Changes in microbial population in the rumen of sheep fed *Acacia mearnsii* tannin extract for methane reduction

by

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Declaration

I, Imrana-Bakare Lawal hereby declare that this thesis, submitted for the Msc(Agric) Animal Science: Animal Nutrition degree at the University of Pretoria, is my own work and has not been previously submitted by me for a degree at any other University.

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Date: 03/07/2022

Dedication

I dedicate this work to Allah, the owner of my soul, The Beneficent, The Merciful, Lord of all the worlds.

To my parents who I can never repay for their love, you mean the world to me.

To my husband you are a blessing to me.

To my girls you have made me stronger and more fulfilled, I love you to the moon and back.

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Abstract

Methane (CH_4) is one of the primary gases that contribute to global warming. It is a by-product of enteric fermentation of ruminant animals, which is produced by microbes (methanogens that belong to domain Archaea) in the rumen. The emission of methane from the ruminant can be reduced to varying degrees through the manipulation of the rumen microbiome by various dietary interventions. It has been well established that diet affects the microbial community structure and composition. Tannins have been shown to directly or indirectly inhibit methanogenesis, thereby reducing methane. However, the effect of encapsulated tannins on the microbial diversity in the rumen has not been fully understood. In this study, 24 rumen samples were analysed from a study where *Eragrostis curvula* based diet was fed to South Africa Mutton Merino sheep, and was supplemented with tannin crude or encapsulated tannin. DNA were extracted and sequenced using shotgun metagenomic and analysed using MG-RAST. Out of the 28 bacteria phyla identified by shotgun sequencing, *Bacteroidetes* (72%) and *Firmicutes* (21%) were the dominant phyla. A total of 500 bacterial genera were recorded, where *Prevotella*, *Bacteroides*, *Eubacterium* and *Clostridium* had the highest abundance. Forty-one archaeal genera were identified with *Methanobrevibacter* having the highest abundance. However, the total bacteria and total methanogen did not significantly differ between the tannin and non-tannin treatments. This shows that tannin in its crude or encapsulated form did not have any effect on the methane producing microbes and the overall microbial community.

Preface

This dissertation is a continuation of a much larger project titled **"Use of lipid encapsulated tannin to replace ionophore in mitigating enteric methane emission and manipulating dietary protein bypass in SA Mutton Merino sheep".** The in vitro gas production and in vivo animal evaluation studies were conducted as part of the PhD research of Mr Shehu L. Ibrahim. The in vivo study was focused on evaluation of the effect of non-encapsulated and encapsulated mimosa (*Acacia mearnsii*) tannins on growth performance, nutrient digestibility, methane and rumen fermentation of South African mutton Merino ram lambs. In the experiment, 40 weaned South Africa Merino sheep weighting between 34 -35 Kg were placed on a total mixed ratio (TMR) formulated by AFGRIFEEDS Ltd, South Africa. Tannin was supplemented in the diet in various forms (encapsulated and un-encapsulated), the findings related to growth performance, nutrient digestibility, methane and rumen fermentation of South African mutton Merino ram lambs were published in Animal Feed Science and Technology, 294 (2022) 155502 (https://doi.org/10.1016/j.anifeedsci.2022.115502). Therefore, the scope of this dissertation is limited on the response of the rumen microbes on tannin (non-encapsulated and encapsulated mimosa (*Acacia mearnsii*) tannins) supplementation.

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List of Abbreviations

Abbreviations	Full name
ATP	Adenosine triphosphate
CDS	Coding DNA sequences
CH_4	Methane
CO_2	Carbon dioxide
CT	Condensed tannins
DMI	Dry Matter Intake
DNA	Deoxyribonucleic acid
EO	Essential oils
FA	Fatty acid
FCE	Feed conversion efficiency
GEI	Gross energy intake
H_2	Hydrogen
HT	Hydrolysable tannins
ITS	Internal Transcribed Spacer
KEGG	Kyoto Encyclopedia of Genes and Genomes
MW	Molecular weight
NGS	Next generation sequencing
OTU	Operational Taxonomic Unit
PAM	Protozoa associated methanogens
PSM	Plant secondary metabolites
PT	Phloro tannins
QIIME	Quantitative Insights into Microbial Ecology MG-RAST
RFI	Residual feed intake
rRNA	Ribosomal ribonucleic acid
VFAs	Volatile fatty acids

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Chapter 1

1 Introduction

Agricultural sources of methane emission accounts for 40 to 60% of the global anthropogenic emissions (Shanmugapriya et al., 2019, Rosentreter et al., 2021). It is estimated that livestock accounts for approximately 40% of the global agricultural gross domestic product GDP (Salmon et al., 2020) and in the developing nations, it is estimated that more than a billion people depend on livestock as means of livelihood. According to Meissner et al. (2013b) the livestock sector in South Africa is a major contributor to food security, social and economic status. The total number of ruminant animals in South Africa are estimated at 12.8 million cattle, 19.4 million sheep and 3 million goats (Casey, 2021) and production can be divided into commercial (intensive and extensive) and subsistence (emerging and communal) systems (Meissner et al., 2013b). Beef cattle production is largely carried out in an extensive production systems and weaners are fattened in the feedlot before slaughter for about 120 days, while those raised solely on pasture alone requires more than 200 days to reach the same body weight (Scholtz et al., 2013, Meissner et al., 2013a, Drouillard, 2018). The low quality of feed on pastures compared to the feedlot diet (high quality feed materials such as maize and other agricultural by-products) imply that cattle raised on pasture end up producing more greenhouse gas (GHG) (Meissner et al., 2013a, Molossi et al., 2020). There are several impacts of livestock activities on the environment, and this include production of GHGs such as methane. It was estimated that the beef cattle on extensive system emitted 83.3% of the 72.6% total enteric methane emission followed by a 13.5% emission from the dairy cattle and 3.2% from the feedlot cattle in 2010 (Meissner et al., 2013a). The small ruminant emitted 15.6% of the total livestock emission of methane in 2010, and of this 91% was emitted from the commercial sheep industry (Du Toit et al., 2013).

Methane (CH_4) is a GHG that is colourless, odourless and mostly produced as a by-product from different sources such as landfills, wetlands, termites and livestock. According to the Environmental Protection Agency (EPA), methane has a Global Warming Potential (GWP) of between 28 and 36 CO_2 equivalent (eq) (Knoell, 2016, O'Bannon, 2021), which implies that methane has the ability to trap radiation and emits it back to surface of the earth 28 to 36 times more compared to carbon dioxide (GWP of 1) (Knoell, 2016). Livestock, especially ruminants produce methane as a by-product of enteric fermentation. The methane in the rumen are product of reaction between carbon dioxide and hydrogen produced during fermentation (Owens and Basalan, 2016, Ungerfeld, 2020b). The process of methane production is aided by the microbes specifically methanogens (Wolin, 1979, McAllister and Newbold, 2008, Buan, 2018). The production of methane in the rumen is associated with a loss of 6 to 15% of gross energy intake of the animal, thereby reducing feed efficiency (Waghorn et al., 2002, Min et al., 2022).

The production of livestock is presently facing a myriad of problems with the rising global population that is expected to reach 9 billion by 2050 (O'Hara et al., 2020). To meet demand, it is expected that food production needs to increase by 70%. The demand for meat and dairy product will also rise which in turn will increase methane emission from livestock. To mitigate this problem, several efforts has been made to reduce methane production from ruminants. The use of chemicals, defaunation, ionophores supplementation, immunization, concentrates and diet supplementation are examples of mitigation strategies that has been explored (Sejian and Naqvi, 2012, Beauchemin et al., 2020, Min et al., 2022). Diet supplementation involves the use of feed additives, halogenated compounds and plant secondary compounds (e.g. tannins, essential oils) to mitigate enteric methane (Faniyi et al., 2016, Min et al., 2020, Honan et al., 2021). Tannins extracted from plants have been widely used as additives in ruminant nutrition (Dhanasekaran et al., 2019). Tannins are found in many nutritionally important legumes and forages, grains and many medicinal plants.

The ruminal microbes that work collectively in breaking down feed materials in the rumen include bacteria, archaea, fungi, protozoa and bacteriophages. These microbes are responsible for generating up to 70 % of the energy requirements of the animal (Knoell, 2021). Complex interactions exist between the microbes and the host, as well as among the microbes, such that intermediate substrates produced by one population of microbes and utilized by another population. The composition and function of rumen microbes are influenced by different factors, and among these factors feed is the most important factor that affects the rumen microbial population. Exploring the compositions of the microbes in animals receiving some form of feed supplementation or diet manipulations for the purpose of mitigating methane is of utmost importance. The composition of the microbes and their response to dietary manipulation can be studied using metagenomics (Malmuthuge and Guan, 2016).

Metagenomics is a culture-independent technique that is used to investigate and characterize microbial communities in terms of their diversity, structure, composition and function (Kibegwa et al., 2020). This high-throughput sequencing technology has gained a lot of attention in the microbiological world in the last decade. The two major sequencing techniques used in the evaluation of rumen microbes include amplicon and shotgun metagenomics (Zhou et al., 2021). The amplicon sequence include 16SrRNA, 18SrRNA and the ITS. This method targets the hypervariable region for sequencing and sequences are aligned to specific databases such as Greengenes and SILVA for taxonomic assignment, while the shotgun metagenomic sequences part or the whole DNA (Rausch et al., 2019)

1.1 Justification

Dietary manipulation can assist to suppress methane emissions from ruminants by inhibiting rumen microbes involved in methane formation, or by diverting hydrogen away from methane production during ruminal fermentation (McGinn et al., 2004, Min et al., 2020). Tannins have been reported to reduce methanogenesis directly or indirectly. It acts by either directly preventing the growth and multiplication of methanogens or indirectly by the defaunation of the ciliate protozoa population that are associated with methanogens or by inhibiting fiber digestion, thereby, causing a reduction or shift in rumen fermentation (Baruah et al., 2019, Adejoro, 2019). Baruah et al. (2019) found that a reduction in methane production observed in sheep fed tannin rich plants- *Syzygium cumini* and *Machilus bombycina* was due to the partial inhibition of the methanogens in the rumen. The direct action of tannin present in Tamarind seed husks reduced the population of methanogens and methanogen-associated protozoa (entodinimorphs and holotrichs) in the rumen of cattle, resulting in a significant methane reduction (Malik et al., 2017b).

Tannins from different plants have been studied in the past to determine their effectiveness on methane reduction as well as their effect on the rumen microbial composition in order to determine mode of action (Dhanasekaran et al., 2019). However, there is limited research on the effect of *Acacia mearnsii* tannin extracts on rumen microbes, though its dose dependent effect on enteric methane emission, feed digestibility and intake are well established. According to a study by Adejoro (2019), it is possible to regulate the release rate of tannin inside the rumen as well as reduce the negative impact of tannin on feed digestibility and intake by micro encapsulation of tannin with oil. However, limited information regarding the effect of micro encapsulation of tannin with oil on rumen microbial population response is available. Therefore, this study was aimed to fill the knowledge gap to improve our understanding of the interactions between tannin and the microbiota in the rumen of sheep fed crude or encapsulated *Acacia mearnsii* tannin extracts.

1.2 Aim

The aim of this research was to establish the changes in microbial composition of crude or encapsulated *Acacia mearnsii* tannin extracts used as methane mitigation additive on rumen microbial diversity in South African Mutton Merino sheep.

The specific objectives included:

i. To describe the microbial population diversity in the rumen of sheep using the metagenomic approach.

ii. To compare differences/changes in microbial diversity in the rumen of sheep supplemented with different dietary additives.

Chapter 2

2 Literature review

2.1 Introduction

Methane is produced in the rumen as a by-product of fermentation. The process of fermentation is dependent on the complex microbial community present in the rumen. These rumen microbes interact with each other and have a symbiotic relationship with the host (Liu et al., 2021). There are different microbes performing different roles in the digestion and fermentation of feed materials. Their composition is affected by feed ingested by the animal among other factors.

