable

bacteria. The variety of functions executed by *Lachnospiraceae* may affect their relative abundance in digestive tract communities of different hosts (Eren *et al.*, 2015). A number of species belonging to the *Lachnospiraceae* family have cellulose-degrading activities and are associated with other cellulolytic microbes. The abundance of *Lachnospiraceae* has been positively correlated with feed efficiency (Shabat *et al.*, 2016) and fermentation in beef cattle (Hernandez-Sanabria *et al.*, 2010), in contrast strains belonging to the family have been found in higher abundance in less efficient animals (Paz et al., 2018). Species of the *Lachnospiraceae* family produce butyrate (Meehan & Beiko, 2014) and a higher butyrate concentration has been reported in more efficient animals (Guan *et al.*, 2008). In the finisher phase, a higher butyrate concentration was observed in MON compared to EO, however EO had a higher butyrate concentration in the starter and grower phases. Various studies (Benchaar *et al.*, 2006; Tomkins *et al.*, 2015) observed an increase in the butyrate concentration when the diet was supplemented with EO. Not all species of this family are butyrate producers (Meehan & Beiko, 2014) and further research is required to investigate the correlation between butyrate-producing microorganisms, such as *Roseburia* and *Eubacterium*, and efficiency.

Within the feedlot period, the starter and grower phase had a more observable difference between MON and EO, while there was no significant variation in phylum abundance between MON and EO in the finisher phase. Adaptation of microbes to EOs can occur, which may elucidate the diminishing effects of EO in a feedlot environment over time (Yang *et al.*, 2010). The effect of EO on microbial fermentation decreased after six to seven days in a dual flow continuous-culture system (Cardozo *et al.*, 2006). Longer exposure of EO to microbes may lead to alterations in the microbiome composition, and the possibility exists that some EOs can be degraded by rumen microbes (Benchaar *et al.*, 2008).

In a meta-analysis of the influence of EOs on the rumen microbial composition, it was observed that the addition of EOs to a diet could lead to a decrease in the protozoa population (Torres *et al.*, 2020). In contrast, this study did not observe any variation in the fungal diversity or phyla abundance between MON and EO in the starter, grower, and finisher phases, this might be due to the similar mode of action between monensin and EO in that they target more permeable bacteria.

Monensin vs probiotic

Although it was indicated through the Proteobacteria ratio that all treatment groups were in dysbiosis within the finisher phase, PRO had a significantly lower Proteobacteria ratio and higher diversity compared to CON. Compared to MON, PRO had a numerically lower Proteobacteria ratio and higher diversity. Cattle are at risk within the finisher phase of a feedlot period as they are fed a diet consisting predominantly of concentrate that can lead to a reduction in pH resulting in dysbiosis in the rumen microbiome (Petri *et al.*, 2013). Dysbiosis is characterized by a low diversity in the rumen microbiome (Petri *et al.*, 2013) and a high Proteobacteria ratio (Auffret *et al.*, 2017). Probiotics are known to have a stabilizing effect on the rumen microbiome composition and are most effective in stressed animals (Riddell *et al.*, 2010). Proteobacteria are mostly amylolytic bacteria, however, this

phylum does contain many pathogenic bacteria (Auffret *et al.*, 2017). As dysbiosis interferes with the stability of the microbial community, pathogenic bacteria subsequently take the opportunity to proliferate resulting in a negative effect on the animal. Such a dysbiosis is well documented in metabolism disorders (Petri *et al.*, 2013; Shin *et al.*, 2015). The supplementation of probiotics is known to influence the diversity, richness and abundance of microbes, resulting in improved immunity, lower occurrence of metabolic disorders and increased nutrient digestion and absorption (Du *et al.*, 2018). A higher diversity has been associated with a balanced and healthy microbiome (Yeoman & White, 2014).

While Proteobacteria was significantly different between PRO and CON, one of its families, *Succinivibrionaceae*, did not differ between the treatment groups. This family is associated with feed efficiency as it produces succinate that can be converted to propionate (Myer *et al.*, 2015). Spirochaetes was higher in abundance within PRO compared to MON. This is in line with a study where calves were supplemented with *Bacillus subtilis* and *B. amyloliquefaciens* (Du *et al.*, 2018). The families, *Lachnospiraceae*, *Clostridiales_XIII*, *Clostridiales_XI* and *Elusimicrobiaceae* were more abundant in PRO compared to MON. Hyper-ammonia producing microbes, including *Clostridium sticklandii*, *C. aminophilum* and *Prevotella ruminicola*, are highly sensitive towards ionophores (Eschenlauer *et al.*, 2002) due in part to their Gram-positive nature.

No *Bacillus* ASVs were identified in this study. This may be due to *Bacillus* not being characterized within the database used or was in such a low abundance that it was not detected by amplicon sequencing. Previous amplicon sequencing-based studies did not detect *Bacillus* species as well (Henderson *et al.*, 2015; O'Hara *et al.*, 2018).

Limited literature could be found on the influence of probiotics on the rumen fungal composition. In this study, Neocallimastigomycota tended towards a higher abundance in PRO compared to MON. The Neocallimastigomycota phylum, which consists of anaerobic fungi, have been indicated to be the primary fungal phylum within the rumen (Gruninger *et al.*, 2014), however Ascomycota was perceived to be the predominant fungal phylum in this study. Ascomycota and Basidiomycota were the predominant phyla in another study that also utilized ITS sequencing (Han *et al.*, 2019).

4.5 Conclusion

Limited differences were noted in the bacterial, archaeal, and fungal rumen population between the MON group and the other treatment groups, CON, EO and PRO. The natural feed additives, EO and PRO, might therefore be considered as possible alternatives to the use of MON. However, large-scale production studies will be required for more conclusive evidence. It was also shown that the probiotic group had a higher alpha diversity within the finisher phase which holds potential as this phase is known to have an increased risk for dysbiosis. A higher diversity is known to be a characteristic of a healthy and resilient rumen microbiome. The effect of MON and EO on the bacterial composition seemed to decrease whereas the effect of the additives on the fungal population seemed to increase as

the feedlot period progressed. Further studies on the adaptation of rumen microbes to diets and dietary components are needed.

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Supplementary Tables

Supplementary Table 4.1 The compositional relative abundance (in percentage) of the bacterial/archaeal phyla and families of the control (CON), essential oil (EO), monensin (MON) and probiotic (PRO) groups in the starter, grower, and finisher phases.

Bacterial/Archaeal Taxa	CON (%)	EO (%)	MON (%)	PRO (%)	p-value
Starter					
Euryarchaeota	1.53	1.20	2.50	1.09	0.188
Methanobacteriaceae	1.52	1.13	2.48	1.05	0.146
Methanomassiliicoccaceae	0.01	0.07	0.02	0.04	0.346
Actinobacteria	1.13	0.54	1.61	0.48	0.009*
Bifidobacteriaceae	0.24	0.04	0.18	0.05	0.014*
Coriobacteriaceae	0.90	0.50	1.43	0.43	0.016*
Bacteroidetes	60.98	62.19	63.06	61.80	0.667
Bacteroidaceae	0.13	0.46	0.19	0.39	0.102
Marinilabiliaceae	0.36	0.27	0.80	0.30	0.682
Porphyromonadaceae	7.29	4.15	7.44	5.81	0.395
Prevotellaceae	36.01	44.53	47.52	45.46	0.231
Rikenellaceae	0.16	2.97	0.20	1.47	0.409
Flavobacteriaceae	1.52	1.50	0.72	1.16	0.140
Sphingobacteriaceae	0.22	0.45	0.11	0.29	0.518
Elusimicrobia	0.11	0.07	0.06	0.14	0.283
Fibrobacteres	2.88	3.13	0.77	9.11	0.010*
Firmicutes	29.04	28.77	24.46	22.57	0.306
Clostridiaceae_1	0.23	0.10	0.15	0.50	0.646
Clostridiales_Incertae_Sedis_XI	0.04	0.06	0.01	0.01	0.133
Clostridiales_Incertae_Sedis_XIII	0.20	0.15	0.13	0.12	0.159
Lachnospiraceae	7.90	9.31	6.33	6.04	0.771
Ruminococcaceae	14.58	11.57	13.04	9.91	0.277
Erysipelotrichaceae	2.39	3.70	2.82	3.84	0.897
Veillonellaceae	0.13	0.11	0.57	0.08	0.028**
Proteobacteria	3.40	2.97	7.22	2.88	0.902
Rhodospirillaceae	0.27	0.18	1.37	0.37	0.148
Bdellovibrionaceae	0.26	0.09	0.31	0.16	0.047*
Succinivibrionaceae	0.97	0.38	4.56	0.45	0.039*
Spirochaetes	0.65	0.91	0.15	1.82	0.131
Synergistetes	0.05	0.02	0.04	0.02	0.142
Tenericutes	0.19	0.05	0.08	0.05	0.074**
Grower					

Euryarchaeota	0.21	0.84	0.89	0.78	0.056**
Methanobacteriaceae	0.21	0.84	0.85	0.77	0.058**
Methanomassiliicoccaceae	0.01	<0.01	0.04	0.01	0.020*
Actinobacteria	1.45	1.40	0.40	0.92	0.043*
Bifidobacteriaceae	0.24	0.04	0.18	0.05	0.014*
Coriobacteriaceae	0.90	0.50	1.43	0.43	0.016*
Bacteroidetes	18.63	28.57	33.41	21.12	0.030*
Bacteroidaceae	0.37	0.13	0.42	0.13	0.188
Marinilabiliaceae	0.17	0.13	0.15	0.08	0.862
Porphyromonadaceae	1.73	3.90	3.93	1.66	0.044*
Prevotellaceae	11.03	19.02	24.76	14.77	0.012*
Prolixibacteraceae	0.08	0.10	0.03	0.12	0.186
Rikenellaceae	0.26	0.56	0.17	1.28	0.149
Flavobacteriaceae	0.09	0.14	0.22	0.14	0.265
Sphingobacteriaceae	0.48	0.14	0.13	0.01	0.216
Elusimicrobia	0.10	0.07	0.24	0.17	0.098**
Fibrobacteres	0.25	0.50	1.24	0.11	0.043*
Firmicutes	31.81	38.83	21.90	33.87	0.054**
Clostridiaceae_1	0.10	0.43	0.17	0.07	0.259
Clostridiales_Incertae_Sedis_XIII	0.08	0.07	0.05	0.03	0.169
Eubacteriaceae	0.05	0.06	0.01	0.05	0.035*
Lachnospiraceae	14.77	15.13	6.92	13.13	0.034*
Ruminococcaceae	14.33	20.33	12.43	17.90	0.178
Erysipelotrichaceae	0.35	0.47	0.40	0.29	0.903
Veillonellaceae	0.11	0.17	0.06	0.09	0.075**
Proteobacteria	47.40	29.15	41.51	42.93	0.378
Rhodospirillaceae	0.53	0.47	0.48	0.58	0.845
Bdellovibrionaceae	0.18	0.29	0.74	0.22	0.081**
Succinivibrionaceae	0.14	0.27	0.87	0.33	0.028*
Orbaceae	1.53	0.53	1.37	0.87	0.277
Spirochaetes	0.06	0.57	0.35	0.04	0.097**
Synergistetes	0.05	0.04	0.03	0.05	0.568
Finisher					
Euryarchaeota	0.23	0.46	1.61	1.51	0.102
Methanobacteriaceae	0.22	0.45	1.60	1.48	0.099**
Actinobacteria	0.30	0.34	0.26	0.74	0.084**
Coriobacteriaceae	0.27	0.33	0.25	0.72	0.090**
Bacteroidetes	23.24	28.38	29.75	31.91	0.195
Bacteroidaceae	0.08	0.05	0.06	0.07	0.693
Bacteroidales_incertae_sedis	0.04	0.06	0.07	0.05	0.395

Marinilabiliaceae	0.10	0.09	0.15	0.46	0.029*
Porphyromonadaceae	1.06	1.66	1.91	1.74	0.272
Prevotellaceae	19.70	22.73	24.89	20.61	0.544
Prolixibacteraceae	0.05	0.05	0.02	0.05	0.218
Rikenellaceae	0.18	0.47	0.11	0.18	0.118
Flavobacteriaceae	0.02	0.05	0.01	0.06	0.146
Sphingobacteriaceae	0.23	0.41	0.14	2.05	0.045*
Elusimicrobia	0.07	0.04	0.04	0.14	0.006*
Fibrobacteres	0.20	0.30	0.22	0.44	0.124
Firmicutes	9.86	11.99	17.60	21.03	0.023*
Clostridiaceae_1	0.17	0.06	0.45	0.21	0.241
Clostridiales_Incertae_Sedis_XIII	0.04	0.04	0.03	0.09	0.086**
Eubacteriaceae	0.02	0.03	0.01	0.04	0.121
Lachnospiraceae	4.39	5.29	3.84	7.95	0.035*
Ruminococcaceae	4.38	5.52	12.42	10.62	0.024*
Erysipelotrichaceae	0.19	0.16	0.26	0.51	0.170
Acidaminococcaceae	0.02	0.04	0.02	0.08	0.040*
Veillonellaceae	0.05	0.09	0,10	0.11	0.122
Proteobacteria	66.00	58.23	50.37	44.12	0.058**
Rhodospirillaceae	0.08	0.14	0.05	0.37	0.080**
Bdellovibrionaceae	0.04	0.19	0.08	0.18	0.046*
Succinivibrionaceae	0.35	0.20	4.63	0.22	0.067**
Orbaceae	1.32	1.39	2.25	2.13	0.608
Spirochaetes	0.06	0.21	0.10	0.03	0.502
Synergistetes	0.04	0.04	0.04	0.07	0.032*

* Significance at $p < 0.05$

** Tendency towards significance at $p < 0.10$

Supplementary Table 4.2 The relative abundance (in percentage) of the fungal phyla and families in the control (CON), essential oils (EO), monensin (MON) and probiotic (PRO) groups in the starter, grower, and finisher phases.