The aim of this review was to provide an overview on the rumen microbiome followed by a discussion on strategies to reduce methane production and particularly, to understand how diet additives like tannin can influence the diversity of microbes in ruminants. The different methods used in analyzing microbes are also reviewed.

2.2 The Ruminant digestive tract and the rumen microbiome

The special ability of the ruminant to efficiently utilize plant materials including agricultural wastes is a unique advantage of ruminant species in livestock production. The ruminant animal possesses a four-stomach chamber, namely the rumen, reticulum, omasum and abomasum (Figure 2-1). The largest of the compartment is the rumen, an oxygen-free environment with a temperature of 39 °C and pH range of 5.8 to 6.8 (Broudiscou et al., 2014, Zhu, 2016).

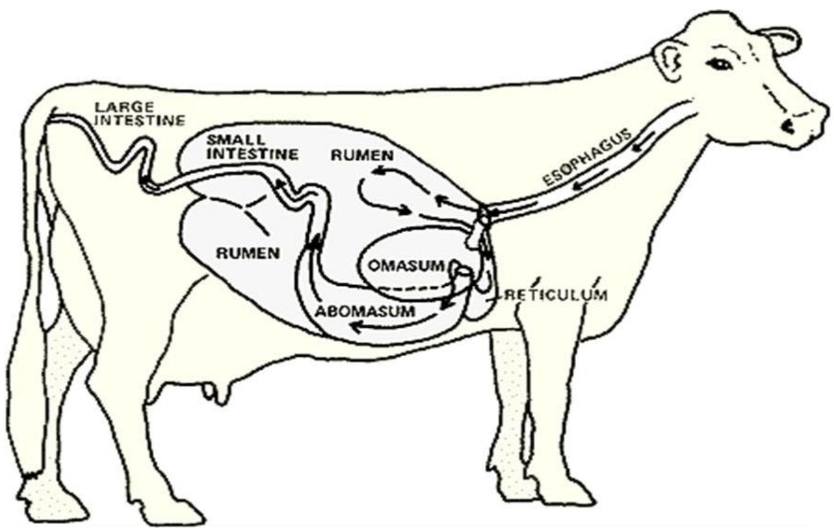

Figure 2-1.: Digestive system of a ruminant animal (Guillermo et al., 2015)

The rumen has a dry matter content of 10 to13% (Welch, 1986, Zhu, 2016). The feed ingested by the animal stratifies into three layers in the rumen (Figure 2-2). The last layer of the rumen is where majority of microbes flourish (Welch, 1986, Zhu, 2016).

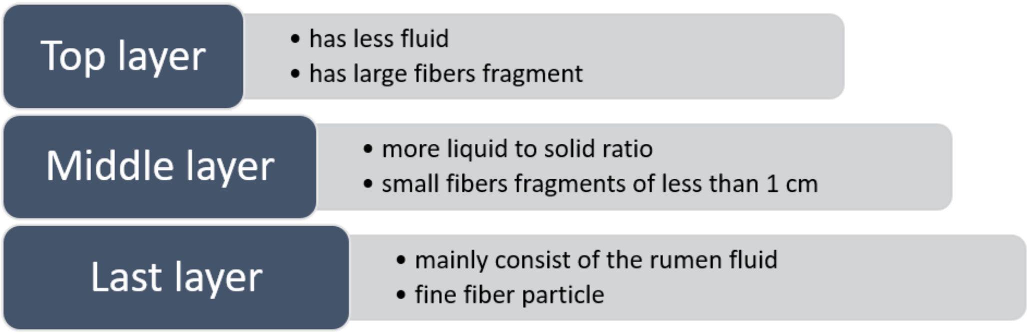

Figure 2-2: The three layers in the rumen (Welch, 1986, Zhu, 2016)

Rumen is a very complex nature, it houses different kinds of microorganisms such as bacteria, archaea, fungi and ciliated protozoa (López-García et al., 2022). Sirohi et al. (2012) reported that the most diverse group are the rumen bacteria, which account for 10^{10} - 10^{11} cells/ml of rumen contents, the archaea represented by methanogens take 10^{7} - 10^{9} cells/ml; the protozoa accounts for 10^{4} - 10^{6} cells/ml and lastly the fungal population accounts for 10^{3} - 10^{6} cells/ml.

The microbes primarily inhabiting the rumen include the bacteria, archaea (methanogens), fungi and protozoa. Bacteria and protozoa make up the larger percent of microbial biomass, while fungi represent a small percentage of about 8 to 12 % (Rezaeian et al., 2004, Matthews et al., 2019).

2.2.1 Bacteria

There are different kinds of bacteria present in the rumen and they are responsible for fermentation and degradation of different plant fiber (Table 2.2-1). It is noteworthy that the population of bacteria associated with feed particles accounts for up to 50 to 75% of the entire microbial population (Puniya et al., 2015). In addition, the attachment of microbes to feed particles is an essential factor to ensure a successful competition and ultimate survival in the rumen. The rumen bacteria can be classified into 13 bacteria types based on the different strata present in the rumen. They can either be in the liquid phase as free-floating, or attached to feed particles, or to the rumen epithelium, or attached to other rumen microbes such as protozoa and fungi (Knoell, 2021). The majority of these bacteria are obligate in nature and are actively involved in the enzymatic digestion of feed materials (Kamra, 2005).

Table 2.2-1: The classification of rumen bacteria (Puniya et al., 2015)

Bacteria types	Genera and species
1. Acetogens	*Acetitomaculum ruminis, Eubacterium limosum*
2. Acid utilizers	*Megasphaera elsdeni, Wolinella succinogenes, Veillonella gazogene, Micrococcus lactolytica, Oxalobacter formigenes, Desulfovibrio desulfuricans, Desulfotomaculum ruminis, Succiniclasticum ruminis*
3. Amylolytic	*Streptococcus bovis, Ruminobacter amylophilus, Prevotella ruminicola*
4. Cellulolytic (fiber degraders)	*Fibrobacter succinogenes, Butyrivibrio fi brisolvens, Ruminococcus flavefaciens, Ruminococcus albus, Clostridium cellobioparum, Clostridium longisporum, Clostridium lochheadii, Eubacterium cellulosolvens*
5. Hemicellulolytic	*Prevotella ruminicola, Eubacterium xylanophilum, Eubacterium uniformis*
6. Lypolytic	*Anaerovibrio lipolytica, Butyrivibrio fibrisolvens*
7. Pectinolytic	*Treponema saccharophilum, Lachnospira multiparus*
8. Proteolytic	*Prevotella ruminicola, Ruminobacter amylophilus, Clostridium bifermentans*
9. Saccharolytic	*Succinivibrio dextrinosolvens, Succnivibrio amylolytica, Selenomonas ruminantium, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus brevis, Lactobacillus helveticus, Bifidobacterium globosum, Bifi dobacterium longum, Bifidobacterium thermophilum, Bifidobacterium ruminale, Bifidobacterium ruminantium*
10. Tanninolytic	*Streptococcus caprinus, Eubacterium oxidoreducens*
11. Ureolytic	*Megasphaera elsdenii, Bacteroides ruminicola*
12. Lactic acid producers	*Streptococcus bovis*
13. Lactic acid utilizers	*Megasphaera elsdenii*

Bacteria are classified into different groups based on enzymatic activities, such as but not limited to fiber-degrading bacteria, starch utilizers, lactic acid utilizers, lactic acid producers and so on (Flint et al., 2012). The sugar and starch digesters accounts for a significant number of the total rumen bacterial population. For example, in the diet of high-producing dairy cows, the sugar and starch content usually exceed 30%, hence these bacteria are seriously needed (Ishler and Varga, 2001). Even in cases where an animal is on an all-straw diet, the fiber-degrading bacteria hardly accounts for more than 25% of the bacterial population in the rumen (Puniya et al., 2015).

2.2.2 Tanninolytic bacteria

A number of studies have shown that some group of microbes are able to degrade tannins (both HT &CT) in the rumen and these has been mostly characterized from the both wild and domesticated ruminants (Osawa et al., 1995a, Osawa et al., 1995b, Odenyo et al., 1999, Goel et al., 2005, Chen et al., 2021b). Patra and Saxena (2011) and Odenyo et al. (1999) reported the existence of rumen microbes that are tannin tolerant/degraders in different ruminants (Goel et al., 2005).

Streptococcus caprinus was isolated from the ruminal fluid of feral goats that browsed on Acacia plant rich in tannins, this species was said to be tolerant of either types of tannins at high concentrations (Brooker et al., 1994). *Selenomonas ruminantium* is yet another bacterium that is capable of degrading tannins, it was also isolated from feral goats which browsed on Acacia (Skene and Brooker, 1995), as well as from other ruminants from East Africa fed different forages rich in tannins (Odenyo and Osuji, 1998). Five different types of bacteria capable of degrading tannin was isolated from Indonesian goats which were fed diet containing tannin-rich Calliandra (which has tannins up to 10% of dry weight) (Wiryawan and Tangendjaja, 1999). It was found that this bacteria are able to reduce the concentration of tannic acid by 52% within 12 h and reduce the concentration of CT by 48% within 72 h. Tjakradidjaja et al. (2000) reported that twenty rumen bacteria (*Lactobacillus, Butyrovibrio, Streptococcus,Clostridium, Megasphaera, Leuconostoc, Enterobacter and Prevotella sp.*) that are tolerant to tannin have been characterized using 16S rRNA.

2.2.3 The rumen protozoa

The rumen protozoa group accounts for about 50% of the microbial biomass (Williams et al., 2020b). They are important in recycling of nitrogen through their intensive bacterial predation (Faciola, 2004), which serves as the main protein source (Williams and Coleman, 2012). They are responsible for the degradation of proteins of microbial origin, especially bacteria, this action in turn affects the overall amount of the microbial proteins or amino acids that is available for intestinal digestion. In addition, it is important to note that a large amount of protein in the biomass of the rumen protozoa may not be accessible for digestion in the intestine (Matthews et al., 2019).

The rumen protozoa are divided into ciliate and flagellate protozoa. However, the most dominant are the ciliates. The ciliate protozoa are classified into two main genera; *entodiniomorphid* and *holotrich*. Examples of ciliate protozoa include; *Entodinium bovis, Isotricha intestinalis, Dasytricha ruminantium*. The ciliate protozoa play a vital role in the digestion of fibers as well as the modulation of the fermentation parameters with the fermentation end-products similar to that of bacteria, most especially acetate, butyrate and hydrogen (Newbold et al., 2015). Methanogens are known to attach and live on the surface of the rumen protozoa to enable them directly access the H_2. They are able to efficiently utilize starch and also store it. This ability can help to slow down the production and build-up of acids that results in reduction of rumen pH. Rumen ciliates multiply slowly, it takes about 15 to 24 h as opposed to the bacterial population. To ensure the protozoa survive in the rumen and are not washed out, they hide in the slower moving (fiber mat) part of the rumen (Williams et al., 2020a). When low-roughage diet is fed to the animal, fiber retention is reduced in the rumen, and this may cause a reduction in the protozoa population.

The anaerobic fungi are active and efficient fibre degraders (they degrade structural carbohydrates of plant cell walls) and are most important in the digestion of poor quality forage consumed by the animal (Varga and Kolver, 1997). They are able to achieve these due to a broad range of potent polysaccharide degrading enzymes (cellulases, hemicellulases, xylanases, avicelases, glycosidases etc) (Williams et al., 1994, Lee et al., 2001). The population size of the rumen anaerobic fungi depends on the type of feed ingested by the animal. An increase in the population of the anaerobic fungi is observed when the animal feeds on high fibre diet as compared to the low population when soft leafy diet is consumed (Denman et al., 2008). Common examples of the anaerobic fungi present in the rumen are *Piromyces communis, Caecomyces communis, Neocallimastix variabilis, Neocallimastix frontalis, Orpinomyces joyonii* etc as shown in *Table 2-2*.2. A number of studies has shown that anaerobic fungi in the rumen interacts with other rumen microbes. Orpin and Joblin (1997) observed that there was significant interaction between fungi (e.g. Neocallimastix and Piromyces species) and the methanogens, a similar observation was reported by (Li et al., 2021). Anaerobic rumen fungi produce hydrogen in large quantities, which attracts the methanogens encouraging them to form a stable association (Millen et al., 2016). Morvan et al. (1996) and Orpin and Joblin (1997) also reported that some rumen bacteria with high hydrogen affinity such as *Eubacterium limosum* and *Acetitomaculum ruminis* also interacts with fungi. They are able to utilize the hydrogen produced by the fungi for their own gain.

2.2.4 Anaerobic fungi

Anaerobi fungi make up 8 to 12% of the microbial population in the rumen (Rezaeian et al., 2004) and are active celluloytic microbes that specifically attach themselves to fibrous plant fragments where they colonize and grow on them. Orpin and Joblin (1997) reported that the rumen fungi have zoospores that are either monoflagellated or polyflagellated and that they also exhibit asexual life cycle.

Table 2-2.2: Protozoa and fungi found in the rumen. Adapted from (Castillo-González et al., 2014)

Protozoa	Genera and species
Cellullolytic protozoa	*Enoploplastron triloricatum*, *Eudiplodinium maggii*, *Diploplastron affine*, *Epidinium caudatum, Diplodinium monacanthum, Diplodinium pentacanthum*
Proteolytic protozoa	*Entodinium caudatum, Eudiplodinium medium*
Fungi	
Cellulolytic fungi	*Neocallimastix frontalis, Piromyces communis, Orpinomyces joyonii*

2.2.5 The rumen methanogens

Rumen methanogens are archaea (Tholen et al., 2007), with small population sizes (<3%) as compared to the entire rumen microbial environment. They are of high importance as they assist in maintaining low hydrogen concentration in the rumen (Belanche et al., 2014, Castillo-González et al., 2014). This is done through a process called methanogenesis, where they use the hydrogen released by other microbes during fermentation to produce methane in the presence of CO_2. The absence of H_2 leads to a more favorable pattern of VFAs formation and also increases the efficiency of fermentation (Wolin, 1979, McAllister and Newbold, 2008).

These rumen archaea are strict anaerobes that do not survive in an oxygen-rich environment (Matthews et al., 2019). Protozoa are oxygen scavengers and reported to provide one of the best environments for methanogens resulting in a symbiotic relationship between methanogens and protozoa (Puniya et al., 2015). The symbiotic relationship between the rumen methanogens and protozoa is further supported by the fact that, protozoa produces H_2 in large quantities and if not removed, becomes inhibitory to their metabolism (Puniya et al., 2015). Protozoa associated methanogens (PAM) make up to 25% of the total rumen methanogen population. PAM produces about 37% of total methane production in ruminants (Belanche et al., 2014, Matthews et al., 2019). During the degradation of plant cell wall in the rumen, hydrogen is produced as an intermediate compound by anaerobic fungi and cellulolytic bacteria (*Ruminococcus flavifaciens, R. albus*) (Hobson and Stewart, 2012). However, this hydrogen produced in the rumen never gets accumulated, due to the vast utilizeation by the methanogens to produce methane and ATP (Wolin et al., 1997, Puniya et al., 2015, Ungerfeld, 2020a).

The rumen methanogens are found in the rumen fluid, attached to particles, attached to rumen epithelium and also attached to protozoa both as endo and ecto symbionts (Janssen and Kirs, 2008).

The most common and important order of rumen methanogens is the *Methanobacteriales*. The most abundant genera are the Methanobrevibacter (63.2%), *Methanosphaera* (9.8%), followed by *Methanomicrobium* and *Methanobacterium* at 7.7% and 1.2% respectively (Patra et al., 2017a). The order *Thermoplasmatales* (formerly Rumen Cluster C) represents 7.4% of the total rumen archaea. The most common example of methanogens present in the rumen are *Methanobacterium bryantii, Methanobrevibacter ruminantium*, *Methanobrevibacter smithii* (Patra et al., 2017b).

2.2.6 Bacteriophages

The rumen bacteriophages are present at >10^9 particles per ml, and one of the most important components of the rumen ecosystem (Matthews et al., 2019). They are known to be obligate pathogens for bacteria and are specific for the different kinds of bacteria in the rumen. The bacteriophages aid in turnover of bacterial mass in the rumen and capable of lysing bacteria (Puniya et al., 2015). Their lysing action on the bacterial cells allows for the easy availability of the bacterial protein to the animal. The bacteriophages specificity for the different bacteria in the rumen can be exploited to inactivate and remove undesired rumen bacteria such as the methanogens or *Streptococcus bovis* (Klieve et al., 1999, Bach et al., 2002). However, limited information exists on the genetic blueprint and functionality of the methanogenic phages, but some discovery has been made with the use of in vitro techniques (Stanton, 2007) and electron microscopy (Ackermann, 2007).

2.3 Methane production in ruminants

In ruminant animals, the fermentation (pre-gastric) of feeds into volatile fatty acids (VFAs), hydrogen (H_2) and carbon dioxide (CO_2) is basically controlled by the rumen microbiome (Li et al., 2021). The VFAs which are the end-products, are transported through the rumen wall and then utilized by the host animal, while the CO_2, a by-product is released through eructation. The second fermentation by-product H_2, can either be incorporated into volatile fatty acids production or is mainly converted to methane (CH_4) (Zhu et al., 2016). The conversion of H_2 to CH_4 is done by the methanogens present in the rumen (Zhu et al., 2016).

2.3.1 Biochemistry of methane

The continuous fermentation of carbohydrates in the rumen leads to accumulation of H_2, which is in turn removed by methanogens through the methanogenesis pathway. This is a crucial and beneficial step that ensures the continuous anaerobic fermentation in the rumen. There are three methanogenic pathways that have been identified, namely; hydrogenotrophic, methylotrophic (conversion of methyl-group containing compounds) and the acetoclastic (Ferry, 2011). The hydrogenotrophic is the most common pathway where the methanogens use, H_2 as an electron donor to reduce CO_2 to CH_4 (Hook et al., 2010). It has also been suggested that there is evidence for a fourth pathway (hydrogen-dependent methylotrophic methanogenesis) (Welander and Metcalf, 2005), where methanogens utilize a range of methyl donor compounds for methane production (Poulsen et al., 2013). However, regardless of the pathway, CO_2 is main electron acceptor in methanogenesis.

Different electron donors utilized include methanol, H_2, acetate, carbon monoxides, methlyamines, although the majority of the known archaea methanogens grow when hydrogen is utilized as electron donors (Kim and Gadd, 2019). In the hydrogenotrophic pathway, hydrogen utilized is typically gotten from both the rumen bacteria and protozoa as a catabolic product. Methanogens take up the released hydrogen to reduce CO_2, producing methane, through the interspecies hydrogen transfer. In this pathway, a cofactor, methanofuran activates the carbon dioxide to form formylmethanofuran (Caspi et al., 2010). The methyl group from 5-methyltetrahydromethanopterin is then transferred to coenzyme M, which results in methyl-CoM producing CO_2 and CH_4 (Caspi et al., 2010). Examples of methanogens that utilizes hydrogen to produce methane belong to the genera Methanobrevibacter, Methanothermobacter, Methanobacterium, Methanothermus, and some belonging to the genus Methanosarcina (Ferry, 2012, Puniya et al., 2015).

The acetoclastic methanogenesis is derived when acetate is converted to acetyl-CoA, then a methyl-group is transferred into the methanogenic pathway (Ferry, 1992). The methyl group is transferred to tetrahydrosarcinaterin and then to coenzyme M, to produce methyl-CoM which is then demethylated to produce methane (Puniya et al., 2015). However, it is believed that only a specific order *Methanosarcinales* utilizes this pathway (Ferry, 1992). Though, these strains that utilizes acetate to produce methane do not multiply quickly (takes up to four days), this makes them slow to colonize and become a successful competitor of substrates in the rumen (Van Soest, 2018). This saves acetate from becoming utilized to produce methane in the rumen, leaving methane mostly to the microbes that utilize H and other substrates (Pesta, 2015).

The use of formate in the production of methane is also quite common. It is suggested that up to 18% of enteric methane in the rumen results from formate (Puniya et al., 2015). Formate is a byproduct, which is formed during fermentation of pyruvate to acetate, and it is only found in small amounts (< 1% of total VFA) (Pesta, 2015) as it is rapidly utilised. Cellulolytic bacteria, ciliate protozoa and fungi all produce formate (HCOO-), which is eventually utilized by methanogens (Ellis et al., 1990). Through formate hydrogenases, formate is converted to H_2 and CO_2 and then ultimately used in methanogenesis to produce methane (Puniya et al., 2015).

Although the three methanogenesis pathway (Figure 2.2-3) may differ from one another, there is a step in the pathways common to all, where methyl-coenzyme M reacts with a thiol coenzyme (coenzyme B), to form methane (Hedderich and Whitman, 2006).

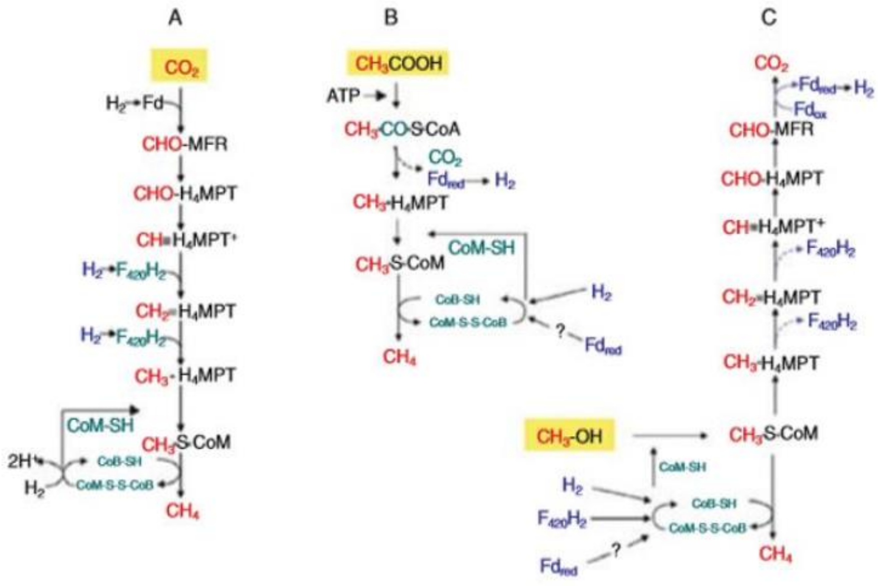

Figure 2.2-3: The methanogenesis pathway (Hedderich and Whitman, 2006)

Due to the use of hydrogen in methane production as the most common substrate, it is important that methane emission strategies are focused on alternative hydrogen sinks in the rumen as well as inhibiting the activities of the methanogens (Adejoro, 2019). Examples of factors that affect the amount of hydrogen produced in the rumen include the diet and the different types of the microbes present in the rumen (Haque, 2018). Sulphate-reducing bacteria are examples of microbes that are able to compete with the ruminal methanogen for H_2 which in turn reduce or inhibit the methanogenesis pathway (Zhao and Zhao, 2022).

2.4 Methane mitigation strategies

The development of an effective methane mitigation strategy must consider some important factors such as the inhibition of pathways for methane production and H_2 utilization for other end products, as well as targeting directly or indirectly the methanogens involve in the production (Martin et al., 2010) of methane. According to Martin et al. (2010), any strategies developed must address one or more of the objectives below;

i. Reduction of H production that should be accomplished without affecting the digestion of feed.

ii. The stimulation of H utilization or consumption towards pathways that produces other end products useful for the animals

iii. In inhibition of methanogens both in numbers and/or activity. This should be done by taking into account actions that stimulate pathways which utilizes hydrogen so as to avoid a buildup of hydrogen in the rumen and its resultant negative impact on fermentation.