Fungal Phyla	CON (%)	EO (%)	MON (%)	PRO (%)	p-value
Starter					
Ascomycota	75.99	73.79	73.07	58.50	0.787
Cladosporiaceae	3.93	3.67	4.22	2.41	0.395
Aureobasidiaceae	1.34	1.28	1.53	1.96	0.792
Dothideales_fam_Incertae_sedis	16.51	11.10	12.65	8.33	0.231
Didymellaceae	34.72	36.55	37.79	20.74	0.235
Didymosphaeriaceae	0.69	0.64	0.63	0.74	0.891
Massarinaceae	0.30	0.18	0.15	0.10	0.111

Montagnulaceae	0.56	0.15	0.30	0.11	0.026*
Phaeosphaeriaceae	0.85	1.04	0.53	0.84	0.402
Pleosporaceae	2.13	2.27	2.00	2.02	0.857
Sporormiaceae	0.21	0.16	0.40	0.25	0.157
Aspergillaceae	9.46	11.07	7.42	12.04	0.544
Trichocomaceae	0.23	0.27	0.30	0.27	0.638
Myxotrichaceae	0.24	0.33	0.07	0,23	0.059**
Phaffomycetaceae	0.11	0.13	0.23	0.13	0.866
Plectosphaerellaceae	0.11	0.39	0.22	3.14	0.012*
Hypocreales_fam_Incertae_sedis	0.69	1.29	0.61	0.94	0.303
Nectriaceae	0.74	0.56	1.03	1.20	0.181
Stachybotryaceae	0.26	0.18	0.33	0.23	0.326
Microascaceae	0.35	0.18	0.27	0.19	0.549
Chaetomiaceae	0.64	0.57	0.59	0.70	0.866
Togniniaceae	0.20	0.16	0.11	0.09	0.977
Trichosphaeriaceae	0.26	0.24	0.18	0.28	0.565
Basidiomycota	4.13	3.45	7.30	6.13	0.287
Phallaceae	0.04	0.02	0.21	0.08	0.041*
Cystobasidiomycetes	0.11	0.03	0.11	0.10	0.080**
Sporidiobolaceae	0.08	0.05	0.23	0.14	0.424
Filobasidiaceae	3.60	3.04	6.30	5.34	0.309
Tremellaceae	0.09	0.06	0.16	0.19	0.273
Ustilaginaceae	0,03	0.06	0.11	0.09	0.152
Mortierellomycota	0	0	0.06	0.13	0.542
Mucoromycota	0.39	0.53	0.56	0.68	0.562
Lichtheimiaceae	0.14	0.29	0.19	0.20	0.687
Mucoraceae	0.24	0.23	0.26	0.41	0.177
Neocallimastigomycota	18.80	21.46	18.43	33.75	0.084**
Anthophyta	0.67	0.77	0.57	0.78	0.902
Grower					
Ascomycota	89.50	91.31	89.47	81.31	0.033*
Phaeococcomycetaceae	0.12	0.11	0.07	0.19	0.717
Cladosporiaceae	2.27	1.37	1.32	1.39	0.750
Teratosphaeriaceae	0.28	0.26	0.08	0.29	0.159
Aureobasidiaceae	0.17	0.19	0.30	0.38	0.740
Dothideales_fam_Incertae_sedis	0.54	0.79	1.18	0.98	0.024*
Didymellaceae	8.69	11.96	10.45	12.60	0.782
Didymosphaeriaceae	3.08	3.48	1.86	3.24	0.258
Phaeosphaeriaceae	3.89	5.44	2.40	5.51	0.647
Pleosporaceae	0.85	0.82	0.92	1.24	0.899

Sporormiaceae	0.29	0.42	0.24	0.30	0.326
Teichosporaceae	0.40	0.43	0.15	0.25	0.025*
Tubeufiaceae	0.06	0.26	0.01	0.08	0.063**
Herpotrichiellaceae	0.12	0.16	0.04	0.14	0.204
Aspergillaceae	46.45	37.18	50.65	30.02	0.034*
Trichocomaceae	3.12	4.72	2.12	2.44	0.012*
Myxotrichaceae	2.30	1.07	2.35	1.96	0.202
Debaryomycetaceae	0.20	0.24	0.26	0.32	0.645
Phaffomycetaceae	0.77	0.38	2.94	0.44	0.133
Saccharomycetaceae	0.04	0.06	0.12	0.20	0.287
Chaetosphaeriaceae	0.10	0.15	0.07	0.11	0.732
Diaporthaceae	0.23	0.27	0.16	0.33	0.775
Plectosphaerellaceae	0.30	0.25	0.15	0.08	0.026*
Bionectriaceae	0.21	0.37	0.13	0.22	0.107
Cordycipitaceae	0.13	0.10	0.06	0.13	0.085**
Hypocreaceae	1.24	1.38	1.03	1.21	0.442
Hypocreales_fam_Incertae_sedis	1.71	1.78	1.15	1.43	0.097**
Nectriaceae	2.22	3.10	2.11	2.75	0.792
Stachybotryaceae	0.17	0.16	0.09	0.13	0.048*
Microascaceae	0.16	0.19	0.29	0.17	0.016*
Myrmecridiaceae	0.16	0.25	0.07	0.11	0.030*
Chaetomiaceae	5.97	9.59	4.32	6.98	0.075**
Trichosphaeriaceae	0.61	0.99	0.77	0.98	0.687
Apiosporaceae	0.11	0.13	0.06	0.13	0.248
Basidiomycota	0.97	1.52	1.61	1.49	0.012*
Cystobasidiomycetes	0.18	0.22	0.08	0.26	0.224
Erythrobasidiaceae	0.12	0.14	0.07	0.10	0.088**
Sporidiobolaceae	0.20	0.42	0.39	0.37	0.567
Filobasidiaceae	0.24	0.55	0.90	0.54	0.017*
Mucoromycota	2.10	2.44	2.43	2.78	0.309
Lichtheimiaceae	1.49	1.48	0.59	0.92	0.035*
Mucoraceae	0.52	0.53	1.71	1.68	0.056**
Neocallimastigomycota	6.76	4.24	6.14	13.45	0.544
Anthophyta	0.57	0.37	0.17	0.85	0.019*
Finisher					
Ascomycota	54.27	35.79	43.97	50.60	0.056**
Cladosporiaceae	0.15	0,21	0.14	0.20	0.860
Aureobasidiaceae	1.38	1.34	0.51	0.52	0.012*
Dothideales_fam_Incertae_sedis	0.76	0.92	0.38	0.61	0.274
Didymellaceae	16.59	17.68	9.58	18.63	0.210

Didymosphaeriaceae	0.78	0.51	0.61	1.03	0.051**
Massarinaceae	0.57	0.38	0.56	0.73	0.042*
Phaeosphaeriaceae	0.30	0.29	0.20	0.30	0.146
Aspergillaceae	23.59	4.29	21.59	21.14	0.018*
Trichocomaceae	0.26	0.19	1.24	0.69	0.018*
Debaryomycetaceae	0.19	0.17	0.16	0.16	0.548
Phaffomycetaceae	0.19	0.39	0,24	0,28	0.831
Diaporthaceae	0.23	0.28	0.19	0.16	0.142
Clavicipitaceae	0.15	0.36	0.10	0.18	0.041*
Hypocreales_fam_Incertae_sedis	0.87	0.45	0.53	0.62	0.047*
Nectriaceae	6.98	6.85	4.26	3.73	0.025*
Chaetomiaceae	0.17	0.09	0.27	0.25	0.052**
Basidiomycota	1.16	1.89	0.63	1.52	0.092**
Sporidiobolaceae	0.47	0.79	0.20	0.51	0.082**
Filobasidiaceae	0.37	0.76	0.18	0.63	0.080**
Ustilaginaceae	0.24	0.03	0.14	0.23	0.015*
Mucoromycota	1.48	11.47	0.90	2.09	0.102
Lichtheimiaceae	0.39	0.13	0.10	0.36	0.009*
Mucoraceae	1.08	11.34	0.79	1.73	0.156
Neocallimastigomycota	42.67	47.82	53.94	45.06	0.350
Anthophyta	0.43	3.03	0.56	0.72	0.390

* Significance at $p < 0.05$

** Tendency towards significance at $p < 0.10$

Chapter 5

The effect of monensin, a *Bacillus*-probiotic, and essential oils on the bacterial and fungal composition in the jejunum of South African Bonsmara cattle

Abstract

Microorganisms in the ruminant gastrointestinal tract affect the efficiency and health of the animal. Much research has been done on the rumen microbiome and factors that might influence the microbial population towards more efficient pathways. Limited knowledge is available on the factors that affect the jejunum microbiome. The microorganisms in the jejunum have been reported to affect the health and production of the animal. The influence of monensin, a *Bacillus*-probiotic, and essential oils on the bacterial and fungal composition of the jejunum in beef cattle under intensive feedlot conditions was investigated in this study. Forty-eight Bonsmara calves were divided into four groups which received the basal diet, the basal diet with monensin, a *Bacillus*-probiotic, or essential oils inclusion. Following a 120-day intensive feeding period, small intestine digesta samples were collected at slaughter. DNA extraction was performed on the samples and submitted for internal transcribed spacer (ITS) and 16S rRNA amplicon sequencing. For the bacterial and fungal populations, 6 968 and 4 990 amplicon sequence variants (ASVs) were detected, respectively. A difference in the bacterial alpha diversity in the control and probiotic groups were observed, which had a significantly higher diversity compared to the monensin and essential oil groups. The Firmicutes phylum was the most abundant within the jejunum digesta followed by Actinobacteria and Proteobacteria. The probiotic group had a higher abundance of *Olsenella*, *Blautia* and *Eubacterium* while a higher abundance of *Roseburia* was found in the control group. These bacteria have been associated with a decrease in inflammation in the animal. Monensin and the essential oils blend decreased the abundance of both beneficial and pathogenic bacteria, such as *Clostridium_sensu_stricto*. This study demonstrates that feed additives influence the composition and diversity of the jejunal microbiome by increasing or decreasing beneficial bacteria.

5.1 Introduction

The microorganisms in the gastrointestinal tract play an essential part in the production, efficiency, and health of ruminants (Ley *et al.*, 2008). Recently, the majority of research has focussed on the microorganisms found within the rumen as it influences the amount of nutrient available to the animal. The remaining part of the gastrointestinal tract microbiome may also contribute to the production and health of the animal because of the role these microorganisms play in nutrient digestion and absorption from feed and in limiting opportunistic pathogens (Donaldson *et al.*, 2016). The microbial composition of livestock can be studied due to advancements in sequencing technology and

results can be applied in strategies that can contribute to increased efficiency and decrease the environmental impact of livestock production (Krause *et al.*, 2013).

The gastrointestinal tract microbiome can be broadly segregated into three different regions: rumen, small intestine, and large intestine with each containing different ecological and microbial characteristics (Wang *et al.*, 2019; Liu *et al.*, 2020). In the ruminant, the role of the small intestine is the chemical digestion of feedstuffs and nutrient absorption. Due to this, a small number of microorganisms, predominantly facultative anaerobes and acid-tolerant microbes (Donaldson *et al.*, 2016), are found within the small intestine, especially the jejunum, that break down proteins and utilizes amino acids or ammonia (Bergen, 2015). Modifying the jejunal microbiome is a way to improve animal productivity and health due to the influence of these microbes on the metabolism of the jejunum, especially energy homeostasis (El Aidy *et al.*, 2013). A correlation exists between the cytokine concentration and the microorganisms found in the jejunum, indicating an interaction between the microbiome in the jejunum and the immune system of the animal (Ye *et al.*, 2022). Pathways such as xenobiotics degradation, biodegradation and metabolism have been reported to be enriched in the small intestine suggesting that microorganisms are involved in regulating host health (Wang *et al.*, 2022). Antimicrobial substances produced by the microorganisms can inhibit the growth of pathogenic microorganisms in the digestive tract resulting in a more efficient animal with improved growth performance (Baümler & Sperandio, 2016).

Feed additives are fed to livestock to increase efficiency and improve the health of the animal (Jouany & Morgavi, 2007). Monensin is commonly used as an antibiotic growth promoter in South African feedlots. It is known to inhibit bacteria with permeable cell membranes, resulting in a lower acetate to propionate ratio (Ogunade *et al.*, 2018). As consumer awareness grows, the use of antibiotic growth promoters is being questioned due to the risk of the development of antibiotic-resistant bacteria and the transference thereof to the human population (Casewell *et al.*, 2003). Alternative feed additives, such as probiotics and essential oils, are being investigated and have been reported to influence the rumen microbiome (Patra & Yu, 2012; Du *et al.*, 2018). Essential oils (EO) are aromatic oils that exhibit a similar mode of action as monensin, in that it targets the bacteria with more permeable cell membranes (Li *et al.*, 2013). Studies have found an increase in butyrate concentration and growth with the addition of EOs to the diet (Benchaar *et al.*, 2008; Patra & Yu, 2012). Probiotics have been shown to increase the diversity within the rumen microbiome as well as the abundance of fibre-degrading microbes (Du *et al.*, 2018). Studies have reported an improvement in animal growth, immune modulation and intestinal development when animals were fed with a *Bacillus*-probiotic (Sun *et al.*, 2016; Du *et al.*, 2018).

While the effect of feed additives have been studied on the rumen microbiome and in monogastric animals, limited literature could be found on their effect on the small intestine microbiome of ruminant animals. Liu *et al.* (2020) has indicated the importance of variation in the abundance of particular taxa in the bacterial community of the jejunum with regard to health in cattle. Therefore, in this study the

focus was on the effect of monensin, a *Bacillus*-probiotic and essential oils on the jejunal microbiome of Bonsmara cattle raised under feedlot conditions.

5.2 Materials & methods

5.2.1 Animals and diet

Ethical approval was conferred by the University of Pretoria's Animal Ethical Committee (NAS445/2019). Forty-eight Bonsmara bull calves (224 ± 22 kg, 10-14 months old) were sourced from one farm and backgrounded on natural grazing for 40 days before they entered the trial. The study was conducted under commercial feedlot conditions in a trial facility in Edenville, Free State, South Africa. The bulls were randomly allocated into four groups and blocked by weight with three animals to a pen (four pens/treatment group). The groups were as follows: a control group with a basal diet (CON), the basal diet with monensin (0.03 g/animal/day) (MON), the basal diet with essential oils (eugenol, capsicum and cinnamaldehyde, 1 g/animal/day) (EO) and the basal diet supplemented with a *Bacillus* probiotic (*Bacillus licheniformis* and *Bacillus subtilis*, 2.75 g/animal/day) (PRO).

5.2.2 Jejunum digesta collection

The animals were fed for a period of 120 days with starter, grower, and finisher diets. The diet compositions were reported in Linde *et al.* (2022) (Chapter 3). After reaching market weight, the animals were transported to the abattoir the day prior to slaughter and the feed was withdrawn for approximately 20 hours prior to slaughter. Standard slaughter procedures were followed; the animals were stunned with a captive bolt and exsanguinated. The gastrointestinal tract was removed from the carcass. The jejunum was identified on visual inspection and an incision made approximately midway. Approximately 10 ml of jejunal digesta was collected, instantly frozen in liquid nitrogen and deposited in a -80 ºC freezer until extraction could be completed.

5.2.3 DNA extraction and amplicon sequencing

DNA extraction was conducted using a Qiagen PowerFecal Pro DNA (Qiagen, Germany) extraction kit following the manufacturer's protocol. A bead-beating step was performed where 300 µl of the sample was homogenized for twelve minutes at maximum speed (400 x 10 rpm) in a Beadbug homogenizer (Benchmark Scientific, USA). A Nanodrop ND-1000 Spectrophotometer (Thermo Scientific, USA) and a Qubit Fluorometer (Invitrogen, USA) were used to quantify the extracted DNA. Samples were submitted for 16S rRNA (V3-V4) using 341F (5'CCTAYGGGRBGCASCAG) and 806R (5'GGACTACNNGGGTATCTAAT) as primers and ITS1 amplicon sequencing with the following primers F-5'GGAAGTAAAAGTCGTAACAAGG and R-5'GCTGCGTTCTTCATCGATGC using a NovaSeq 250 sequencer (Illumina, USA). Data was received with primers removed.

5.2.4 Bioinformatic and statistical analyses

Bioinformatic and statistical analyses were performed using various packages within R v4.0.2 (R Core Team, 2013). On average per sample, 198 162 ± 11 918 and 194 734 ± 10 955 reads were generated from the 16S rRNA and ITS sequencing, respectively. Quality control of the reads and removal of chimeras were conducted with DADA2 (Callahan *et al.*, 2016). Data was rarefied to minimum sequence reads for both 16S rRNA and ITS data. Reads were grouped into amplicon sequence variants (ASVs) and were discarded if they were detected less than ten times in 5% of the samples. The phyloseq (McMurdie & Holmes, 2013) package was used to estimate the abundance of the taxa to genus level and the alpha diversity (observed number of ASVs, Chao1, Shannon and Simpson indices). Beta diversity was plotted with a principal coordinate analysis (PCoA) based on weighted (quantity) and unweighted (quality) UniFrac distances. UniFrac is a phylogeny-based method that takes phylogenetic variation among ASVs into consideration when determining differences within the microbiome community from each treatment group (Lozupone *et al.*, 2007). The Ribosomal Database Project was used for 16S rRNA ASVs annotation (Cole *et al.*, 2014) while the UNITE database was used for fungal annotation (Nilsson *et al.*, 2019). A significant difference between the feed additive treatment groups were calculated based on permutational analysis of variation (PERMANOVA) using vegan (Oksanen *et al.*, 2020). The betadisper procedure in vegan was used to test the homogeneity of variance assumption. If the assumption was violated, analysis of similarities (ANOSIM) was used to determine significance between microbial profiles, with $R < 0.25$ indicating similar microbial profiles (Clark, 1993).

Significant differences in the relative abundance of the taxa and the alpha diversity indices were calculated using the Kruskal Wallis and the Wilcoxon-rank sum tests. Multiple-test correction for the p-values were done with the Benjamini-Hochberg procedure. A significant difference was recognized at $p < 0.05$ and trends were recognized at $0.05 < \text{p-value} < 0.1$.

5.3 Results

After quality control and chimera removal, 109 615 ± 19 997 and 154 342 ± 10 187 reads remained for 16S rRNA and ITS sequencing, respectively. From these reads, seven bacterial phyla, 24 bacterial families, 60 bacterial genera, five fungal phyla, 35 fungal families and 43 fungal genera were identified from 6 968 bacterial and 4 990 fungal ASVs. Alpha diversity for the bacterial population, but not for the fungal population, differed significantly between the treatment groups (Table 5.1).

Table 5.1 The alpha diversity indices (Observed number of ASVs, Chao1, Shannon and Simpson indices) of the bacterial and fungal composition in the jejunum of the control (CON), monensin (MON), essential oils (EO) and probiotic (PRO) groups.

Alpha diversity indices	CON	MON	EO	PRO	p-value
16S rRNA					
Observed number of ASVs	708 ± 195^{a}	509 ± 99^{b}	500 ± 101^{b}	633 ± 96^{a}	0.004*
Chao1	715 ± 196^{a}	516 ± 99^{b}	506 ± 101^{b}	647 ± 95^{a}	0.004*
Shannon	3.77 ± 1.04^{ac}	2.94 ± 0.50^{a}	2.85 ± 0.60^{a}	3.85 ± 0.58^{bc}	0.003*
Simpson	0.90 ± 0.08^{ac}	0.83 ± 0.06^{a}	0.82 ± 0.09^{a}	0.92 ± 0.04^{bc}	0.002*
ITS					
Observed number of ASVs	257 ± 114	168 ± 78	190 ± 40	163 ± 54	0.796
Chao1	257 ± 113	168 ± 78	190 ± 40	163 ± 54	0.811
Shannon	2.20 ± 0.84^{ab}	1.42 ± 0.69^{a}	2.03 ± 0.37^{b}	1.60 ± 0.57^{ab}	0.128
Simpson	0.69 ± 0.19^{ab}	0.50 ± 0.20^{a}	0.70 ± 0.11^{b}	0.57 ± 0.16^{ab}	0.198

* indicate significant difference between treatment groups at $p < 0.05$

^{abcd} different superscripts across columns indicates significant differences at $p < 0.05$

Beta diversity for the bacterial composition was analysed using unweighted and weighted UniFrac distances and plotted with a principal coordinate analysis (PCoA) (Figure 5.1). Clustering of samples indicates a higher similarity in the microbial composition. A significant difference in the bacterial beta diversity was observed using PERMANOVA ($p = 0.001$; betadisper, $p = 0.128$).

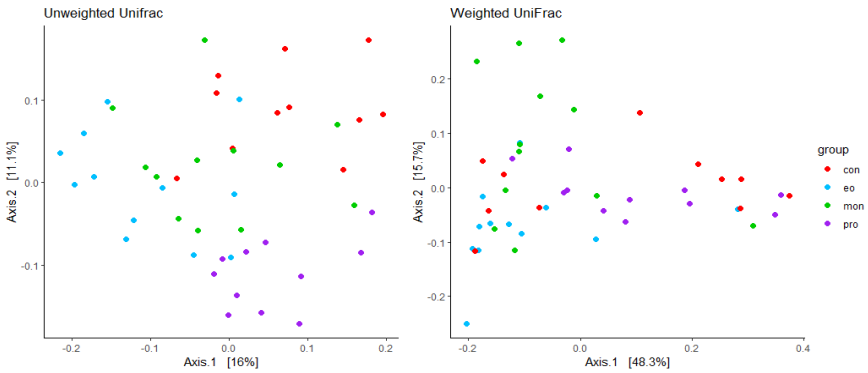

Figure 5.1 A PCoA plotted using unweighted (left) and weighted (right) UniFrac distances of the jejunal bacterial/archaeal population of the control (CON, red), essential oils (EO, blue), monensin (MON, green) and probiotic (PRO, purple) groups.

The PCoA plot based on unweighted and weighted UniFrac distances illustrated no clustering in the fungal composition between the treatment groups. A significant difference between the treatment groups for beta diversity was determined by PERMANOVA ($p = 0.034$), however the assumption of

homogeneity of variation was violated (betadisper, p = 0.003) and analysis of similarities (ANOSIM) indicated a similar microbial profile between the feed additive groups (R = 0.108).

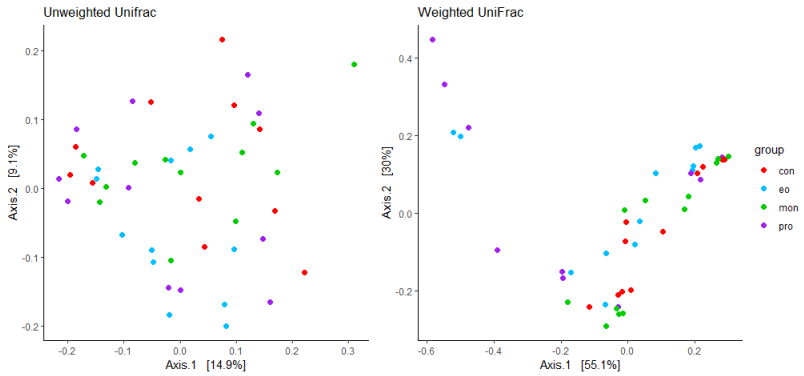

Figure 5.2 A PCoA plotted using unweighted (left) and weighted (right) UniFrac distances of the jejunal fungal population of the control (CON, red), essential oils (EO, blue), monensin (MON, green) and probiotic (PRO, purple) groups.

In Figure 5.3, the relative abundance of the phyla, families, and genera of the four treatment groups are illustrated. Firmicutes (87.81%) was the most predominant bacterial phylum, followed by Actinobacteria (2.99%) and Proteobacteria (0.69%).

Only Actinobacteria showed a significant difference in abundance (p = 0.001) between the treatment groups, with the phylum being more abundant in PRO compared to EO and MON. The most abundant families observed in the jejunum was *Peptostreptococcaceae* (59.78%), *Lachnospiraceae* (10.81%), *Ruminococcaceae* (5.10%) and *Clostridiales_Incertae_Sedis_XIII* (4.82%). A significantly higher abundance of *Peptostreptococcaceae* (p = 0.021) was observed in MON and EO in comparison with PRO and CON while *Lachnospiraceae* (p = 0.005) was significantly more abundant in PRO and CON in comparison with MON and EO. A significantly higher abundance of *Clostridiales_Incertae_Sedis_XII* (p = 0.014) was observed in CON in comparison with MON. Of the genera, *Clostridia_XI* (39.19%) and *Romboutsia* (20.48%) was the most abundant. *Clostridial_XI* (p = 0.002) was significantly higher in abundance in MON in comparison with PRO while *Romboutsia* (p = 0.074) tended towards a higher abundance in EO compared to PRO. The Euryarcheaota phylum made up 7.49% of the 16S rRNA reads and did not differ between the treatment groups. The relative abundance of the various taxa can be found in Supplementary Table 5.1.

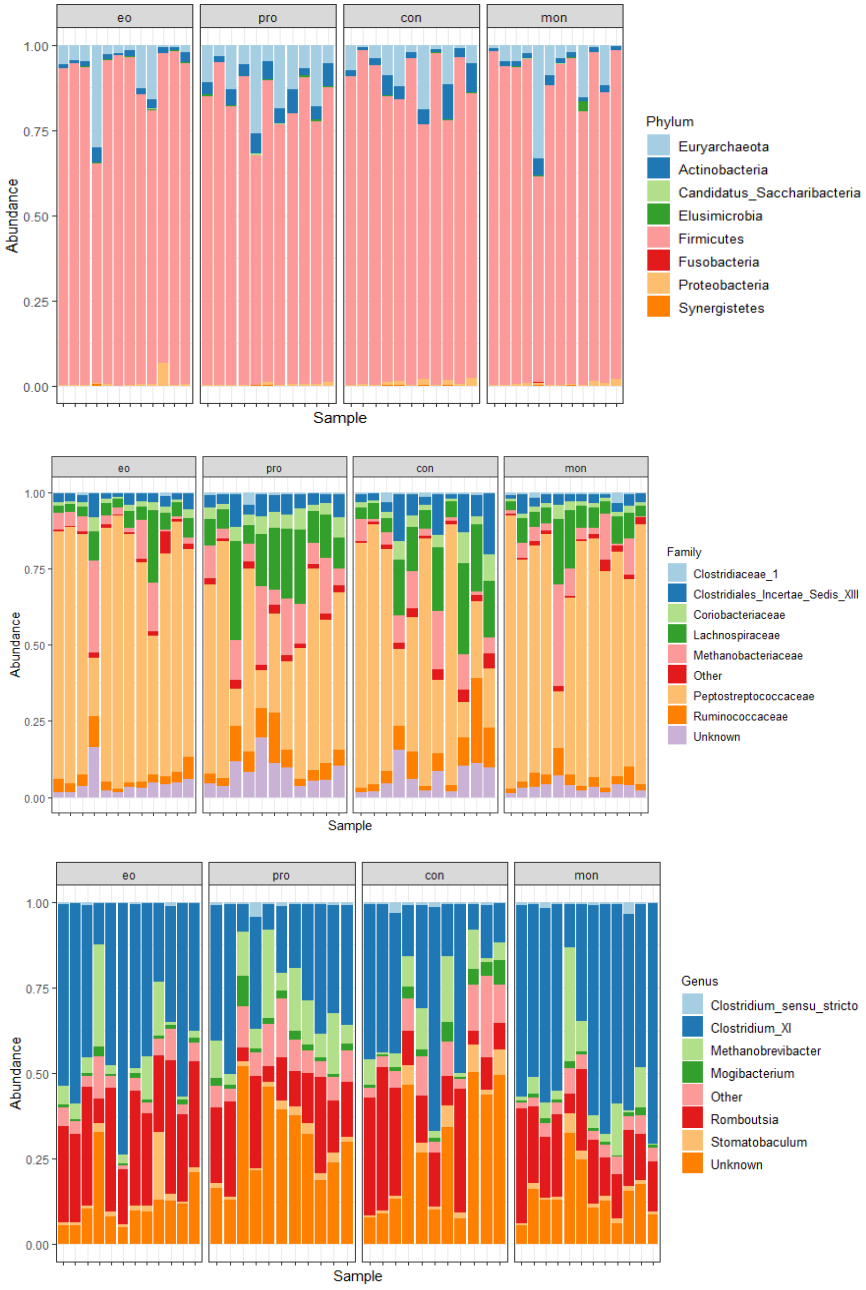

Figure 5.3 The relative abundance of the bacterial/archaeal phyla (top), families (middle) and genera (bottom), depicted as different colours as shown in the legend on the right of the graphs, found in the jejunum of the control (CON), monensin (MON), essential oils (EO) and probiotic (PRO) groups.

The Ascomycota (93.74%) phylum was the most predominant fungal phylum followed by Mucoromycota (4.03%) and Neocallimastigomycota (3.01%) (Figure 5.4).