Methane mitigation strategies (Figure 2.2-4) in ruminant can be classified into three broad headings namely; management, nutritional and advanced strategies.

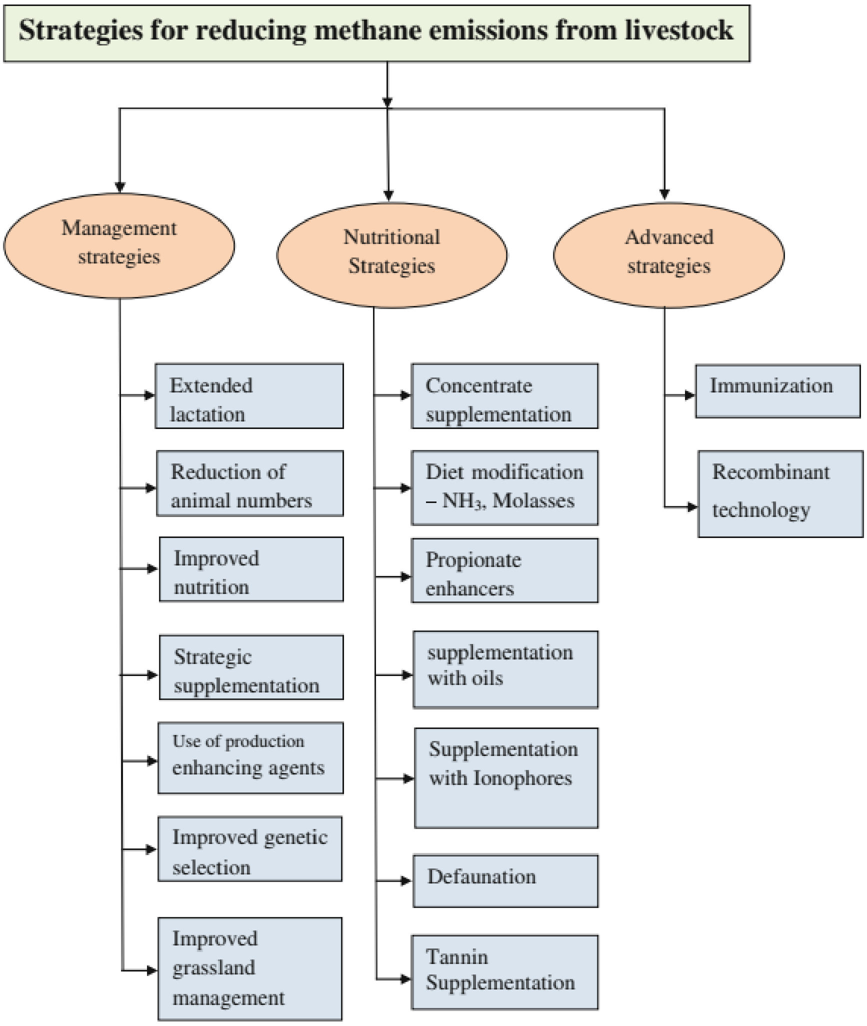

Figure 2.2-4: Mitigation strategies (Sejian and Naqvi, 2012)

2.4.1 Management strategies

According to Sejian and Naqvi (2012), implementation of the basic management principles of livestock offers a good opportunity at methane reduction as well as improving animal production. Some of the strategies that can be used to reduce methane emissions include the following (Hogan, 1993, Sejian and Naqvi, 2012);

i. Improved nutrition through chemical and mechanical feed processing
ii. Strategic supplementation through balanced nutrition
iii. Improved rangeland and grassland management
iv. Improved genetic selection
v. Use of production enhancing agents and
vi. Improved reproduction and extended lactation.

These management strategies have various ways for reduction of methane in the ruminant. Genetic selection can be applied to select animals that produce less methane per unit of feed consumed (higher feed efficiency). In general, improving the production efficiency of the animal will lead to a reduced methane production per animal product (Sejian and Naqvi, 2012). Furthermore, Ulyatt and Lassey (2001) reported that genetic selection contributes to methane reduction in two ways; the genetic improvement of animals to realize more products per unit of feed intake (feed efficiency) and secondly, the dietary manipulation through increased feed intake and right feed composition (Brito et al., 2021). It was reported by Arthur et al. (2001) that cattle with lower dry matter intake than their peers of the same live weight and average daily gain, have a low residual feed intake, this makes them more feed efficient. The residual feed intake (RFI) is calculated according to Hegarty et al. (2007) as "the difference between actual feed intake and the expected feed requirements for maintenance of body weight and a certain level of production".

Implementation of appropriate grazing management practices that improve the pasture quality increases animal productivity as well as reduce methane arising from enteric fermentation (DeRamus et al., 2003). The maintenance of soil fertility in an extensive production system is one way to ensure the production of a high quality forage for the animals, and accordingly a 22% reduction in methane production was observed in cattle that grazed on such forage (DeRamus et al., 2003). The reduction in the enteric methane production was related to an improved digestibility of the high quality forage leading to a better feed efficiency traits (Du Toit, 2017). In a study by (Maas, 1987), a decrease in methane production was reported in animals fed fresh grass with higher nitrogen concentrations. A low nitrogen concentration pasture of 3% produced methane losses of 6.5% of gross energy intake (GEI), whereas pastures with 4.5% nitrogen concentration yielded methane losses of 5.2% of gross energy intake. Pastures with a high nitrogen concentration was associated with increased digestibility (Tamminga et al., 2007).

Mitigation of methane through microbial intervention has been reported by several researchers, this intervention include; defaunation (removal of protozoa from the rumen), killing or reducing the number of methanogens using antibiotics, phages or bacteriocins, as well as developing alternative hydrogen sinks such as reductive acetogenesis (Ulyatt and Lassey, 2001, Sejian and Naqvi, 2012).

The advance strategies includes immunization and recombinant technologies. According to Gworgwor et al. (2006), immunization one of the most remarkable methods of methane mitigation strategies developed is the use of vaccine which contains antigens from both methanogens and protozoa. This vaccines is a cost-effective treatment that is potent in reducing methane production in animals. Taking these into consideration, Shu et al. (1999) and Baker et al. (2004) suggested that it may be possible to immunize the ruminant animals against their own methanogens and protozoa, and that such approach could also bring about a decrease in the population of streptococci and lactobacilli present in the rumen. A lot of study is still required in this field to ensure an effective strategy is developed, as there exist several strains of methanogens present in the rumen. If this development becomes successful, vaccination would be an indispensable and valuable strategy, as the whole of the ruminant population could be vaccine. It is anticipated that vaccination could bring about a 70% reduction in methane production in ruminants (Iqbal et al., 2008).

2.4.2 Nutritional strategies

i. Supplementation with oils

Several nutritional strategies had been exploited, this includes the addition of oils or fats to the ruminant animal's diet. This is known to decrease CH_4 production in vitro by up to 80% (Fievez et al., 2003) and 25% in vivo (Machmüller et al., 2000). The suppression of methane by fats or oils is achieved via direct or indirect mechanisms (Iqbal et al., 2008). The direct mechanism is characterized by the fatty acid (FA) toxicity of the methanogens. While the indirect mechanism consists of reduction of double bonds in unsaturated fatty acids, protozoal inhibition, increased productivity and enhanced propionate production (Beauchemin et al., 2009, Bayat et al., 2018). The use and effect of different vegetables oils on methane reduction has been reported by several researchers. Czerkawski et al. (1966) explained that there was a suppressive effect observed by long chain unsaturated FAs on methanogenesis in sheep, which was further supported by the competition between the two processes of biohydrogenation and methanogenesis. A lower acetate to propionate ratio was also observed when sunflower oil was added in the diet of cattle (McGinn et al., 2004). Machmüller et al. (1998) reported the detrimental effects on the rumen protozoa by certain oils. These authors reported that the rumen protozoa were reduced by 88 to 97% when canola oil was included in sheep's diet at 0%, 3.5% and 7%. The direct toxic effect by unsaturated fatty acids on methanogens was also observed when coconut oil inhibited the methanogens in the rumen. It was suggested that this was done by changing their metabolic activity and composition (Machmüller et al., 2003). However the efficacy of different oils varies, Machmüller et al. (1998) observed that rape seed, linseed and sunflower seed oil are all not good methane inhibitors as coconut oil, which he described to be an effective inhibitor. A decrease of 22% in methane emission was also reported by McGinn et al. (2004) when sunflower oil was added in the diet of cattle.

However, there are several negative effects associated with the use of fats and oils on the animals. Significant reduction in fiber digestibility was reported when sunflower oil was added to the diet (McGinn et al. (2004), as well as the high cost and negative impact of milk fat concentration observed by Zheng et al. (2005) when oils were added to diet of lactating cows. Jordan et al. (2006) also reported that finishing animals (beef heifers) required longer time to attain common carcass weight (750 pounds) (Holland et al., 2014) when the diet was supplemented with coconut oil.

ii. Concentrates supplementation

Negative correlation between proportion of concentrates in a diet and methane emissions was reported by (Yan et al., 2000, Iqbal et al., 2008). The nature of carbohydrates as well as the rate of its fermentation has been known to affect the proportion of VFAs produced in the rumen (Boadi et al., 2004, Du Toit, 2017) and the available amount of excess hydrogen that is converted to methane (Mirzaei-Aghsaghali et al., 2008). A comparison between structural and non-structural carbohydrates on methanogenesis showed that there is increased methane production during fiber fermentation as compared to fermentation of soluble carbohydrates (Moe and Tyrrell, 1979). Acetate production is accompanied by production of H_2, whereas the production of propionate is associated with a net uptake of H_2 (Du Toit, 2017). The formation of propionate competes directly with methane production in the rumen. The ratio of acetate to propionate has been reported to increase with increase in the fiber content of the diet, a negative correlation was also observed between proportion of acetate in rumen fluid and the efficiency of metabolizable energy (Du Toit, 2017).

Starch-rich diet tends to favour propionate producing microorganisms and therefore hydrogen is diverted away from methanogens (Janssen, 2010). A roughage-based feed tends to favour the acetate production and therefore there is an increase in methane production per unit of fermentable organic matter in the rumen (Johnson and Johnson, 2002). Fermentation of fiber results in a larger loss of gross energy intake in the form of methane than fermentation of starches and sugars (Boadi et al., 2004). This is mainly because the rate of ruminal fermentation decreases, which is accompanied with a decreased rate of passage out of the rumen, which in turn favors a higher acetate to propionate ratio (Hegarty and Gerdes, 1999). When more propionate is formed in starch based diet, the supply or availability of H_2 for methane production is limited. This results in decrease in pH causing a reduction in methanogenic activity (Iqbal et al., 2008). Furthermore, a concentrate based diet has shown to reduce methane production by decreasing the protozoal population (Iqbal et al., 2008, Van Soest, 2018).