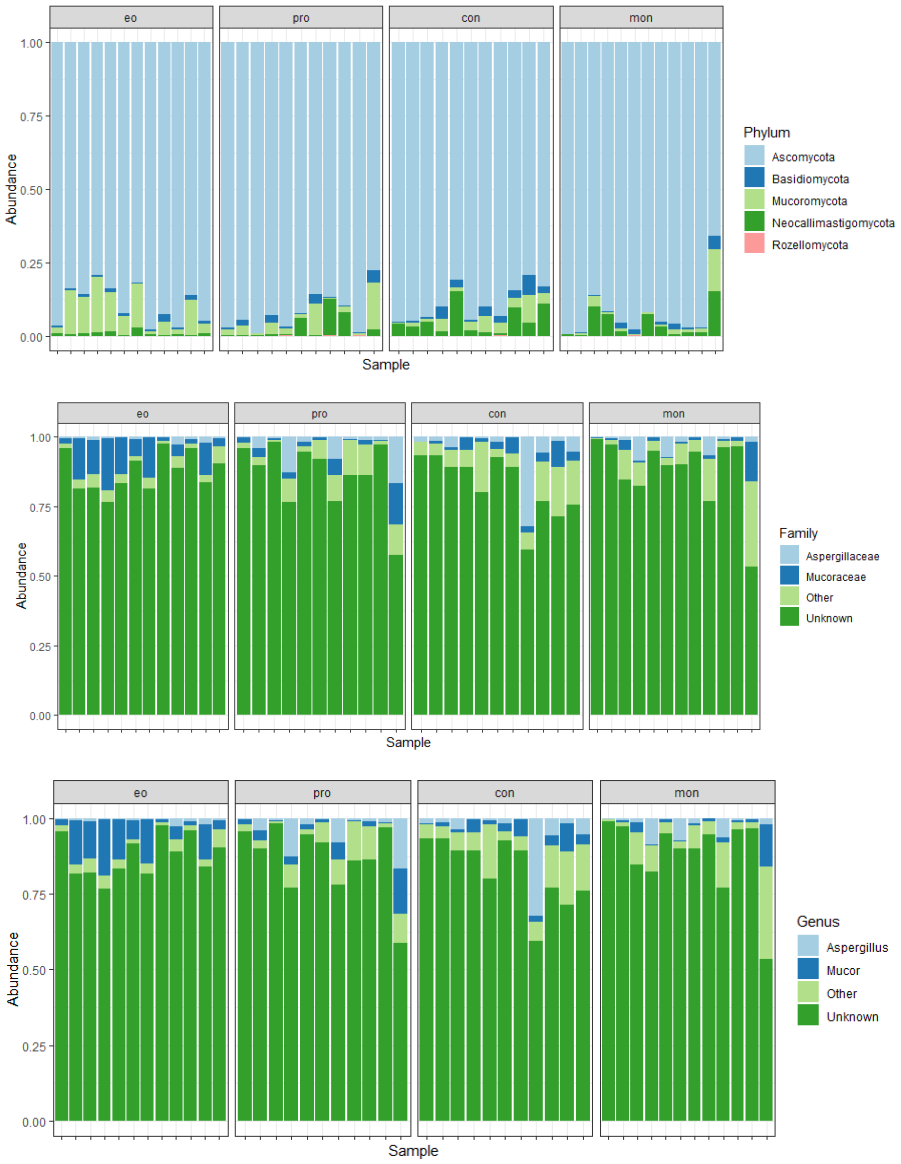

Figure 5.4 The relative abundance of the fungal phyla (top), families (middle) and genera (bottom), depicted in different colours as indicated by the legend on the right of the graphs, observed in the jejunum of the control (CON), monensin (MON), essential oils (EO) and probiotic (PRO) groups.

Ascomycota (p = 0.052) had a tendency towards a significant difference with a higher abundance in PRO and MON compared to EO. Mucoromycota (p = 0.002) was significantly higher while Neocallimastigomycota (p = 0.034) was significantly decreased in EO compared to the other treatment groups. Of the families, 87% was not taxonomically identified. *Mucoraceae* (3.77%), *Neocallimastigaceae* (3.62%) and *Aspergillaceae* (2.70%) was the most predominant families. Within the *Neocallimastigacea* family, the most abundant genus was *Caecomyces* (2.92%). The relative abundance of the fungal taxa in the jejunum can be found in Supplementary Table 5.2.

5.4 Discussion

Despite reports indicating the importance of the jejunum microbiome in efficiency (Myer *et al.*, 2016; Perea *et al.*, 2017) and health (Hooper & MacPherson, 2010; Baümler & Sperandio, 2016), there remains a dearth of information on the microbial composition and the effect of nutrition thereupon. In this study, the results indicate that different feed additives can influence the bacterial, and fungal population in the jejunum microbiome of Bonsmara cattle raised under feedlot conditions.

The bacterial alpha diversity was higher in the CON and PRO groups in comparison with the EO and MON groups. A high diversity aids in the balance of the gastrointestinal tract microbiome (Khafipour *et al.*, 2016) and has been suggested as an indicator of an animal's health status. A high diversity, richness, and evenness, within the microbiota is deemed advantageous because it improves the stability of the microbiome, particularly during nutritional challenging environments as it permits it to use restricted resources more effectively (Russell & Rychlik, 2001; Khafipour *et al.*, 2016).

Probiotics, consisting of *Bacillus* strains, such as within this study, have been reported to increase the rumen microbiome diversity by their antimicrobial activity and promoting the proliferation of advantageous bacteria (Du *et al.*, 2018). Although a high diversity and richness is correlated with a healthy microbiome, other studies have indicated that an increase in richness can also be due to intestinal and extra-intestinal inflammation and infections (Lee *et al.*, 2014; Khafipour *et al.*, 2016).

The decrease in the diversity within the EO and MON groups can be due to the inhibition of permeable microorganisms, this is known to be part of the mode of action of these feed additives (Patra & Yu, 2012; Reti *et al.*, 2013). Antibiotic growth promoters, such as monensin, and essential oils, produce antimicrobial enzymes that can inhibit opportunistic pathogens resulting in a decrease in the diversity within the microbiome (Dibner & Richards, 2005).

Beta diversity differed significantly between the treatment groups for the bacterial composition of the jejunum. Differences observed might be attributed to the higher diversity in the PRO and CON groups in comparison to the EO and MON groups. Firmicutes, Actinobacteria, and Proteobacteria were the most abundant phyla in the jejunum in this study. This is similar to studies conducted in Hu sheep (Zhong *et al.*, 2022), Angus cattle (Liu *et al.*, 2020) and a Nellore steer (De Oliveira *et al.*, 2013). Patescibacteria has also been noted to be abundant (Wang *et al.*, 2022; Zhong *et al.*, 2022), but was not found to be present in this study. Different databases used to identify the various taxa as well as different methods applied could lead to differences among studies. The Firmicutes phylum plays a vital part in carbohydrate degradation and its increase in abundance in the rumen is correlated with energy harvesting and a higher fat deposition (Jami *et al.*, 2014), but its role in the small intestine remains uncertain (Myer *et al.*, 2016). The Bacteroidetes phylum has been reported to be abundant in the small intestine (Liu *et al.*, 2020; Zhong *et al.*, 2022), however its presence was not observed in this study and was also not observed to be abundant in a study conducted by Wang *et al.* (2022). This might be caused by the concentrate-based diet that was fed to the feedlot animals in this study as high concentrate diets

are known to affect the jejunal microbiome, such as a reduction in the Firmicutes abundance in jejunal digesta (Plaizier *et al.*, 2021; Zhong *et al.*, 2022). A diet composed predominantly of concentrates might result in a substantial quantity of undegraded carbohydrates entering the jejunum and influencing the microorganisms.

The abundance of the Actinobacteria phylum was significantly higher in PRO in comparison with EO and MON. The abundance of both Actinobacteria and Patescibacteria were identified as being characteristic of the small intestine (Wang *et al.*, 2022). The *Olsenella* genus from the Actinobacteria phylum ferments carbohydrates to volatile fatty acids, especially acetate and butyrate, and enhances the anti-inflammatory function of the animal (Wang *et al.*, 2019). *Olsenella* was significantly higher in abundance within PRO compared to EO and MON. *Eggerthellaceae* and *Nocardiaceae*, also from the phylum Actinobacteria, were identified as being predominant microbes for the small intestine and can produce bioactive metabolites that can be used as naturally derived antibiotics (Barka *et al.*, 2016). These genera may therefore play important roles in maintaining homeostastis in the jejunum (Wang *et al.*, 2022). Despite the general low abundance of *Eggerthellaceae* observed within the jejunum, it had a tendency towards a higher abundance in EO and PRO compared to CON and MON.

Lachnospiraceae, *Ruminococcaceae* and *Clostridiales_Incertae_Sedis_XIII* were found to be the most abundant families in the jejunum of the cattle in this study. *Ruminococcaceae*, *Lachnospiraceae* and *Christensenellaceae* have been observed to be abundant in all regions of the gastrointestinal tract (Wang *et al.*, 2022). *Peptostreptococcaceae* was significantly more abundant in MON and EO in comparison with PRO and CON. *Peptostreptococcaceae* was observed to be the predominant bacterial family in the jejunum of crossbred heifers (Wang *et al.*, 2022). Members from this family have been identified as hyper ammonia-producing microbes that can utilize amino acids and could therefore be involved in feed digestion and efficiency (Kim *et al.*, 2014). *Lachnospiraceae* was significantly more abundant while *Ruminococcaceae* tended towards being significantly more abundant in PRO and CON compared to MON and EO. The bacterial species within the *Lachnospiraceae* family have fibre and protein degrading properties, whereas the majority of species belonging to *Ruminococcaceae* are primary degraders of resistant polysaccharides by producing enzymes that breakdown the plant cell walls (Wang *et al.*, 2017).

Among the genera, *Clostridia_XI* and *Romboutsia* were the most abundant in this study, similar to a study conducted in Nubian goats where *Romboutsia* was reported as one of the most abundant genera (Wang *et al.*, 2019). A study in Hu sheep observed *Ruminococcus*, *Lachnospiraceae*, *Olsenella*, *Acetitomaculum* and *Candidatus Saccharimonas* as the most abundant genera found in the jejunal digesta (Zhong *et al.*, 2022) while a study with three-week old Holstein bulls found that genera *Sharpea*, *Butyrivibrio*, *Ruminococus* and *Lactobacillus* were the most abundant (Malmuthuge *et al.*, 2014).

Mogibacterium, *Eubacterium*, *Pseudoramibacter*, *Blautia* and *Ruminococcus* were significantly higher in abundance in PRO compared to MON and EO. *Eubacterium* and *Ruminococcus* aids in hemicellulolytic digestion and biohydrogenation within the digesta (Xue *et al.*, 2020). The *Blautia*

genus has been reported to have a probiotic role within the gastrointestinal tract as a decrease in its abundance has been associated with inflammatory diseases (Liu *et al.*, 2021). A study with goats supplemented with different *Bacillus*-strain probiotics reported that *Bacillus* was possitively correlated with *Faecalibacterium*, *Blautia* and *Roseburia* (Zhang *et al.*, 2020b). *Roseburia* protects the epithelial cells from damage caused by inflammation (Quan *et al.*, 2018) and was significantly higher in abundance within CON compared to the other treatment groups. *Turibacter* was significantly higher within CON and PRO compared to MON and EO. *Turibacter* is known to decrease the animal's vulnerability to *Salmonella* infection (Zhuang *et al.*, 2020). In general, the supplementation of MON and EO decreased the abundance of potentially pathogenic, commensal, and beneficial bacteria in the jejunum microbiome.

The effect of EO on the jejunal microbiota was minimal compared to the other groups with the abundance of few microbes observed to have a significant difference between EO and the other treatment groups. The genus, *Clostridium_sensu_stricto*, was significantly lower in abundance within EO compared to the other treatment groups. *Clostridium_sensu_stricto* has been correlated with various diseases (Call *et al.*, 2018; Dong *et al.*, 2019) and has been reported to increase in abundance during stressful situations (Zhang *et al.*, 2021). Its decrease is therefore beneficial towards the animal. The *Romboutsia* genus tended towards a significantly higher abundance within EO compared to PRO. Species from *Romboutsia* are associated with carbohydrate usage, degradation of amino acids and anaerobic respiration (Gerritsen *et al.*, 2019). *Romboutsia* is also positively correlated with body weight and might therefore have a positive influence on the animal's production. In a study on broilers, no effect was found in the small intestine microbiome when supplemented with an essential oils blend (Altop *et al.*, 2018). The effect of EOs on the microbiome can be influence by various factors such as the active ingredient of the essential oils and the dosage (Calsamiglia *et al.*, 2007).

There is limited literature on the role of fungi in the jejunum as most fermentation and fibre-degradation occurs in the rumen of the ruminant animal. Fibre degradation also occurs in the intestinal tract, however the transit time within the jejunum might be too short to see significant fibre digestion (Myer *et al.*, 2016). Despite no difference between the groups in the alpha diversity of the fungi population in this study, numerically the diversity was higher in CON and EO compared to PRO and MON. The use of monensin is known to decrease the fungal and protozoal population within the rumen (Elliott *et al.*, 1987). However, it is uncertain as to why the fungal diversity would be lower in PRO as well. In this study, Ascomycota was the primary fungal phylum followed by Mucoromycota and Neocallimastigomycota. Ascomycota had a tendency towards a significant difference with a higher abundance in PRO and MON compared to EO. Ascomycota is a known oxygen scavenger within the rumen (Zhang *et al.*, 2020a). As the jejunum is lower in oxygen concentration compared to the rumen (Donaldson *et al.*, 2016), it is possible that the abundance of the fungi is residue from the rumen. To be able to determine if the fungi is active within the jejunum, a metatranscriptomics study is recommended. In EO, Mucoromycota was significantly higher while Neocallimastigomycota was significantly lower

in comparison with the other treatment groups. Mucoromycota is also an aerobic fungi, such as Ascomycota (Zhang *et al.*, 2020a).

It is should be mentioned that the abundance of some of the microorganisms might be due to spillage from the rumen, either as an active organism or as residue, however this might be a small percentage (Myer *et al.*, 2016). Although this study only focussed on the abundance of the microorganisms in the jejunal digesta, various studies have reported that the mucosa-associated microbes are diverse and play an essential part in the immunity and health of the animal (Malmuthuge *et al.*, 2014; Zhang *et al.*, 2021). A mechanisms that protects the animal is the mucus that is secreted from goblet cells in the gastrointestinal tract (Atuma *et al.*, 2001). Throughout the gastrointestinal tract, the mucus layers vary in thickness and bacteria colonize the loosely adhesive layer, however, no colonization takes place in the inner layer (Hansson & Johansson, 2010). The bacteria that colonize the adherent layer possibly stimulate the host immune response through pattern recognition receptors (Malmuthuge *et al.*, 2014). Additional studies are required to investige the influence of nutritional components on the mucosa-associated microbiota.

5.5 Conclusion

This study confirmed that the inclusion of monensin, probiotics, and essential oils can influence the composition and diversity of the jejunal microbiota. The probiotic and control group had a high abundance of beneficial bacteria including *Olsenella*, *Eubacterium*, *Blautia*, and *Roseburia*. In contrast, the supplementation of monensin and essential oils decreased the jejunal microbiome diversity and the abundance of pathogenic and beneficial microbes. Additional research is required to investigate the function of fungi within the jejunal microbiome and to determine the influence of feed additives on the mucosa-associated microorganisms.

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Supplementary Tables

Supplementary Table 5.1 The relative abundance (as percentage) of the bacterial and archaeal phyla, with the respective families and genera, in the jejunum of the control (CON), monensin (MON), essential oils (EO) and probiotic (PRO) groups.

Bacterial/Archaeal taxa	CON (%)	MON (%)	EO (%)	PRO (%)	p-value
Euryarchaeota	6.81	7.49	7.04	11.46	0.148
Methanobacteriaceae	6.81	7.48	7.04	11.44	0.148
Methanobrevibacter	6.79	7.43	6.99	11.35	0.141
Methanosphaera	0.05	0.05	0.04	0.09	0.176
Methanosarcinaceae	0.004	0.01	0.001	0.02	0.196
Methanosarcina	0.003	0.01	0.001	0.02	0.196
Actinobacteria	3.87	1.83	1.84	4.45	0.001*
Coriobacteriaceae	3.87	1.84	1.84	4.45	0.001*
Adlercreutzia	0.41	0.21	0.20	0.34	0.093**
Atopobium	0.02	0.03	0.01	0.02	0.257
Denitrobacterium	0.06	0.02	0.04	0.10	< 0.01*
Eggerthella	0	0	0.01	0.004	0.113
Enterorhabdus	0.01	0.004	0.01	0.02	0.124
Olsenella	1.22	0.49	0.44	1.48	0.001*
Senegalimassilia	0.12	0.05	0.03	0.06	0.042*
Slackia	0.05	0.04	0.05	0.25	0.001*
Candidatus_Saccharibacteria	0.011	0.02	0.03	0.08	0.141
Elusimicrobia	0.12	0.36	0.12	0.20	0.558
Firmicutes	88.19	89.60	90.11	83.34	0.108
Christensenellaceae	0.03	0.01	0.02	0.02	0.149
Clostridiaceae_1	0.96	0.96	0.48	1.04	0.009*
(Clostridium_sensu_stricto)					
Clostridiales_Incertae_Sedis_XIII	7.79	2.93	3.21	5.35	0.014*
Butyricicoccus	0.02	0.001	0.001	0.002	0.010*
Clostridium_IV	0.40	0.19	0.17	0.40	0.254
Eubacteriaceae	0.99	0.32	0.41	1.27	< 0.001*
Anaerovorax	0.68	0.30	0.75	0.90	< 0.001*
Mogibacterium	2.77	1.15	1.12	2.33	0.022*
Eubacterium	0.83	0.27	0.31	0.85	0.001*
Pseudoramibacter	0.16	0.05	0.10	0.42	< 0.001*
Lachnospiraceae	13.13	8.35	6.21	15.57	0.005*
Acetatifactor	0.02	0.01	0.01	0.01	0.469
Blautia	0.05	0.03	0.01	0.12	0.004*

Cellulosilyticum	0.02	0.1	0.01	0.02	0.076**
Clostridium_XlVa	0.71	0.06	0.3	0.26	0.001*
Clostridium_XlVb	0.004	0	0.001	0.01	0.147
Coprococcus	0.03	0.01	0.002	0.02	0.036*
Dorea	0.10	0.04	0.09	0.14	0.009*
Howardella	0.44	0.01	0.01	0.02	0.179
Lachnobacterium	0.001	0.002	0.003	0.02	0.031*
Lactonifactor	0.29	0.23	0.15	0.58	0.003*
Moryella	0.02	0.004	0.004	0.01	0.572
Pseudobutyrivibrio	0.05	0.03	0.03	0.04	0.831
Roseburia	0.48	0.003	0.001	0.002	< 0.001*
Stomatobaculum	3.31	1.63	2.83	1.84	0.695
Syntrophococcus	0.26	0.25	0.11	0.25	0.008*
Asaccharospora	0.05	0.11	0.02	0.08	< 0.001*
Peptostreptococcaceae	51.88	70.29	71.65	45.30	0.021*
Clostridium_XI	31.70	52.23	44.48	28.34	0.002*
Romboutsia	20.10	17.91	27.12	16.81	0.074**
Ruminococcaceae	6.91	3.38	3.69	6.43	0.088**
Ruminococcus2	0.03	0.004	0.003	0.06	0.017*
Gemmiger	0.02	0.002	0.002	0.02	0.099**
Intestinimonas	0.46	0.16	0.06	0.29	0.011*
Oscillibacter	0.04	0.004	0.001	0.01	0.555
Pseudoflavonifractor	0.02	0.002	0.001	0.001	0.074**
Ruminococcus	0.28	0.19	0.18	0.70	0.004*
Saccharofermentans	0.59	0.17	1.07	0.22	0.024*
Sporobacter	0.01	0	0.001	0.02	0.304
Erysipelotrichaceae	0.04	0.08	0.12	0.05	0.058**
Turicibacter	0.12	0.05	0.04	0.08	0.058**
Fusobacteria	0.002	0.004	0	0	0.287
Proteobacteria	0.90	0.64	0.78	0.43	0.175
Bradyrhizobiaceae (Bradyrhizobium)	0.005	0.004	0.004	0.01	0.924
Methylobacteriaceae (Methylobacterium)	0.02	0.02	0.02	0.04	0.951
Acetobacteraceae	0.01	0.001	0.005	0.001	0.199
Rhodospirillaceae	0.39	0.32	0.07	0.05	0.172
Aestuariispira	0.14	0.25	0.03	0.02	0.168
Sphingomonadaceae (Novosphingobium)	0.004	0.004	0.005	0.01	0.995
Bdellovibrionaceae (Vampirovibrio)	0.03	0.01	0.02	0.06	0.848
Campylobacteraceae (Campylobacter)	0.17	0.09	0.47	0.01	0.018*

Helicobacteraceae (Helicobacter)	0.02	0.03	0.05	0.04	0.326
Succinivibrionaceae (Succinivibrio)	0.15	0.02	0.01	0.06	< 0.001*
Enterobacteriaceae	0.05	0.12	0.13	0.08	0.027*
Escherichia/Shigella	0.004	0.02	0.02	0.007	0.026*
Synergistetes	0.09	0.05	0.08	0.05	0.911
Synergistaceae	0.09	0.05	0.08	0.05	0.911
Cloacibacillus	0.05	0.03	0.06	0.04	0.825
Pyramidobacter	0.02	0.01	0.01	0.01	0.610
Synergistes	0.01	0.004	0.001	0.001	0.329

* Significance at $p < 0.05$

** Tendency towards significance at $p < 0.10$

Supplementary Table 5.2 The relative abundance (as percentage) of the fungal phyla, with the respective families and genera, in jejunum of the control (CON), monensin (MON), essential oils (EO) and probiotic (PRO) groups.

Fungal taxa	CON (%)	MON (%)	EO (%)	PRO (%)	p-value
Ascomycota	90.61	93.74	89.34	93.33	0.052**
Cladosporiaceae (Cladosporium)	0.14	0.01	0.03	0.03	0.085**
Aureobasidiaceae (Aureubasidium)	0.003	0.003	0.01	0.006	0.682
Didymellaceae	0.04	0.01	0.02	0.02	0.675
Neoascochyta	0.003	0.003	0.01	0.01	0.575
Didymosphaeriaceae	0.02	0.03	0.01	0.04	0.490
Paraconiothyrium	0.007	0.02	0.007	0.03	0.584
Paraphaeosphaeria	0.01	0.005	0.007	0.01	0.643
Massarinaceae	0.01	0.002	0.002	0.005	0.994
Pleosporaceae	0.01	0.004	0.003	0.004	0.069**
Epicoccum	0.01	0.01	0.001	0.006	0.561
Stagonospora	0.01	0.002	0.002	0.005	0.994
Alternaria	0.01	0.004	0.003	0.004	0.069**
Sporormiaceae	0.08	0.04	0.02	0.04	0.203
Preussia	0.05	0.03	0.01	0.04	0.180
Aspergillaceae (Aspergillus)	4.79	2.27	0.77	2.96	0.303
Trichocomaceae (Thermomyces)	0.02	0.02	0.01	0.07	0.087**
Phaffomycetaceae (Wickerhamomyces)	0.27	0.91	0.25	0.60	0.393
Saccharomycetaceae	0.01	0.01	0.01	0.03	0.032*
Torulaspora	0	0.001	0	0.005	0.068**
Saccharomycetales_fam_Incertae_sedis (Candida)	0.07	0.25	0.45	0.02	0.684
Hypocreales_fam_Incertae_sedis (Harzia)	0.01	0.02	0	0	0.023*

Nectriaceae	0.03	0.03	0.01	0.006	0.870
Fusarium	0.01	0.001	0.004	0.006	0.423
Gibberella	0.02	0.03	0.004	0	0.082**
Microascaceae	0.02	0.02	0.02	0.12	0.329
Microascus	0.001	0.003	0.005	0.04	0.127
Chaetomiaceae	0.03	0.03	0.02	0.02	0.912
Chaetomium	0.02	0.003	0.01	0.006	0.893
Mycothermus	0.01	0.02	0.01	0.008	0.793
Thielavia	0.0004	0.001	0.002	0.001	0.899
Sordariaceae (Sordaria)	0.006	0.17	0.001	0.002	0.337
Sordariales_fam_Incertae_sedis (Remersonia)	0.03	0.02	0.22	0.005	0.214
Togniniaceae (Phaeoacremonium)	0.06	0.002	0.10	0.005	0.44
Trichosphaeriaceae (Nigrospora)	0.01	0.01	0.0001	0.003	0.607
Basidiomycota	1.99	0.96	0.94	0.93	0.186
Lycoperdaceae (Lycoperdon)	0.01	0.01	0.01	0.02	0.163
Strophariaceae (Psilocybe)	0.01	0.001	0.001	0.004	0.327
Geastraceae (Nidulariopsis)	0.02	0.002	0.002	0.001	0.629
Sphaerobolaceae (Sphaerobolaceae)	0.08	0.02	0.04	0.01	0.031*
Phallaceae	0.06	0.02	0.11	0.01	0.006*
Lysurus	0.06	0.01	0.11	0.01	0.004*
Malasseziaceae (Malassezia)	0.09	0.001	0.002	0	0.038
Sporidiobolaceae (Rhodotorula)	1.26	0.73	0.53	0.62	0.659
Filobasidiaceae (Naganishia)	0.40	0.15	0.23	0.14	0.354
Ustilaginaceae (Ustilago)	0.06	0.03	0.01	0.11	0.169
Mucoromycota	2.90	1.68	8.91	2.61	0.001*
Lichtheimiaceae	0.23	0.07	0.12	0.51	0.05**
Lichtheimia	0.03	0.02	0.04	0.22	0.11
Mucoraceae	2.66	1.61	8.74	2.09	0.001*
Rhizomucor	0.20	0.05	0.05	0.22	0.262
Mucor	2.66	1.61	8.74	2.09	0.002*
Rhizopodaceae	0.005	0.001	0.05	0.01	0.016*
Neocallimastigomycota	4.49	3.62	0.82	3.13	0.034*
Neocallimastigaceae	4.49	3.62	0.82	3.13	0.034*
Caecomyces	4.25	3.56	0.77	3.08	0.055**
Neocallimastix	0.23	0.06	0.04	0.05	0.182
Rozellomycota	0.005	0.002	0	0.006	0.066**

* Significance at $p < 0.05$

** Tendency towards significance at $p < 0.10$

Chapter 6

Non-invasive approaches for sample collection in rumen microbiome studies

Abstract

The most common method used for rumen sample collection through a cannula which is an invasive technique that can limit the samples size of a study. Alternative less invasive techniques, such as the use of a stomach tube or collecting sample content at slaughter, needs to be investigated. The aim of this study was to investigate the rumen microbiome composition of samples collected using a stomach tube and at slaughter after feed was withdrawn. Rumen content was collected via a stomach tube from sixteen Bonsmara bulls in the finishing phase of a feedlot period and at slaughtered. DNA extraction was performed on the samples and sent for 16S rRNA and internal transcribed spacer (ITS) sequencing. Phyloseq and DADA2 were used for bioinformatic analyses in which 11 773 and 11 021 amplicon sequence variants (ASVs) were identified for 16S rRNA and ITS sequencing, respectively. A significant difference in the rumen microbial composition between the samples collected using the two different techniques were observed. Higher bacterial diversity was found within the samples collected at slaughter compared to the samples collected using a stomach tube. A significant difference in beta diversity between the two methods indicated different microbial profiles. Proteobacteria was the predominant phylum in the samples collected by stomach tube while Bacteroidetes was more abundant in the samples collected at slaughter. Diet can influence the microbial composition and feed was withdrawn from the animals before slaughter. It is recommended that studies investigating the influence of dietary components on the rumen microbiome utilize a stomach tube to collect samples or if collecting rumen content at slaughter, not to withdraw the feed from the animals.

Keywords: microbiome composition, sampling, slaughter, stomach tube

6.1 Introduction

Researchers have new approaches to study the rumen microbiome, on account of the availability of next-generation sequencing (NGS) technology (Deusch *et al.*, 2015). As ruminants are labelled for contributing to methane emissions, an increased understanding of the rumen microbiome and associated interactions are essential (Wang *et al.*, 2017).