However, there is higher chance of increased health risk such as acidosis when a concentrate based diet is fed (Chen et al., 2021a). The high cost of concentrates will also make this an expensive option for most farmers in developing nations.

iii. Defaunation

The term defaunation refers to the elimination of protozoa from the rumen. According to Fonty et al. (1988) ciliate protozoa only begin to establish in rumen of the newborn three weeks after birth, as the ciliate protozoa are not present at birth. Ivan et al. (1986) reported that if newborn are not allowed to mingle or graze with older animals, and are reared in complete isolation of the old animals, no protozoa will colonize their rumen. Since protozoa are closely associated with methane production, defaunation have been widely practiced (Morgavi et al., 2010b). The defaunation techniques which include the use of synthetic chemicals (e.g. calcium peroxide, copper sulphate and detergents), dietary manipulation (virginiamycin, milk fat) and natural compounds (e.g. ecdysones; which is a steroidal compound that causes skin shedding in insects, vitamin A and non-protein amino acids) have all been reviewed by (Hegarty, 1999). Defaunation reduces methane production through one of the following methods: (1) a decrease in the population of the methanogens associated with protozoa (Machmüller et al., 2000), (2) a decrease in fiber digestion (Machmüller et al., 2003), (3) an increase in the partial pressure of oxygen in the rumen (Iqbal et al., 2008), and (4) a reduced H_2 transfer (Finlay and Fenchel, 1993). Newbold et al. (1995) reported that methanogens that are in symbiotic relationship with protozoa are responsible for 9-25% of rumen methanogenesis. Dohme et al. (1999) found that the production of methane decreased by 61% in defaunated rumen fluid. Iqbal et al. (2008) also reported in vivo results, however the resultant effect of defaunation on methane production is quite variable, as dietary effect has a strong role to play. For example, in a study where defaunated cattle were placed on high concentrate diet, an approximate of 50% methane reduction was observed (Kreuzer et al., 1986), however in another study, there was no methane reduction in defaunated sheep fed diets based on hay, maize silage and concentrates (Machmüller et al., 2003).

Some challenges of defaunation for methane reduction has been reported. Machmüller et al. (2003) observed that digestion (most especially fiber and protein) was negatively affected in a completely defaunated animal. Other studies reported that a decrease in methane production in defaunated animals was only temporary (Ranilla et al., 2004). Which limits the use of defaunation as mitigation strategy.

iv. Supplementation with Ionophores

Ionophores are produced by soil microbes as polyether antibodies that helps to modulate the movement of cations like potassium, calcium and sodium across cell membranes. The most common examples of ionophores that have been used extensively in manipulation of ruminal fermentation are lasolid and monensin. Ionophores' mode of action on methane production is as follows; Ionophores (1) increases the feed conversion efficiency (FCE) (Goodrich et al., 1984), (2) selective reduction of acetic acid production (Slyter, 1979), though a shift in population of bacteria (from gram positive to gram negative bacteria) which in turn prompts the production of propionic acid (Moss et al., 2000, Kumar et al., 2009), (3) inhibit hydrogen release from formate (Van Nevel and Demeyer, 1979) and (4) suppress the growth in population of ciliate protozoa (Guan et al., 2006). In a study by O'Kelly and Spiers (1992), a 55% methane reduction in Brahman steers was reported, when monensin was supplemented in their diet, however, the reduction was attributed to a reduced feed intake (an anoretic effect) and 45% to the specific rumen activity effect. Supplementation with monensin at 24ppm in diet of dairy cows resulted in a decreased methane production by 28% (Kinsman et al., 1997). It has also been reported that there was a reduction in the loss of gross energy to methane by a 9% when monensin was supplemented in the diet of beef cattle (McGinn et al., 2004). A review by Van Nevel and Demeyer (1979) on data gathered from in vitro studies showed a wide range in the percentage (0-76%) of methane inhibition effect by monensin. However, Omar (2004) found that the effect of monensin on methane suppression could not be maintained for repeated applications. In his study, he reported that the rumen microbe were able to adapt to the ionophores within a period of 45 days. This is further supported by the reports of (Johnson et al., 1994, McCaughey et al., 1997), who found that suppression of methane production by monensin in cattle was short-lived. Another limitation to the use of ionophores is the increasing awareness and resistance of consumers to the routine use of ionophores, as they are also considered a type of antibiotics. It use as growth promoter has currently been prohibited in some countries (Iqbal et al., 2008).

v. Plant secondary metabolites (PSM)

According to Wallace (2004) and Adejoro (2019), plants produce a number of secondary compounds known as phytochemicals, which serves as protection against insects, microbes, animals and other plants. Examples of PSM include terpenes, protein inhibitors, organosulphur and polyphenolic compounds (mainly saponins, tannins and essential oils) (Adejoro, 2019). PSMs have only been recently recognized for their potential effect on methane reduction (Beauchemin et al., 2008). This effect of methane suppression by PSMs is associated with the presence of antimicrobial properties that destroy protozoa (Hristov et al., 2003), fungi (Patra and Saxena, 2009) as well as bacteria (Bodas et al., 2012) present in the rumen.

Plants saponins have been demonstrated to have potentials for methane reduction. Beauchemin et al. (2008) found that the effectiveness of saponins on methane reduction varies among the different sources. Kumar et al. (2008) reviewed some saponins sources as well their percentages for methane reduction and they include; *Quillaja saponaria* (10%), *Medicago sativa* (3-5%), *Yucca schidigera* (4%), *Sapindus rarak* and *Emblica officinalis*. The anti-protozoal effect of saponins and the resultant defaunation improved the production of propionate with a subsequent reductions in butyrate and acetate in an vitro study (Hess et al., 2003). Kumar et al. (2008) also reported that saponins decreased the production of enteric methane from 20-60% when tested on different substrates, which was accompanied by a reduction in ammonia nitrogen as well as decrease in the protozoal population. The decrease in the population of protozoa reduces the inter species transfer of hydrogen to the methanogens associated with protozoa; this affects the availability of hydrogen to methanogens for methane production.

Essential oils from plants have been used to manipulate the rumen microbial activity due to their anti-microbial characteristics (Benchaar et al., 2008). The anti-microbial effect of EO has been attributed to their interactions with cell walls of bacteria, including the electron transport, phosphorylation, ion gradients, protein translocation and other enzyme dependent reactions (Dorman and Deans, 2000, Benchaar et al., 2008). Essential oil extracted from *Origanum* and *Thymus* showed a strong inhibiting effects on methane in an in vitro study, although the concentration of propionate and acetate was reduced (Evans and Martin, 2000). Kamra et al. (2006) reported a methane reduction of 64% when garlic extracts was used in an in vitro study, with no negative effect on feed digestibility. However, Benchaar et al. (2008), warned that the use of EO may not have a long term applicability as extended use of EO could result in the total inhibition of fermentation process in the rumen.
Tannins have also been studied widely as they are known to have methane mitigation potential due to their antimicrobial properties. Tannins affects both the rumen functions and methanogens depending on the type, source and level of inclusion (Malik et al., 2017a). A detailed discussion of type of tannins and their mode of action is discussed below.

2.5 Types, sources and mode actions of tannins

Tannins are water-soluble polyphenols (Jerónimo et al., 2016) that have high complexity and high molecular weight between 500-20,000 Da (Junior et al., 2017). These compounds can be classified into hydrolysable tannins (HT) and condensed tannins (CT) (Figure 2-5) (Frutos et al., 2004), there is also a third class known as phlorotannins (PT) only found in marine algae (brown and red algae) (Aboagye and Beauchemin, 2019). HT and CT also referred to as terrestrial tannins are widely spread in the plant kingdom and abundant in a lot of shrubs, medicinal herbs, cereals and forages. Condensed tannins also referred to as proanthocyanidins have high molecular weight (MW) of 1900 – 28,000 Da (Aboagye and Beauchemin, 2019). Tannins sources in temperate regions include *Vicia sativa*, *Lotus corniculatus, Onobrychis coronarium, Lotus pedunculatus* etc, while in tropical regions, tannins are commonly found in both leguminous and non-leguminous shrubs and trees such as *Argania spinosa*, *Acacia angustissima*, *Ceratonia silique*, *Acacia mearnsii* etc. Both HT and CT differ in concentrations depending on the growing conditions of the plant, the part of the plant as well as the stage of growth (Adejoro, 2019, Piluzza et al., 2014)

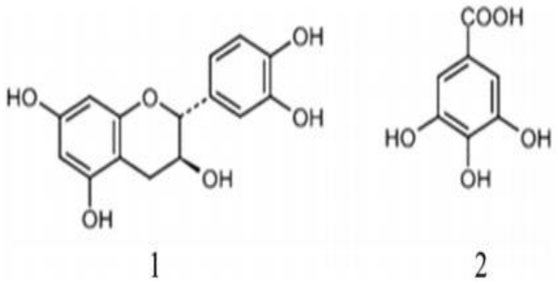

Figure 2-5: The chemical structure of typical example of tannins. (1) Condensed tannin (CT) (Epicatechin) and (2) Hydrolysable tannin (HT) (Gallic acid) (Lamy et al., 2011)

The concentrations of tannins from tropical plant sources are generally higher than that of temperate plant (Berard et al., 2011). This high concentrations have been associated with the higher temperatures and effects of drought on the chemical compositions of the plants (Aboagye and Beauchemin, 2019). It is suggested that the higher concentrations of tannin is a form of defense in plants that are environmentally stressed.

Tannins contains a phenolic hydroxyl group which gives them the ability to be able to bind with several macromolecules especially proteins, and some instances carbohydrates, metal ions and nucleic acids (Makkar, 2003). These interactions between other molecules and tannins determine the metabolic effect on the animals. The interaction between tannins and proteins is considered the most important determining factor of either the nutritional value of potential toxicity of tannins in the animals (Aboagye and Beauchemin, 2019). The protein in the tannin-protein complex may come from microbial, dietary, mucous or endogenous sources in the rumen.

2.6 Effect of tannins on methane

In vitro and in vivo studies have shown that tannins can act as rumen modifiers, however, the exact mechanisms in which methanogenesis is affected is inconclusive (Aboagye and Beauchemin, 2019). There are different proposed mode of actions of how tannins affects methane production and they include; (1) the direct actions of tannins on methanogens (Field et al., 1989, Diaz Carrasco et al., 2017) (2) they affect fibrolytic bacteria and cause a decrease in degradation of fiber (Carulla et al., 2005) (3) they affect protozoa that are associated with methanogens (Bhatta et al., 2009) and (4) acts as hydrogen sink (Becker et al., 2014). Tannins may act in one or all of the proposed modes of actions mentioned above. The mechanisms by which tannins affects methane production also differs based on tannin type (source, molecular weight), concentration of tannin, the animal type and dietary substrate. For example, Beauchemin et al. (2007) reported that when Quebracho (*Schinopsis quebracho-colorado*) condensed tannins were supplemented in diet of beef steers at 2% DM, there was no effect on methane production, although the faecal excretion of nitrogen was reduced, in contrast, tannins from carob (*Ceratonia* *siliqua*) pulp in the diet of lambs at 2.5% DM, severely affected weight gain in lambs (Priolo et al., 2000).

Carulla et al. (2005) reported that a 13% reduction in enteric methane production when *Acacia mearnsii* CT was supplemented at 2.5% DMI in diet of sheep fed rye grass, a small drop in digestibility of feed was observed, but overall the growth rate of the animal was not affected. Similarly, Adejoro et al. (2019) reported a 30% and 19% decrease in methane production when crude tannins diet and encapsulated tannin diet were fed to South African Merino sheep, without affecting the DMI. In another study, Williams et al. (2020c) reported an 11% reduction in enteric methane production when *Acacia mearnsii* CT was supplemented at 400g/day in the diet of Holstein-Friesian cows.

2.7 Tannins and the rumen microbes

2.7.1 Effect of tannins on methanogens and protozoa

According to Jayanegara et al. (2012) tannins affected methane production either through a direct effect on the methanogens or indirectly by affecting the ruminal degradation of nutrients. An example of the direct effect on rumen methanogens was demonstrated by Tavendale et al. (2005) when he reported the effect of CT extracted from *Lotus pedunculatus* on *Methanobrevibacter ruminantium* in an *in vitro* study. It was suggested that this effect may be due to the deactivation of the *mcr* enzyme which is an important enzyme in methane production (Juottonen et al., 2006). Tan et al. (2011) also reported a linear reduction in the population of the methanogens and decrease in methane production when CT extracts of *Leucaena leucocephala* hybrid-Rendang was studied *in vitro*. The *Methanobrevibacter spp*. is gram-positive methanogens that is known to have a higher susceptibility to the effect of tannins as compared to the gram negative methanogens such as *Methanomicrobium* and *Methanimicrococcus* (Field and Lettinga, 1992). According to Smith et al. (2005) the binding ability of tannins to proteins leads to both unavailability of substrates for digestion as well as inhibition of extracellular enzymes which results in the deactivation and the subsequent death of the microbes.