The collection of representative rumen content samples has been widely debated as different techniques have certain advantages and limitations (Steiner *et al.*, 2015). Three common methods used to sample the rumen for microbiome studies are, through a cannulated animal (Anderson *et al.*, 2016;

Martinez-Fernandez *et al.*, 2019), using a stomach tube (Baek *et al.*, 2020; Stergiadis *et al.*, 2021) and at slaughter (Du *et al.*, 2019; Lopes *et al.*, 2021). Sampling using a rumen cannula has been the standard method of collection of rumen digesta for fermentation characteristics and microbiome community analyses (Nocek, 1997). Despite the advantage of direct collection of digesta from the rumen, this method is invasive, costly and has animal welfare implications (de Assis Lage *et al.*, 2020).

Oesophageal or stomach tubing is non-invasive, less expensive and can be applied to a number of animals, resulting in the increase in the statistical power of the study. Possible saliva contamination, variable positioning within the rumen of the tube and erratic sampling of the liquid and solid fractions (Duffield *et al.*, 2004; Shen *et al.*, 2012) have restricted the use of oesophageal tubes for rumen microbiome composition studies. According to Paz *et al.* (2016), representative rumen samples can be collected using an oesophageal tube as long as rumen fluid and rumen particles are included in the samples.

The collection of rumen samples at slaughter is non-invasive and representative rumen samples containing both solid particles and fluid can be collected (Roehe *et al.*, 2016). This technique is convenient when the microbiome of complete gastrointestinal tract is studied as all samples can be collected at once (Myer *et al.*, 2015; Freetly *et al.*, 2020).

Studies have reported that both collection of rumen content at slaughter as well as through a stomach tube can be compared to cannulated animals when investigating the rumen microbiome composition (Wallace *et al.*, 2014; Song *et al.*, 2018). Due to the cost of cannulation and the welfare of the animal with regard to care and infection, the effect of non-invasive methods, such as tubing and collection at slaughter, on the rumen microbiome were the focus of this study.

6.2 Materials & methods

This research forms part of a larger study with ethics approval (NAS445/2019) in which the effect of different feed additives was investigated on the rumen microbiome of 48 Bonsmara bull calves under intensive feeding conditions. Details of the methodology in regard to the animals, diets and statistical analyses can be found in Linde *et al.* (2022) and Chapter 4. For this analysis, data originating from sample collection at finisher (n = 16) and at slaughter (n = 16) were used.

One animal from each pen (n=16) was randomly selected to have rumen content collected via stomach tube one week before slaughter approximately two hours after feeding. A flexible tube was inserted into the animal's mouth by a trained veterinarian until it reached the ventral sac of the rumen. Negative pressure was then applied to the tube to force out rumen content (both particles and fluid). The first 50 ml of rumen content was discarded, and visual examination done to prevent saliva contamination. The next 50 ml was immediately frozen in liquid nitrogen. The stomach tube was flushed with warm water between being inserted into each animal to prevent contamination.

Feed was withdrawn 24 hours before slaughter to obtain an empty live weight as per the feedlot's regulations. At slaughter, a cut was made with a sterilized knife in the ventral sac of the rumen and whole rumen content (fluid and solid particles) was collected from all animals and immediately frozen in liquid nitrogen. The rumen content would have mixed sufficiently through the slaughter process to take representative samples. All whole rumen content samples were deposited in a -80 ºC freezer until DNA extraction could be completed.

Extracted DNA was submitted for 16S rRNA (V3-V4) and ITS amplicon sequencing using Illumina NovaSeq 250 (Illumina, USA). The primers used for 16S rRNA sequencing was 341F (5'CCTAYGGGRBGCASCAG) and 806R (5'GGACTACNNGGGTATCTAAT) and for ITS sequencing F-5'GGAAGTAAAAGTCGTAACAAGG and R-5'GCTGCGTTCTTCATCGATGC. Average reads generated was 197 234 ± 14 702 and 191 785 ± 22 672 for 16S rRNA and ITS sequencing, respectively. DADA2 (Callahan *et al.*, 2016) was primarily used for bioinformatic analyses. The Ribosomal Database Project (Cole *et al.*, 2014) was used for 16S rRNA annotation and the UNITE database (Nilsson *et al.*, 2019) for the fungal annotation. Taxonomy was assigned to genus level.

The data was rarefied to minimum sequence reads and amplicon sequence variants (ASV) detected in 5% of the samples less than ten times were removed. Alpha diversity (observed number of ASVs, Shannon diversity and Chao1 richness indices) was calculated with phyloseq (McMurdie & Holmes, 2013), while beta diversity was determined by using weighted and unweighted UniFrac distances illustrated with a principal coordinate analysis (PCoA) plot (Lozupone *et al.*, 2007). Permutational multivariate analysis of variance (PERMANOVA) (Oksanen *et al.*, 2020) and analysis of similarities (ANOSIM) were performed to ascertain the differences between the microbial compositions. For ANOSIM, an R value between 0.5 and 0.75 indicated different microbiome profiles, between 0.25 and 0.5 different profiles with some overlap and between 0.1 and 0.25 similar profiles.

Significant differences in the relative abundance of the taxa and in alpha diversity were determined by the Kruskal Wallis test. Multiple test correction was performed using the Holm-Bonferroni procedure. Significance was recognized at $p < 0.05$ and trends at $0.05 < p < 0.1$. Correlation in the abundance of the taxa between the samples collected using stomach tubing and immediately after slaughter was tested using Spearman's correlation.

6.3 Results

After quality control and chimera removal, 127 106 ± 12 387 and 146 502 ± 17 550 reads remained for 16S rRNA and ITS sequencing, respectively. The amount of amplicon sequence variants (ASV) identified with 16S rRNA sequencing was 11 773 and with ITS sequencing 12 327.

The alpha diversity parameters shown in Table 6.1 revealed a significant difference ($p < 0.05$) between the two methods. It should be noted that the alpha diversity parameters did not differ for the feed additives (data not shown).

Table 6.1 The average and standard deviation of the alpha diversity (observed number of ASVs, Chao1 and Shannon indices) of the rumen samples collected using a stomach tube or at slaughter for the 16S rRNA and ITS rumen microbial populations.

Alpha diversity indices	Stomach tube	At Slaughter	p-value
16S rRNA			
Observed number of ASVs	749 ± 98	842 ± 88	0.013*
Chao1 Index	752 ± 97	845 ± 87	0.014*
Shannon Index	3.62 ± 0,55	4.64 ± 0.49	0.0001*
ITS			
Observed number of ASVs	150 ± 13	106 ± 23	<0.0001*
Chao1 Index	150 ± 13	107 ± 23	<0.0001*
Shannon Index	3.22 ± 0.16	2.19 ± 0.62	0.0001*

* Significance at $p < 0.05$

Both the sampling method ($p = 0.001$) as well as the feed additive ($p = 0.014$) revealed significant difference in beta diversity for the 16S rRNA microbial population with PERMANOVA. This was confirmed in the principal coordinates plot (PCoA) using unweighted UniFrac distances (Figure 6.1). However, in the PCoA plotted using weighted UniFrac distances no clustering of the feed additive treatment groups were observed. The axes of the unweighted UniFrac PCoA plot explained 25.9% while the axes of the weighted UniFrac PCoA 55.2% of the variation. Analysis of similarities (ANOSIM) showed that the samples from the different sampling methods had different microbial profiles ($R > 0.56$) in terms of phylum composition, but samples obtained from the animals supplemented with different feed additives showed similar microbial profiles ($R < 0.25$).

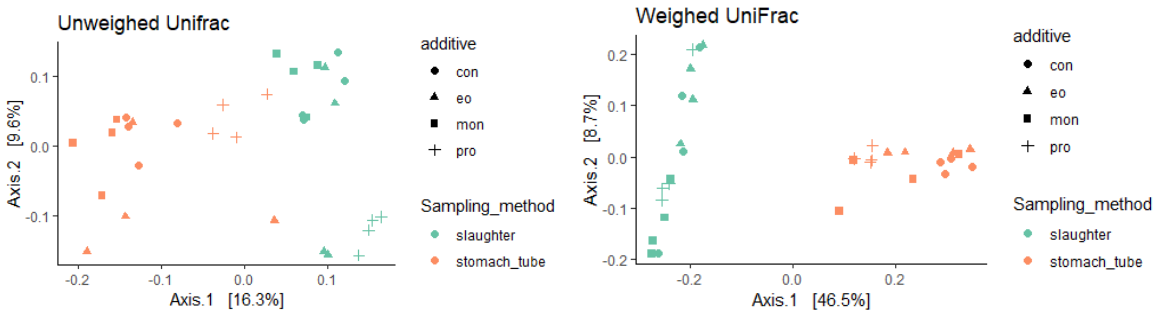

Figure 6.1 Principal coordinate analyses (PCoA) plots depicting the unweighted (left) and weighted UniFrac (right) analyses of the feed additives and sampling method used in the 16S rRNA population with sampling methods depicted by different colours and the feed additives by different shapes.

The beta diversity of the rumen fungal population differed significantly between the two sampling techniques (p = 0.001) but not between the additives (p = 0.214). The PCoA plot using unweighted and weighted UniFrac distances indicated clustering of the samples collected by stomach tube (Figure 6.2). The PCoA axes of the unweighted and weighted UniFrac analysis of the fungal composition explained 38.1% and 56.9% of the variation, respectively. Analysis of similarities also showed that the fungal population of the samples collected with two different sampling methods had different microbial profiles (R > 0.5) while the fungal population of the animals fed different feed additives showed similar microbial profiles (R < 0.25).

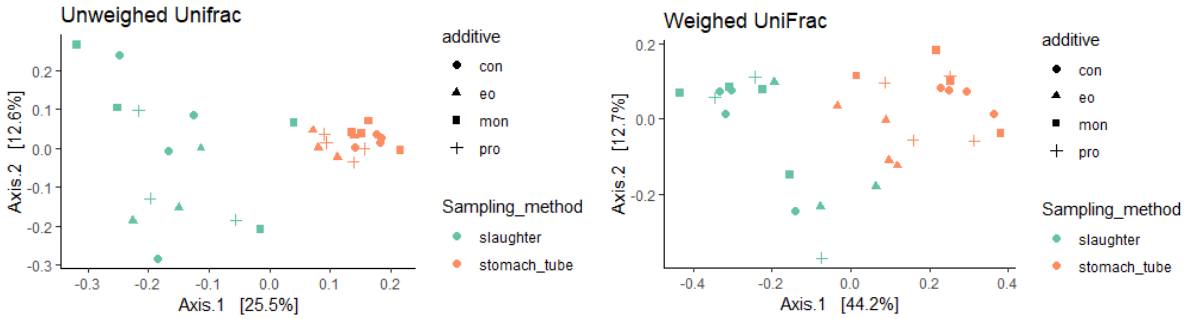

Figure 6.2 The PCoA plot based on unweighted (left) and weighted (right) UniFrac distances of the ITS rumen population of samples collected with different techniques, depicted in different colours, and collected from animals fed different feed additives (depicted in different shapes).

Differences between the sampling techniques were observed in individual taxa (Figure 6.3). The most abundant phyla were Proteobacteria (54.02%), followed by Bacteroidetes (28.60%) and Firmicutes (15.43%) in the samples collected using a stomach tube, while Bacteroidetes (56.97%) followed by Firmicutes (25.67%) were the most predominant phyla in the samples at slaughter. The relative abundance of the bacterial/archaeal phyla and families can be found in Supplementary Table 6.1. *Ruminococcaceaea* and *Prevotellaceae* were the most abundant families for both collection methods.

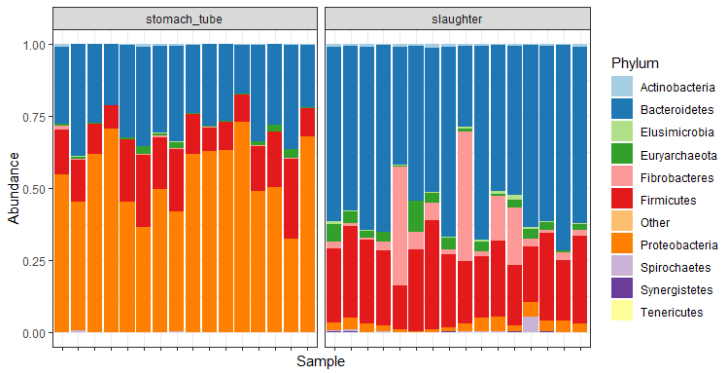

Figure 6.3 The relative abundance of the phyla, depicted in different colours, of the 16S rRNA rumen microbial population of samples collected via stomach tube or at slaughter.

Most of the fungal phyla, Ascomycota ($p < 0.001$), Basidiomycota ($p = 0.028$), Chytridiomycota ($p = 0.025$) and Neocallimastigomycota ($p = 0.014$), indicated a significant difference in abundance between the sampling techniques (Figure 6.4). Ascomycota (47.13%) and Neocallimastiogmycota (47.06%) were the most predominant phyla in the samples collected using a stomach tube. The samples collected at slaughter had a higher abundance of Neocallimastigomycota (74.29%). The relative abundance of the fungal phyla and families are attached as Supplementary Table 6.2.

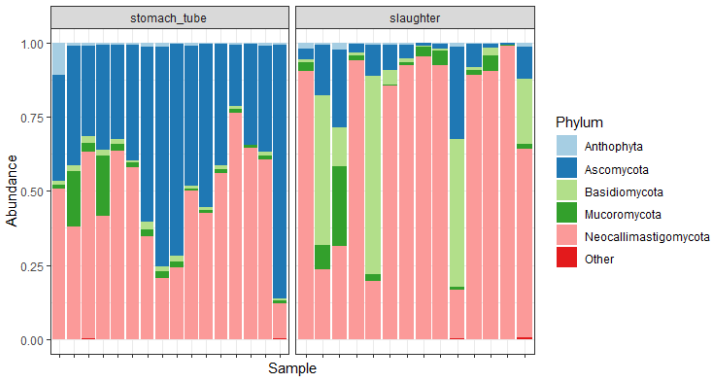

Figure 6.4 The relative abundances of the fungal phyla from samples collected using a stomach tube or at slaughter. The phyla are depicted as different colours indicated in the legend to the right of the graph.