The effect of condensed tannins on rumen protozoal population and diversity has been reported. Cieslak et al. (2012) verified that significant reduction was observed in the population of protozoa when the animal's diet were supplemented with tannins. Saminathan et al. (2016) explained that since there exist a symbiotic relationship between the rumen protozoa and the methanogens, especially due to the production of formate and hydrogen by the protozoa, it is possible that a reduction in the population of protozoa by action of tannin also directly affect the population of the methanogens associated with it. The indirect reduction in methanogens (brought about by defaunation) (Bhatta et al., 2013) population may affect methane emission (Cieslak et al. (2012). Methanol and ethanol extracted tannins of *Terminalia chebula* has been reported to decrease the total protozoa population inclusive of both the small and large entodiniomorphs (Patra et al., 2006). Kobe lespedeza (a tannin rich plant) supplemented to goats, linearly decreased the population of the rumen protozoa while increasing the feed intake without affecting the total bacterial counts (Animut et al., 2008). Few studies however reported the increase in protozoal population as an effect of tannin. For example, Chiquette et al. (1989) reported an increase in number of protozoa in the rumen of sheep fed CT from sulla (*Hedysarum coronarium*) and birdsfoot treefoil (*L. corniculatus*). Similarly, Salem et al. (1997) found that when *Acacia cyanophylla Lindl* leaves (which contains 4.5% tannins) was added in increased portion to a lucern-hay based diet of sheep, the protozoa population in the rumen increase linearly. These studies are evident that the different tannins types and sources do not have the same effect on protozoa.

2.7.2 Effects of tannins on rumen bacteria and fungi

Generally, it has been discovered that tannins inhibit the growth and/or activities of the microorganisms. Tannins inhibitory effect on bacteria as explained by Smith et al. (2005) is due to the ability to form protein complexes with the bacterial cell wall and membrane as well as the resulting of morphological changes in extracellular enzymes secreted. The growth as well as rate of proteolysis in *B. fibrisolvens, F. succinogenes, S. bovis, Clostridium proteoclasticum, R. albus, and Eubacterium spp* was found to be inhibited by CT of *L. corniculatus* in an in vitro study (Min et al., 2005). In another study by Jones et al. (1994) CT from sainfoin (*Onobrychris viciifolia*) inhibited the growth of the following proteolytic bacteria *R. amylophilus, S. bovis* and *B. fibrisolvens*, however a strain of *P. ruminicola* was tolerant to the effect of the CT. However, the supplementation of 30% Calliandra leaves that contains tannins did not have any effect on the total proteolytic bacteria or rumen fungi, nor was the efficiency of the microbial protein synthesis inhibited, although significant reduction in the population of cellulotytic bacteria such as *F. succinogenes* and *Ruminococcus spp*. was observed (McSweeney et al., 2001). These studies demonstrated that the different sources of tannins affects the microbial species in different ways.

Fewer studies on the effect of tannins on rumen fungi have been reported. McSweeney et al. (2001) found that the activities of the fiber-degrading fungi was less sensitive to the effects of condensed tannins compared to cellulolytic bacteria. Paul et al. (2003) further confirmed that rumen fungi was not adversely affected and could grow in concentrations of up to 20 g/l of tannic acid. It seems that effect of tannins on rumen fungi may not be as pronounced as that of the rumen bacteria.

2.8 Techniques for analyzing the rumen microbiome

There are two types of techniques used in analyzing rumen microbiome namely; the culture dependent and culture independent methods. In culture dependent technique, the GIT of the ruminants possess a range of extremities that makes it difficult to replicate the conditions outside of it. While some of the rumen microbes are aerobic and can grow outside the rumen, a large number of the microbes are anaerobic. This makes them difficult to culture in laboratory media (Matthews et al., 2019). Culture-dependent techniques are quite tedious as it requires different kinds of selective and enrichment culturing conditions so as to be able to replicate the microbes' natural environment (Matthews et al., 2019). Anaerobic microbes are difficult to culture, as oxygen must be excluded as well as other complex growth requirements must be present for the microbes (Rufener et al., 1963). To replicate the environment of the rumen, a continuous culture system was developed by Rufener et al. (1963), however, this method along with other similar methods was used for identification and enumeration of the ruminal microbes.

The classification of the rumen bacteria using the traditional methods were based solely on the standard bacterial identifications technique which includes the shape of the bacteria, morphology, and gram stain (Matthews et al., 2019). The nutritional needs as well as the fermentation end products were also studied and used as a means of classification. A technique where roll tubes was used to grow anaerobic species became popular in the mid-1900s and replaced by the use of the conventional agar plates (Hungate, 1966). However, after several large projects in the late twentieth century by Robert Hungate and other researchers, it was concluded that although culture techniques could identify the major taxonomic groups in rumen, they did not provide an accurate representation of the microbial diversity present in the rumen. Krause et al. (2013) further supported that only an estimated 20% of the entire rumen microbiome can be cultured using the standard techniques, and more recently, McCabe et al. (2015) suggested that less than 1% of total microbial species are culturable.

The culture independent technique also known as the DNA-based approach of detection and identification of microbes can be used for examination of the microbial communities from different environments at molecular level (Matthews et al., 2019). High throughput sequencing technologies are used for the description and identification of the different microbial communities. Examples of the use of high throughput technology include 16S rRNA, Internal Transcribed Spacer (ITS) amplicon sequencing which are used for bacterial and fungal communities (De Filippis et al., 2017) respectively, as well as (metagenomic sequence) shotgun sequencing which involves the sequencing of DNA fragments in a random manner, without taking into consideration which microbe they come from (Clark and Pazdernik, 2013).

Targeting the mcrA gene to identify methanogens has also been suggested (Luton et al., 2002). All the methods mentioned above employ bioinformatics tools to analyze and /or compare different microbes' diverse ecosystem. One importance of this technique, is that it allows the identification of the unculturable microbe. With the use of DNA sequencing technologies, research of microbial and animal ecosystem has evolved (Hardison, 2003).

The 16S amplicon sequencing is a technique that has been used extensively and it involves the uses of the variable regions (V1-V9) of the bacterial 16 rRNA gene for taxonomic assignment (Chakravorty et al., 2007). It is used for microbial diversity analysis of samples collected from different environment such as human gut (Dethlefsen et al., 2008), rumen, soil (Chong et al., 2012) etc. 16S uses specific primers to identify specific archaea and bacteria present in a sample. The advantages and limitations are summarized in Table 2-3.

The 18S rRNA is one of the primary components that contains both the hypervariable and conserved regions of the eukaryote cells. 18S can be used to identify protozoa, but there is high chance of amplifying the animal DNA which in turn affects the results.

Table 2-3: Advantages and limitations of 16S rRNA Amplicon

Limitations of 16S Amplicon	Advantages of I6S Amplicon	Reference
• Artificial sequence may be produced due to sequence error • Wrongly assembled Amplicon - chimeras • Leading to difficulty in identification	• It amplifies the specific microbes, therefore no host contamination.	• Wylie et al. (2012)
• Inaccurate classifications of organism's different species, due to same 16S rRNA gene sequence. • Reduced accuracy at species level.	• Relatively inexpensive compared to shotgun metagenome.	• Sharpton (2014).
• Provides information only about taxonomic composition and none on functional composition.		• Sharpton (2014).
• Highly diverged microbes such as viruses or novel microbes may be difficult to study, due to unavailability of taxonomically informative markers.		• Sharpton (2014).

The ITS gene is found between the 18S and the 5.8S rRNA genes, and has degree of sequence variation (Ghosh et al., 2019). It is mainly used to study diversity of fungi in environmental samples (Bromberg et al., 2015).

2.9 Metagenomic analysis (Shotgun)

Metagenomic analysis is mostly utilized to study the complex microbial population sampled from the environment directly, without a single organism culturing or isolation. In different environments, microbes have important roles to play, but many remain to be characterized in depth. Handelsman (2004) coined the word metagenomics. Metagenomics assist in identifying various characteristics of a sample, and allows microbes characterization to be done in any given environmental sample. Shotgun metagenomics can be defined as the untargeted sequencing of all microbial genomes that is present in a sample (Quince et al., 2017). Shotgun sequencing can assists in the identification of different species available in the community (both culturable and unculturable), and also gives more understanding to the metabolic activities and functional roles of the microbes in the environmental sample (Langille et al., 2013). Shotgun can be used to recover whole genome sequence.

According to Madhavan et al. (2017) shotgun metagenome is divided into two types;

(1) Sequence based, whereby the microbial diversity and genomes of an environmental samples is described and,
(2) Functional, whereby the functional gene are identified without determining from which species the genetic material originated from.

Some of the disadvantages of using shotgun compared to amplicon sequences in that they generate large complex data that are quite difficult to analyze, and shotgun metagenome is expensive especially when the DNA of the host significantly outnumbers the DNA of the microbes. The identification and removal of sequence contaminants is also quite difficult in shotgun data (Kunin et al., 2008, Quince et al., 2017), although contamination is a general challenge common to environmental sequencing (Degnan and Ochman, 2012, Quince et al., 2017). For example, if a contaminant contains lots of genes that are not common in the community it is easy for the contaminant to mislead the analysis of the microbial diversity (Sharpton, 2014). Furthermore, metagenomic data especially microbiota can contain unwanted DNA of the host and can sometimes overwhelm the microbial community DNA.

On the other hand, shotgun metagenome can identify all microbes (viruses, eukaryotes). There is no primer bias. It also provides functional information of the microbial species (Xing et al., 2020).

2.10 Bioinformatics for Amplicon-based and shotgun metagenome

The steps for analyzing the amplicon-based sequences include: pre-processing of read, OTU picking, taxonomic assignment and statistical analysis. Common tools used for the analysis are summarized in Table 2-4. Despite that there exists so many tools for analysis of the 16S, QIIME2 is considered the ‘gold standard’ (Bolyen et al., 2019)

Table 2-4: Features of tools used in analysis of 16S rRNA, Adapted from Plummer et al. (2015)

Feature	QIIME	Mothur	MG-RAST
License	Open-source	Open-source	Open-source
Implemented in	Python	C++	Perl
Website	http://qiime.org/	http://www.mothur.org/	http://metagenomics.anl.gov
Web-based interface	YES (http://www.n3phele.com/) Not supported/maintained by the QIIME team	NO	YES (at website above)
Primary usage	Command line	Command line	GUI (at website above)
Amplicon analysis	YES	YES	YES
Whole metagenome shotgun analysis	YES – experimental only	NO	YES
Sequencing technology compatibility	Illumina, 454, Sanger, Ion Torrent, PacBio	Illumina, 454, Sanger, Ion Torrent, PacBio	Illumina, 454, Sanger, Ion Torrent, PacBio
Quality control	YES	YES	YES
16S rRNA gene Databases searched	RDP, SILVA, Greengenes and custom databases	RDP, SILVA, Greengenes and custom databases	M5RNA, RDP, SILVA and Greengenes
Alignment Method	PyNAST, MUSCLE, INFERNAL	Needleman-Wunsch, blastn, gotoh	BLAT
Taxonomic analysis/assignment	UCLUST, RDP, BLAST, mothur	Wang/RDP approach	BLAT
Clustering algorithm	UCLUST, CD-HIT, mothur, BLAST	mothur, adapts DOTUR and CD-HIT	UCLUST
Diversity analysis	alpha and beta	alpha and beta	alpha
Phylogenetic Tree	FastTree	Clearcut algorithm	YES
Chimera detection	UCHIME, chimera slayer, BLAST	UCHIME, chimera slayer, and more	No
Visualisation	PCA plots, OTU networks, bar plots, heat maps	Dendrograms, heat maps, Venn diagrams, bar plots, PCA plots	PCA plots, heat maps, pie charts, bar plots, Krona and Circos for visualisation
User Support	Forum, tutorials, FAQs, help videos	Forum, SOPs, FAQs, user manual	Video tutorials, FAQs, user manual, ‘How to’ section on website

For the statistical analysis, a taxonomic tree can be obtained in QIIME and can be visualized using with any tree display tool e.g FigTree (availabele at http://tree.bio.ed.ac.uk/software/figtree/). Variability within a single population such as dominance, evenness and richness can be measured using Alpha diversity. Other diversity metrics that can be measured include Phylogenetic Diversity (Chao, 1984, Tucker et al., 2017), Shannon entropy (Gorelick, 2006).

Beta diversity measures diversity across populations or samples, it is calculated using matrices such as weighted and unweighted Unifrac and Principal Coordinate Analysis (PCoA) (Lozupone et al., 2006). It helps to measure the relative or absolute overlap between samples to determine the taxa shared among them. Both alpha and beta diversity is supported on QIIME.

Figure 2-6 shows a simplified steps involved in analysis.

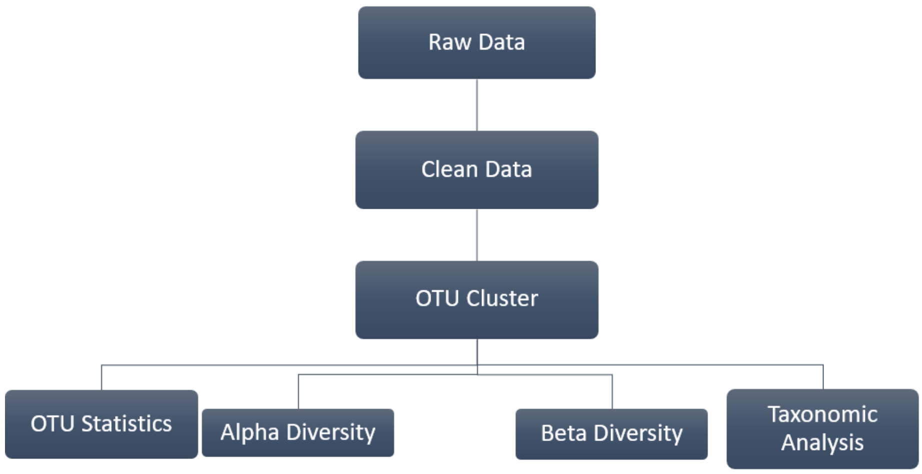

Figure 2-6: Overview of the steps in 16S rRNA Analysis (Lischer and Shimizu, 2017)

The analysis of shotgun metagenomics as shown in Figure 2-7 are as follows.

The raw reads undergoes pre-processing stage (trimming, filtering and de-replication), which is followed by assembly. Assembly involves the combination of similar sequence reads to form a contig (contiguous sequence). There are two strategies that can be used to assemble reads: Co-assembly which is reference-based assembly, and De novo assembly (Lischer and Shimizu, 2017). There are handful of tools use for sequence assembly. Some of the commonly used metagenomic assembly tools are: metaSPAdes, Meta-IDBA SOAP MetaVelvet, MetaVelvet-SL and Meta-Ray (van der Walt et al., 2017). The next stage is binning, at this stage contigs or reads are clustered into similar groups (metagenomic sequence to a taxonomic group). Composition-based binning and similarity-based binning are the two types of algorithms that are used. The fourth step is gene prediction. Coding DNA sequences (CDS) prediction helps in deciding which metagenomic reads has coding sequences. Unassembled or assembled metagenomic sequences can be used for gene prediction.

There are three ways to carryout genes predict: a). "gene fragment recruitment", b) "protein family classification", and c) "de novo gene prediction" (Sharpton, 2014). Annotation is done once coding sequences are predicted. The genes that are predicted are annotated to detect homologous genes, KEGG pathways, clusters of orthologous genes, Gene ontology terms, orthologous families or (COGs/KOGs) or TIGRfams or protein families using Pfam and functional motifs using InterPro (Wheeler et al., 2007, Ashburner et al., 2000, Kanehisa et al., 2010, Bateman et al., 2004, Haft et al., 2003, Tatusov et al., 2003, Jensen et al., 2007, Hunter et al., 2009).

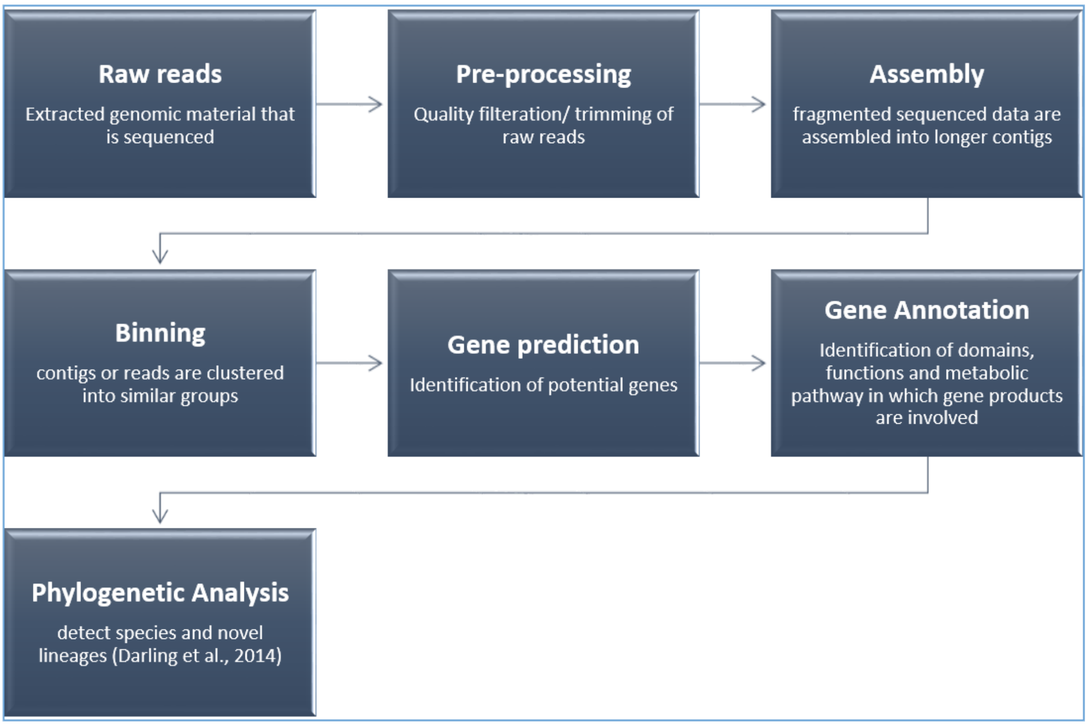

Figure 2-7: The workflow of shotgun analysis

2.10.1 Metagenomics Phylogenetic Analysis

The taxonomic approaches can give specific taxonomic hierarchy information, e.g. "phylum, class, order, family, genus and species" (Darling et al., 2014, Kayani et al., 2021), while phylogenetic methods assist at the level of taxonomic to detect species and novel lineages (Darling et al., 2014). Different tools such as AmphoraNet, TIPP ("taxonomic identification and phylogenetic profiling"), and Phylosift and so on have been reported used for the phylogenetic analysis of metagenomes (Darling et al., 2014, Kerepesi et al., 2014, Nguyen et al., 2014). The PhyloSift database comprises a set of "elite" gene families of Archaea and Bacteria, and also it comprises further four sets of gene families ("16S and 18S ribosomal RNA genes, mitochondrial gene families, eukaryote-specific gene families, and viral gene families"). The metagenome reads are run against a known set of gene database for taxonomy prediction.

2.11 Conclusion

Methane mitigation using dietary supplementation has shown to be effective. In this chapter we have shown reports that composition of microbial community present in the rumen can be affected by factors such as feed and diet quality. It was also established that examining the effect of feed additive supplementation on microbial structure could lead to identification as well as better understanding of the methane reduction. Several studies have also reported changes in the microbial diversity using metataxonomic approaches. This chapter also shows that there are limited information on the in-depth description of microbial population with using metagenomics.

Chapter 3

3 Materials and methods

3.1 Introduction

An animal trial was conducted with approval from the Animal ethics committee (EC075-17) at the small stock unit of the Hillcrest experimental farm of the University of Pretoria, South Africa. Rumen samples were collected at the end of the experiment and stored in the Nutri-lab freezer. These samples were made available for the current research to study the rumen microbiome of the animals fed crude and encapsulated *Acacia mearnsii* tannin extract.

The current study was approved (NAS250/2020) by Animal Ethics Committee (AEC) University of Pretoria for the use of the samples for DNA extraction and generation of sequencing data for analyses.

3.2 Background on the origin of the samples

The study was carried out at the small stock unit of the Hillcrest experimental farm of the University of Pretoria, South Africa. The study was carried out during October 2018 to January 2019. The average for the minimum and maximum temperatures during this period were 13.5°C and 29.8°C.
Forty South African Mutton Merino sheep (4 months old males) with an average body weight of approximately 28 kg were used in this experiment. The animals were allocated to 20 pens in randomized completely block design (RCBD), 10 animals per treatment. The study lasted for 103 days with 26 days' adaptation period. Animals were stratified first according to their body weight and similar animals were randomly assigned to one of the four dietary additive treatments, in a randomized completely block design, and two animals were housed per pen. Feed was offered to the animals in the morning and the evening. Methane measurement was carried out in methane chamber and lasted for six days (Ibrahim and Hassen, 2022). All experimental sheep were vaccinated, dewormed and their wool shorn prior to the commencement of the study (Ibrahim and Hassen, 2022).

The four dietary additive treatments were:

- T1: TMR + distilled water (negative control),
- T2: TMR + 75 mg/kg DM of Monensin (positive control),
- T3: TMR + 20 g/kg DM *Acacia mearnsii* tannin extract
- T4: TMR + 29 g/kg DM of Acacia tannin extract micro encapsulated with Sunflower oil (Ibrahim and Hassen, 2022).

3.2.1 Microencapsulation of the Tannin extract

The encapsulation of tannin extract with sunflower oil was based on the solid/oil/water (S/O/W) technique as described by Adejoro (2019) with a few modification. The water solution was prepared by measuring 300 mL distilled water containing 1% (w/v) Tween80 emulsifier into a 500 mL beaker and then an iron rod homogenizer (PRO400DS, Pro Scientific Inc., Oxford CT 06478 USA) was used to homogenize the mixture at 20,000 revolutions per minute (rpm) for 3 minutes till a foamy mixture was obtained. Next was the preparation of the solid-in-oil solution which was prepared by measuring 8.5 g of tannin extract powder into a 100 mL beaker that contained 30 mL sunflower oil solution and 50 mg/mL DCM mixed with 0.5% (w/v) Span80 as a surfactant. The mixture was then stirred with a magnetic stirrer at 400 rpm for 2 minutes. To produce the final solid/oil/water solution, the solid-in-oil solution was added to the water solution and homogenized at 20,000 rpm for 3 minutes. Afterwards, magnetic stirring plate was used to stir the mixture for 3 hours at 800 rpm so as to completely evaporate the DCM. The microcapsules of the sunflower oils produced were squeezed through a four layers of clean cheese cloth, then rinsed with 100 mL distilled water before transferring it to an aluminium container to freeze-dry for 5 days. The sunflower encapsulated tannins extract were ground to powder and stored in refrigerator.

3.2.2 Rumen sample collection

At the end of the experiment, the animals were slaughtered and rumen samples was collected from 24 animals (6 animals per treatment). At the slaughter house, rumen contents for each animal were emptied into a bucket and hand mixed thoroughly before a representative sample was taken. Rumen samples were then filtered through four layers of cheesecloth and then stored at -20°C afterwards.