The *Neocallimastigaceae* family was the most abundant family regardless of sampling technique. The samples taken using a stomach tube had a higher abundance of the *Aspergillaceae* family ($p < 0.001$). The genus *Caecomyces* was significantly more abundant ($p < 0.001$) in the samples collected at slaughter compared using a stomach tube while *Neocallimastix* was numerically ($p = 0.158$) more abundant in the samples collected using a stomach tube.

There was no correlation between the phyla abundance of the 16S rRNA and ITS populations between the two techniques.

6.4 Discussion

Although cannulation is regarded as a standard in livestock studies to collect rumen samples, the method required is costly, requires surgical insertion of a cannula and limits the sample size (Ramos-Morales *et al.*, 2014). Alternative techniques such as collecting samples by stomach tube and at slaughter have been reported to have similar microbial profiles compared to cannulation (Terré *et al.*, 2013; Wallace *et al.*, 2014). A significant difference in the rumen microbiome between the samples collected at slaughter and the samples collected using a stomach tube was observed in this study.

Saliva can possibly contaminate samples taken via stomach tube resulting in incorrect measurements (Garrett *et al.*, 1999). In this study, the first 50 ml was disposed of to safeguard against saliva contamination and visual examination was conducted. Stomach tube collection should be done by an experienced and qualified individual to ensure that the tube remains stable while inserted, and the procedure is done swiftly to keep saliva contamination minimal (Lodge-Ivey *et al.*, 2009) .

Previous research reported that the diversity and richness of the microbial population found in the rumen microbiome decreased after slaughter (Wallace *et al.*, 2014) even without the withdrawal of feed before slaughter. In this study, the alpha diversity of the samples collected at slaughter had a significantly higher diversity and richness of bacteria and significantly lower fungal diversity and richness compared to the samples collected via stomach tube. It was expected that the diversity of the bacterial population would be decreased in the samples collected after slaughter as feed was withdrawn from the animals for approximately 20 hours before slaughter. A decrease in feed intake results in a decrease in passage rate and in the diversity of the rumen microbiome as no substrates enter the rumen for microbial growth (de Assis Lage *et al.*, 2020; Freetly *et al.*, 2020). However, studies with feed withdrawn from animals (Freetly *et al.*, 2020; Welch *et al.*, 2020) had a similar rumen microbial composition compared to studies in which the animals were slaughtered without the withdrawal of the feed (Wallace *et al.*, 2015; Martínez-Alvaro *et al.*, 2020). The samples collected via stomach tube originated from animals in the finisher phase of a feedlot period in which a high concentrate diet was fed resulting in a lower microbial diversity (Petri *et al.*, 2020). The low Shannon diversity index in the samples collected via stomach tube could be an indication of dysbiosis (Du *et al.*, 2018). The withdrawal of the high concentrate feed, and therefore a possible increase in pH, could have resulted in an increase in the rumen diversity which was observed in the samples collected at slaughter. This may indicate the resiliency and plasticity of the rumen bacterial population to return to normal conditions, as determined by host genetics (Roehe *et al.*, 2016), after a disturbance has been removed. The rumen microbiome community is known to be compositionally and functionally resilient (Weimer, 2015).

Samples collected via stomach tube have been reported to contain less particles due to larger pieces getting fixed in the tube which could lead to underestimating the number of microorganisms attached to the particles and reporting a lower microbial diversity (Ramos-Morales *et al.*, 2014). As more bacteria can attach to particles compared to free-flowing in liquid, the richness in the particle fraction is higher in comparison to the liquid fraction in samples collected from animals fed roughage based diets (Kong *et al.*, 2010). However, other studies observed that the two fractions had similar community profiles in animals fed predominantly concentrate rations (Schären *et al.*, 2017; McGovern *et al.*, 2018) possibly due to the decrease in particles in the rumen (Sadet *et al.*, 2007). Collecting representative rumen samples that consist of particles and fluid is important to define the microbiome composition (Paz *et al.*, 2016). In this study, care was taken with the samples collected via stomach tube to include solid particles.

The specific rumen location sampled can additionally influence the results generated from samples collected via stomach tube (Shen *et al.*, 2012). Saliva and water provided to the reticulo-rumen by the oesophagus may result in the dilution of rumen contents when sampled from that point using stomach tubing. The presence and weight of a tube in the mouth and oesophagus can apply pressure across the ruminoreticular fold resulting in the stimulation of saliva production over time. The ventral rumen sac was reported to be the ideal place for rumen content sampling, as the saliva content there is minimal with a high volume of rumen fluid (Dirksen & Smith, 1987). The higher fluid volume, fewer large particles and lower dry matter content in the rumen ventral sac avoids the obstruction of the stomach tube and the resulting delay in sampling (Zebeli *et al.*, 2007). However, studies have also shown that sampling from different rumen sites did not result in a difference in microbial composition (de Assis Lage *et al.*, 2020).

A significant difference was observed between the feed additive groups in the beta diversity of the bacterial population according to PERMANOVA but not in the fungal population. More specific differences in the rumen bacteria composition due to feed can be detected through stomach tubing compared to the use of cannulae (Ramos-Morales *et al.*, 2014). From the unweighted Unifrac distances plotted through a principle coordinates analysis (PCoA), it can be seen that the probiotic group clustered separately from the rest of the samples, however this is not observed in the PCoA of the weighted Unifrac distances where the abundance of the taxa in the samples is considered (Lozupone *et al.*, 2007). The difference in microbiome composition due to the feed additives are as a result of the higher diversity and lower Proteobacteria abundance found in the animals supplemented with the probiotic. This indicates that a trace of the effect of feed additives can still be found after feed is withdrawn from the animals.

Although studies have indicated a general similarity with regard to the microbiome composition when comparing samples collected through a stomach tube and rumen canula, using different sampling methods can lead to a differentiation in the relative abundance of certain taxa (Lodge-Ivey *et al.*, 2009; Henderson *et al.*, 2013). The predominant phyla were Proteobacteria, Bacteroidetes and Firmicutes in the samples collected using a stomach tube while Bacteroidetes followed by Firmicutes were the most predominant phyla in the samples collected at slaughter. The microbial composition found in the samples collected at slaughter are more in line with what other studies have reported for the microbial composition in the rumen (Freetly *et al.*, 2020; Zhou *et al.*, 2021). However, this difference is most possibly as a consequence of the withdrawal of feed and not due to sampling method.

In contrast to Henderson *et al.* (2013) who reported no influence of sampling method on the anaerobic fungi in samples collected through a cannula and stomach tube, this study indicated that the fungal phylum Ascomycota was more abundant in the samples collected using a stomach tube versus at slaughter. The Neocallimastigomycota phylum is noted to be the predominant anaerobic fungi in the rumen (Gruninger *et al.*, 2014) which is in line with the fungal population found in the samples collected

at slaughter. The abundance of Neocallimastigomycota and Ascomycota are negatively correlated with each other due to one being aerobic and the other anaerobic (Fliegerova *et al.*, 2021).

A variety of factors can affect the rumen community composition and can be categorized into two group: genuine differences in the composition and the differences due to experimental methodology used (Weimer, 2015). Standardization of rumen microbiome studies are necessary as caution should be used when interpreting abundance data across studies due to different methods employed to study the rumen microbiome composition. To compare studies across the world, sampling of the rumen, the DNA extraction method and the target gene used should be the same or at least shown to have comparable results (Henderson *et al.*, 2013).

The technique employed to sample rumen content is largely determined by the aim of the study or trial. Samples collected by tubing permits an easier analysis of the rumen microbial differences in animals that have been selected for important traits (Henderson *et al.*, 2013) and allows a large number of individuals to be sampled. Determining the influence diet has on the rumen microbiome composition can also be more easily observed with a stomach tube. Studies investigating the whole gastrointestinal tract, from the rumen through to the large intestine, collect samples at slaughter as all samples are collected at the same time point (Myer *et al.*, 2016; Freetly *et al.*, 2020). The effect of nutrition on the microbiome composition of the gastrointestinal tract can also be investigated through this method, however it is recommended not to withdraw feed from the animals before slaughter. If feed is withdrawn, a trace of the effect of the dietary components on the microbial composition can be detected, however it might be different from when the animal is actively consuming the diet. As withdrawal of feed before slaughter is a common procedure in commercial feedlots, it might not be practical in commercial feedlot studies.

6.5 Conclusion

In conclusion, the rumen microbial composition of the samples collected using a stomach tube and at slaughter differed significantly. It is recommended that studies investigating the effect of different nutritional components on the rumen microbiome composition utilize a stomach tube with the correct protocol to collect rumen fluid. Samples collected after slaughter can also be used, but feed should not be withdrawn.

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Supplementary Tables

Supplementary Table 6.1 The relative abundance (as percentage) of bacterial and archaeal phyla, with the respective families, in the rumen samples collected via stomach tube and at slaughter.

Taxa	Slaughter (%)	Stomach tube (%)	Adjusted p-value
Euryarchaeota	3.22	0.94	<0.001
Methanobacteriaceae	3.05	0.92	<0.001
Methanomassiliicoccaceae	0.17	0.02	<0.001
Actinobacteria	0.78	0.43	0.001
Corynebacteriaceae	0.03	0.01	<0.001
Micrococcaceae	0.03	0.02	0.359
Coriobacteriaceae	0.71	0.39	0.001
Bacteroidetes	56.97	28.60	<0.001
Bacteroidaceae	0.04	0.03	0.848
Bacteroidales_incertae_sedis	0.04	0.05	0.018
Marinilabiliaceae	1.37	0.20	<0.001
Porphyromonadaceae	2.45	1.71	0.070
Prevotellaceae	18.08	22.13	0.029
Prolixibacteraceae	0.04	0.03	0.266
Rikenellaceae	1.36	0.23	<0.001
Flavobacteriaceae	0.15	0.03	0.001
Sphingobacteriaceae	15.86	0.67	<0.001
Elusimicrobia	0.47	0.08	<0.001
Fibrobacteres	9.43	0.33	<0.001
Fibrobacteraceae	9.43	0.33	<0.001
Firmicutes	25.67	15.43	<0.001
Christensenellaceae	0.04	0.01	<0.001
Clostridiaceae_1	0.04	0.22	0.002
Clostridiales_Incertae_Sedis_XIII	0.26	0.05	<0.001
Eubacteriaceae	0.10	0.04	<0.001
Lachnospiraceae	7.67	5.40	0.012
Ruminococcaceae	13.19	8.33	0.003
Erysipelotrichaceae	0.47	0.29	0.051
Acidaminococcaceae	0.04	0.05	0.895
Veillonellaceae	0.08	0.12	0.038
Proteobacteria	2.81	54.03	<0.001
Rhodospirillaceae	0.76	0.20	0.002
Bdellovibrionaceae	0.27	0.16	0.356
Succinivibrionaceae	0.20	1.26	0.002
Spirochaetes	0.49	0.10	0.019

SR1	0.01	<0.01	0.429
Synergistetes	0.12	0.05	<0.001
Tenericutes	0.03	0.02	0.056

* Significance at $p < 0.05$

** Tendency towards significance at $p < 0.10$

Supplementary Table 6.2 The relative abundance (as percentage) of fungal phyla, with the respective families, in the rumen samples collected via stomach tube and at slaughter.

Fungal Taxa	Slaughter (%)	Stomach tube (%)	Adjusted p-value
Ascomycota	7.80	47.13	<0.001
Cladosporiaceae	0.06	0.16	0,003
Aureobasidiaceae	0.05	0.98	<0.001
Dothideales_fam_Incertae_sedis	0.14	0.69	<0.001
Didymellaceae	0.97	15.31	<0.001
Didymosphaeriaceae	0.06	0.75	<0.001
Phaeosphaeriaceae	0.02	0.27	<0.001
Aspergillaceae	1.44	18.65	<0.001
Trichocomaceae	0.04	0.56	<0.001
Debaryomycetaceae	0.02	0.18	<0.001
Phaffomycetaceae	3.57	0.27	0.008
Diaporthaceae	0.01	0.21	<0.001
Hypocreales_fam_Incertae_sedis	0.04	0.69	<0.001
Nectriaceae	0.25	5.53	<0.001
Microascaceae	0.03	0.13	0.900
Chaetomiaceae	0.02	0.22	<0.001
Trichosphaeriaceae	0.02	0.15	<0.001
Basidiomycota	14.21	1.34	0.028
Sporidiobolaceae	1.01	0.47	0.001
Filobasidiaceae	0.57	0.45	0.183
Trichosporonaceae	11.85	0	0.002
Ustilaginaceae	0.02	0.19	0.003
Mortierellomycota	0.03	0.01	0.300
Mucoromycota	3.06	3.45	0.131
Lichtheimiaceae	3.00	3.19	0.005
Mucoraceae	3.00	3.19	0.077
Neocallimastigomycota	74.29	47.06	0.013
Anthophyta	0.59	1.01	0.724
Poaceae	0.59	1.01	0.724

* Significance at $p < 0.05$

** Tendency towards significance at $p < 0.10$

Chapter 7
Critical Review and Discussion

Efficient ruminant production relies on the knowledge of factors that can influence the production of the animal. One of the factors that determine the efficiency of the animal that has obtained more attention in recent years is the microorganisms that are found in the rumen and gastrointestinal tract. A large number of microorganisms in the rumen cannot be cultured which makes it challenging to study their presence and role in the rumen microbiome (Weimer, 2015). Due to the decrease in the cost of next generation sequencing and the simplification of bioinformatic pipelines, metagenomic sequencing techniques are being increasingly used for rumen microbiome studies.

The rumen microorganisms are partially responsible for the amount of energy (approximately 70%) the animal can use for maintenance and production (Firkins & Yu, 2015). The energy available to the animal is determined by fermentation products, such as volatile fatty acids and H_2, and by-products, such as methane, produced by microorganisms during the fermentation process. Certain microorganisms that have been linked with high feed efficiency in the animal, such as *Succinivibrionaceae*, produces propionate which in turn releases more energy for the animal to use (Hernandez-Sanabria *et al.*, 2012). Methanogens, which are mostly from the Euryarchaeota phylum, use energy the animal could have used to produce methane. Various factors may influence the pathways within the rumen towards more energy being available to the animal. Factors known to modify the rumen microbiome include the quantity and quality of the diet, feed additives and the genetics of the animal (Henderson *et al.*, 2015). The aim of this study was to investigate the effect of feed additives on the rumen and jejunal microbiome of Bonsmara cattle raised under intensive feedlot conditions using amplicon sequencing.

The proportion of roughage and concentrate in the diet can affect the composition of amylolytic, cellulolytic, Gram-positive and Gram-negative bacteria (Stanton *et al.*, 2020). This in turn affects the fermentation characteristics, the proportion and concentration of the volatile fatty acids as well as the production of by-products, such as methane. In Chapter 3 of this study, amplicon sequencing was used to study the rumen microbiome composition in Bonsmara cattle during a 120-day growth trial. The bacterial, archaeal, and fungal composition changed as the diet increased in concentrate and decreased in roughage portion from backgrounding and the starter phase through to the finisher phase. As the feedlot period progressed, the abundance of fibrolytic bacteria, such as Bacteroidetes, decreased while the abundance of amylolytic bacteria, such as Proteobacteria increased. Some pathogenic species also belong to the Proteobacteria phylum and its abundance has been suggested as an indicator for dysbiosis (Shin *et al.*, 2015; Auffret *et al.*, 2017). The finisher phase is known to be a period of risk in which the rumen microbiome can develop subacute and acute acidosis as a result of an unbalanced rumen (Khafipour *et al.*, 2016). As Proteobacteria are more abundant in the animals within the finisher phase,

precision nutrition strategies to decrease the abundance of pathogenic species but maintain the beneficial microbes in the finisher phase should be investigated. This could be in the form of supplementing feed with specific feed additives at a time point where possible dysbiosis can occur.

As discussed in Chapter 3, the alpha diversity of the samples decreased from backgrounding to finisher as the fraction of concentrates in the diet increased. A wider range of carbohydrate substrates can be found in predominantly roughage diets, such as during backgrounding, resulting in a more diverse rumen microbial composition (Belanche *et al.*, 2012). A lower microbial diversity has been associated with a more efficient animal as it is a more specialized rumen microbiome that does not produce fermentation products that are not utilized (Zhou *et al.*, 2009; Shabat *et al.*, 2016). A higher diversity has been associated with a healthy microbiome (Yeoman & White, 2014) due to its redundancy and resilience (Weimer, 2015). The moment dysbiosis occurs and the redundancy decreases, opportunistic pathogens can increase in abundance resulting in a negative effect on the animal (Krause *et al.*, 2013). This brings to question how much farmers and animal scientists can manage the animals to be productive while still maintaining the health of the animal. In this study (Chapter 3), the animals in the finisher phase had the lowest diversity and while no signs of acidosis were observed, the possibility exists that sub-clinical acidosis might have occurred. As a lower alpha diversity of the microbiome composition within the rumen is associated with a higher efficiency in the animal but a higher alpha diversity is associated with a healthier, balanced rumen microbiome composition, a balance between the efficiency of the animal and its resilience due to the redundancy in the microbiome composition needs to be investigated. Studies will have to be conducted to determine the level at which a low alpha diversity can be considered as dysbiosis that will negatively affect the animal.

One of the ways in which dysbiosis can be prevented and the efficiency of an animal can increase, is by using feed additives that modify the rumen microbiome towards more favourable pathways. Monensin is an ionophore that is used in feedlots to increase the production and efficiency of the animal (Samuelson *et al.*, 2016). Its mode of action entails the inhibition of acetate-producing microbes while aiding the growth of propionate-producing microbes resulting in a lower acetate to propionate ratio (Duffield *et al.*, 2012; Azzaz *et al.*, 2015). However, the European Union (EU) banned its use due to the threat of the development of antibiotic-resistant bacteria. Although the use of ionophores have not yet been banned in South Africa, the possibility exists that in the near future its use will be banned. For such an eventuality, alternatives should be in place. Neighbouring countries and farmers that export meat to the EU cannot use ionophores and alternatives need to be considered to increase the production of the animals.

Natural feed additives, probiotics and essential oils, were investigated as possible alternatives to the use of monensin in this study by investigating their effect on the rumen microbiome composition (Chapter 4). There are studies that have reported an increase, decrease or no effect in production parameters when these feed additives were fed to the animals. No substantial significant differences in the rumen bacterial and fungal population between the feed additive groups were found in this study,

indicating the possibility of using probiotics and essential oils in place of monensin. The probiotic group had a significantly lower richness in the grower phase, but a significantly higher diversity in the finisher phase compared to the other treatment groups. As mentioned previously, a high alpha diversity has been linked to a healthy and resilient microbiome (Yeoman & White, 2014). Supplementing livestock with probiotics have been reported to maintain a healthy intestinal microbiome (Krehbiel *et al.*, 2002). The rumen microbiome diversity should therefore be managed carefully to maintain sufficient diversity to remain healthy and in balance while low enough to be more efficient.

The composition of the fungi in the rumen samples did not show any difference between the feed additive treatment groups in the starter phase (Chapter 4). It did show a tendency to differ in the grower phase while the composition did differ significantly in the finisher phase. Fungi do have a longer growth period compared to other rumen microbes (Theodorou *et al.*, 1996; Belanche *et al.*, 2012) and could therefore take longer to adapt to the diet or the feed additives. This brings to question the ability of microbes to adapt to feed and feed additives. The adaptation of the rumen microbes plays an essential role in ruminant production systems as it can affect the efficiency of the animal. It might also pose a problem in the future as the mode of action of most feed additives involves the inhibition of microorganisms. Both monensin and essential oils target microbes with more permeable cell membranes (Calsamiglia *et al.*, 2007) and their effect on the rumen microbiome population within this study (Chapter 4) decreased as the feedlot period progressed. The possibility exists that vulnerable microbes can acquire the ability to resist these feed additives through lateral gene transfer from invulnerable bacteria (Franzosa *et al.*, 2015). There are studies that show the efficacy of monensin has decreased over recent years (Meyer *et al.*, 2009; Weiss *et al.*, 2020). This decrease in efficacy could be partially explained by the increase in energy-efficient diets with high concentrate portions (Duffield *et al.*, 2012), however the adaptation of microbes to monensin should also be considered. Studies have also reported the possibility that microorganisms can adapt to the use of essential oils (Benchaar *et al.*, 2008). If microorganisms do gain the ability to resist certain feed additives, the efficacy of feed additives in generating a positive effect on the production of the animals might decrease or cease. Feed additives with different modes of action than the inhibition of microorganisms will have to be investigated.

Feed additives can also affect the microorganisms in the remaining gastrointestinal tract as discussed in Chapter 5. The microorganisms in the remaining section of the gastrointestinal tract, such as the small and large intestine, also influences the efficiency of animals (Myer *et al.*, 2017). More beneficial microorganisms, such as *Olsenella*, *Blautia* and *Eubacterium*, were observed to be abundant in the probiotic supplemented animals. However, the control group which received no feed additive also had a higher abundance of beneficial bacteria compared to the essential oil and monensin supplemented groups. Essential oils and monensin are known to inhibit pathogenic bacteria, as mentioned earlier, however other bacteria are also inhibited (Ogunade *et al.*, 2018). The small intestine microbiome has a high functional efficiency (Donaldson *et al.*, 2016) and does not have the redundancy and resilience of the rumen. The possibility exists that a small perturbation in the small intestine

microbiome can lead to dysbiosis and a negative effect on the animal. As the microorganisms in the small intestine have not been studied as extensively as the microorganisms in the rumen, the effect of these microbes in the small intestine on the animal, as well as their interactions, roles and factors that can influence it, should be further researched.

In this study (Chapter 6), it was observed that methods that are used to collect rumen content samples can influence the results obtained from a study. Studies have shown that the composition of samples collected via a stomach tube and at slaughtered can be compared to the composition of samples taken via cannula (Terré *et al.*, 2013; Lopes *et al.*, 2021). Samples were collected via stomach tube approximately five days before slaughter, and immediately after slaughter where the animals were withdrawn from the feed in this study. The rumen microbiome composition, calculated as alpha and beta diversity, for the two different methods, differed significantly. This difference was probably mostly due to the withdrawal of feed before the animals were slaughtered which is a standard operating procedure for a commercial feedlot. As samples taken with different collection methods differed and other studies have reported various factors that can influence the results from rumen microbiome studies, a standardized protocol could lead to the comparison of studies across the world and clearer knowledge regarding the roles of microorganisms in the gastrointestinal tract of ruminants.

The samples collected via stomach tube were characterised by a high abundance of Proteobacteria and Ascomycota, while the samples taken after slaughter had an abundance of Bacteroidetes and Neocallimastigomycota. The composition of the samples collected at slaughter were similar to what other studies reported (Freetly *et al.*, 2020; Zhou *et al.*, 2021). As the feed was withdrawn from the animals for approximately 20 hours as per standard feedlot operation, it is expected that the diversity within the rumen would decrease as indicated by other studies (Wallace *et al.*, 2014), however the diversity increased in this study. This leads once again to questions on the adaptability and resilience of the rumen microbes. There were indications, such as a low alpha diversity and a high Proteobacteria ratio, that the animals were in dysbiosis during the finisher phase. It is possible that the composition returned to balance after the feed, which caused the dysbiosis, was withdrawn and the diversity increased. It is recommended that feed is not withdrawn from the animals if the effect of diet on the rumen microbial composition is to be studied as a decrease in substrates for the microorganisms can influence the microbial composition. This might, however, be difficult in commercial feedlot operations.

7.1 Recommendations

This was the first study investigating the rumen microbiome in South African Bonsmara cattle; however, it had some limitations. Unfortunately, due to infrastructure constraints this study was limited to four pens per treatment group with three animals per pen. Studies have indicated that having four or more samples for sequencing studies is sufficient (Trapnell *et al.*, 2013; Wu *et al.*, 2021), however, due

to the variation found in the microbiota and the complexity of factors that can influence the rumen microbiome, follow-up studies using a larger sample size is recommended. These studies should include a functional component such as shotgun metagenomics or metatranscriptomics to investigate the function of the microbes within the gastrointestinal tract of ruminants. A larger sample size is also needed to investigate the effect of natural feed additives on the production of the animals.

The association between the microbes and feed efficiency should be researched further. There are studies that have reported differences (Shabat *et al.*, 2016; Lopes *et al.*, 2021) and studies that found no differences (Kenny *et al.*, 2018; McGovern *et al.*, 2020) in feed efficiency phenotypes. Feed efficiency is a complex trait with environmental and genetic influences. The host's genetics has been reported to influence the rumen microbiome composition (Li *et al.*, 2019). Breed has also been shown to influence the microbiome composition in the rumen (Auffret *et al.*, 2017; Parmar *et al.*, 2017) and as South Africa has many indigenous breeds, between-breed differences in the rumen microbiome composition might be observed. A greater understanding of the relationship between the rumen microbiome and feed efficiency can be gained if different omics approaches, such as metagenomics, metatranscriptomics, metaproteomics and metabolomics are used together.

Utilizing omics approaches would limit biases that can be introduced by amplicon sequencing, such as the hypervariable regions or gene markers used. The hypervariable region of the 16S rRNA gene can affect the microbes found in the samples. Many studies have suggested using the V1-V3 region as it is highly conserved (McCann *et al.*, 2014). However, sequencing the V3-V4 region can result in simultaneous amplification of bacteria and archaea. Although this hypervariable region is a good indication of the archaeal population and the composition could be studied in relation to the bacterial composition (Roehe *et al.*, 2016), the V6-V8 region would have yielded more accurate abundance results for the archaeal population. The use of the ITS region has also been debated due to the heterogeneity of the ITS1 region resulting in false higher diversity in community composition analysis (Edwards *et al.*, 2019), however it is the most commonly used gene marker for studying the rumen fungi (Fliegerova *et al.*, 2021). There are studies that have used the D1/D2 region of the 28S rRNA gene for the identification of fungi (Fliegerova *et al.*, 2021) and this might be a possible alternative to ITS primers.

The samples in this study had a high abundance of the fungal phylum, Ascomycota. This is in contrast to other studies which found Neocallimastigomycota to be the predominant fungal phylum found in the rumen (Gruninger *et al.*, 2014; Zhang *et al.*, 2020). Microbes from the Neocallimastigomycota phylum are anaerobic fibre-degrading fungi (Gruninger *et al.*, 2014) while microbes from Ascomycota and Basidiomycota are aerobic (Zhang *et al.*, 2020). As the rumen has an anaerobic environment, microbes from Ascomycota and Basidiomycota, scavenge for free oxygen to ensure it stays anaerobic. However, their predominant abundance may suggest a different role, or it might be due to the collection of samples via stomach tube, sequencing the ITS region, breed, or

geographical differences. This needs to be studied further as fungi and the oxygen concentration in the rumen can influence the efficiency of the animal.

The microbial composition found in the small intestine of a ruminant can also influence its efficiency as it is where most of the absorption of proteins and vitamins take place (Myer *et al.*, 2016). The small intestine is also known to influence the health of the animal (Donaldson *et al.*, 2016). While this study observed differences in the small intestinal microbiome of animals supplemented with different feed additives, more questions arose compared to answers, such as if fungi play a role in the jejunum microbiome. Limited literature exists as to the effect of feed and feed additives on the small intestinal microbiome and should therefore be studied. The effect of feed additives on the epithelial mucosa microbiome should also be researched as this microbiome has a direct effect on the health of the animal. Much research has been done on the factors that can influence the rumen microbiome (Henderson *et al.*, 2015; Zhou *et al.*, 2021) and fewer on the jejunal microbiome (Mao *et al.*, 2015; Han *et al.*, 2021). However, the question still remains as to what a good and healthy gastrointestinal tract microbiome consists of. To be able to answer this question the interactions between the microbes need to be investigated as well as the effect of the host on the microbiome. The redundancy within the rumen microbiome can complicate the characterization of a healthy microbiome as many microbes perform the same function (Weimer, 2015). To define the composition of a healthy microbiome within the jejunum might be less complicated as the microbes have a high functional efficiency where a few microbes perform various functions.

In conclusion, the rumen microbiome composition of the animals supplemented with the natural feed additives, essential oils and a probiotic, did not differ substantially from those supplemented with monensin in this study. The jejunal microbiome of animals supplemented with the probiotic had a higher abundance of beneficial microbes while those supplemented with essential oils and monensin had a lower abundance of pathogenic, commensal, and beneficial microbes. Essential oils and probiotics might therefore be considered potential alternatives to the use of monensin. However, large scale production studies are needed to validate that essential oils and probiotics are viable alternatives.

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