3.3 Methodology for microbiome composition

3.3.1 DNA extraction

Twenty-four (24) rumen fluid samples collected from 24 sheep were stored at -20°C at Nutrilab freezer in the Department of Animal Science, University of Pretoria. The stored rumen fluid samples were defrosted at room temperature. DNA extraction was carried out using D4300 ZymoBiomics miniprep (Zymo Research group, U.S.A) following the manufacturer’s protocol (see Appendix). DNA extraction was done in the Animal Genetics laboratory, Department of Animal Science, University of Pretoria.

The concentration of all DNA extracted was determined using high sensitivity scale on the Qubit 2.0 fluorometer (Invitrogen Life Technologies, Carlifornia). The purity was also determined by loading 1 μL of the DNA sample on NanoDrop spectrophotometer (Thermo Scientific™, South Africa). A gel electrophoresis (1% agarose gel, run at 80V for 45mins) was carried out to also confirm the quality of the DNA sample using 2 µL loading dye and 3 µL of the DNA sample.

3.3.2 Polymerase Chain Reaction (PCR)

A polymerase chain reaction (PCR) was performed to test for successful amplification. Universal primers flanking the 16S rRNA sequence was used as adapted by Edwards et al. (1989). Primer sequences used are as follows: 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5'-GGT TAC CTT GTT ACG ACT-3'). A 10 µL master mix consisting of 8 µL of Taq (dNTP, $MgCl_2$ and Taq) (Qiagen, South Africa), 0.3 µL forward primer, 0.3 µL reverse primer, 1.4 µL molecular grade water and 5µL DNA. A BIO-RAD T100™ Thermal Cycler was used for the PCR reactions. Initial denaturation occurred at 94 ºC for 10 min, which was then followed by 30 cycles of denaturation at 94 ºC for 1 min, annealing at 58 ºC for 1 min, extension at 75 ºC for 1 min and a final extension at 75 ºC for 5 min. Samples were kept at 4 ºC after the 30 min cycle.

Agarose gel electrophoresis (3% gel run for 45mins at 80V) was performed on the PCR product to determine whether the amplification was successful. The DNA were stored at -20°C until shipment to a laboratory for sequencing.

3.4 Metagenome library preparation and Sequencing

Twenty- four (24) DNA samples consisting of approximately 20 µL each with concentration range 40-120 ng/µL (details of the samples is in Appendix Table 1). This represented six samples per treatment were shipped to Novogene laboratories in Singapore (Novogene Co. Singapore) for shotgun sequencing.

Metagenome library preparation was done following the standard protocol at the laboratory (Figure 3-1). In brief, the protocol followed by Novogene consist of quality control where twenty-three (23) out of the twenty-four (24) DNA samples passed before proceeding with library preparation. A Covaris sonicator (Covaris Inc. Massachusetts, USA) was used to randomly fragment DNA to produce fragments at 300bp. The fragmented DNA were then end-repaired, A-tailed, purified and then PCR amplification was done. The libraries were diluted to 2 ng/ul and then checked for the insert size (library fragment size) using the Agilent 2100 Bioanalyzer (Agilent Technologies, California, USA). The quality of the library (effective concentration > nM) was determined using Q-PCR to meet the 3nM specification. The qualified libraries were then fed into Ilumina sequencers after pooling according to its effective concentration and expected data volume (See Figure 3-1 for the flow chat). The total number of raw reads acquired was 62.2 gb.

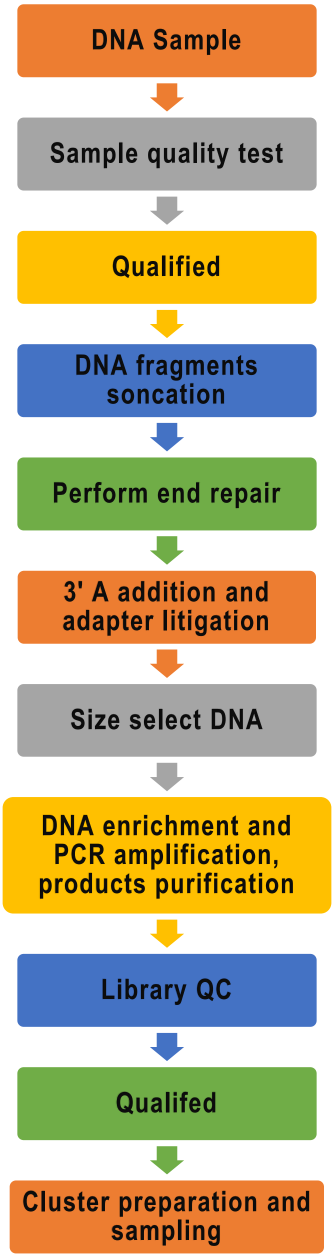

Figure 3-1: The NOVOGENE Sequencing Pipeline

3.5 Bioinformatics and statistical analysis

The data received from Novogene labs after sequencing was in the fastq. format. The bioinformatics was done using MG-RAST (see Figure 3-2 for the pipeline). The raw data files were uploaded to the MG-RAST version 4.0.3 (Meyer et al., 2008), an online server and analysis was carried out using the default parameters. First, the read 1 and read 2 of each sample (in Fastq format) were merged using the 'join paired-ends'. Next was detecting and removing of the adapter sequences using a bit-masked k-difference matching algorithm. The quality control step include a pre-processing step that filtered the sequences based on length and number of ambiguous reads using the software fastq-mcf (Aronesty, 2013) also within the MG-RAST server.

All sequence with low quality having more than five bases and phred score lower than 15 were excluded from the analysis (Unclassified reads). A de-replication step that involves the removal of artificial replicate sequences produced during sequencing was carried out (Gomez-Alvarez et al., 2009). The replicates are identified by binning reads with identical first 50 bp and then one copy of each identical bin is retained. Bowtie2 (Langmead and Salzberg, 2012) was used to remove contaminant that is host specific species sequence (e.g mouse, plant, human). The sequences were then clustered at a 97% identity using the software CD-HIT (Fu et al., 2012). Sequences were annotated using the BLAT algorithm (Kent, 2002) against M5NR which is a non-redundant database. The Refseq database (NCBI Reference Sequence Database) (Pruitt et al., 2007) was used for taxonomic identifications. The data was downloaded from MG-RAST software and relative abundance was generated.

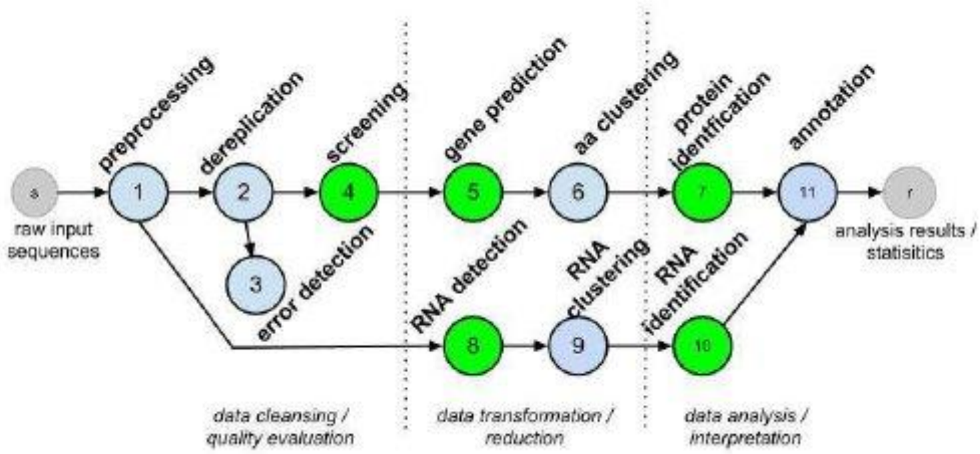

Figure 3-2 The MG-RAST Pipeline used for data analysis

Results from the relative abundance of microbes at different taxonomic levels (phylum, family and genus) were analysed using microiomanalyst.ca to determine the effect of the different treatment on rumen microbes. The low count filter was set to 20% prevalence in samples, where a total of 1094 low abundance features were removed. A total of one low variance feature was also removed based on inter-quartile range. Data normalization using the default setting for data scaling (Total Sum Scaling - TSS) was retained and also no data transformation was done. Pie chart, stacked bar chart and boxplots were generated for visualization.

The α-diversity of each sample was calculated using the Shannon and Simpson indices as well as ACE and Chao1. Principal component analysis was used to visualize Jaccard and Bray-Curtis indices for the beta diversity.

Chapter 4

4 Results

The sequence data counts obtained from all samples ranged from 28,405,982 to 48,140,126 and the average read length was 188 ± 55 to 187 ± 5bp. The reads after quality filtering ranged from 22,963,377 to 36,437,658, with an average read length of 190 ± 52 to 189 ± 52 as obtained from MG-RAST shown in Table 4-1. These reads were processed in MG-RAST and aligned to refseq (NCBI) for taxonomy.

Table 4-1: Sequence details of the 23 samples processed in MG-RAST

Sample ID	Treatments	MG-RAST ID	Initial Reads	QC reads passed	Identified rRNA reads	Initial bp count	Post QC bp count
1B	1	4922806.3	46,071,778	35,670,274	13,454	8,285,734,996	6,484,873,753
2B3	1	4922796.3	47,509,799	34,040,487	11,778	8,711,142,772	6,333,705,968
3B	1	4922788.3	37,414,294	30,367,599	12,974	7,041,590,904	5,741,130,227
4B	1	4922789.3	33,732,157	26,123,881	10,377	6,294,499,107	4,923,964,603
5B	1	4922809.3	37,992,845	29,324,160	10,948	6,812,996,581	5,309,531,967
21B	1	4922798.3	28,405,982	22,963,377	11,510	5,381,913,534	4,963,901,504
6B	2	4922807.3	48,140,126	36,437,658	13,327	8,995,111,871	6,866,385,740
7B	2	4922791.3	32,268,571	26,221,388	10,886	6,069,037,898	4,963,901,504
9B	2	4922795.3	38,853,563	30,591,649	12,335	7,089,113,616	5,628,983,003
10B	2	4922810.3	34,028,378	26,955,497	11,156	6,393,722,803	5,108,963,586
26B	2	4922800.3	31,705,844	25,538,973	13,348	5,844,691,377	4,726,782,766
11B	3	4922799.3	37,734,150	28,552,161	10,546	6,954,278,758	5,326,199,720
12B	3	4922806.3	34,748,608	27,982,492	14,696	6,452,547,677	5,219,400,903
13B	3	4922801.3	32,193,777	25,952,114	12,834	6,079,107,740	4,928,469,641
14B	3	4922794.3	38,745,409	31,932,336	15,217	6,971,501,485	5,772,192,079
15B	3	4922803.3	30,139,724	25,144,400	12,384	5,635,097,953	4,715,583,259
22B	3	4922793.3	34,258,752	27,674,207	12,140	6,276,199,733	5,090,550,164
16B	4	4922804.3	45,109,398	35,792,832	14,536	8,325,082,390	6,642,260,145
17B	4	4922808.3	39,225,660	32,231,637	16,135	7,269,413,505	5,997,745,986
19B	4	4922790.3	39,782,736	30,879,941	13,382	7,557,595,646	5,921,301,649
20B	4	4922797.3	34,092,188	27,213,279	12,019	6,544,053,823	5,249,961,601
23B	4	4922792.3	32,702,845	25,641,354	12,784	6,122,592,104	4,841,822,953
30B	4	4922802.3	33,515,401	27,601,514	14,046	6,247,018,527	5,164,023,369

4.1 Bacterial and Archaeal community composition

4.1.1 Bacteria

The taxonomic composition and abundance of the four dietary treatments consisting of 23 samples for the domain bacteria consisted of several phyla some of which were Actinobacteria, *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Fusobacteria*, *Verrucomicrobiota***.** The domain bacteria had 334,881,038 counts with an average of 14,560,045 per sample. A total of 28 bacteria phyla were identified with *Bacteroidetes* (72%) and *Firmicutes* (21%) as the predominant phyla as shown in Figure 4-1.

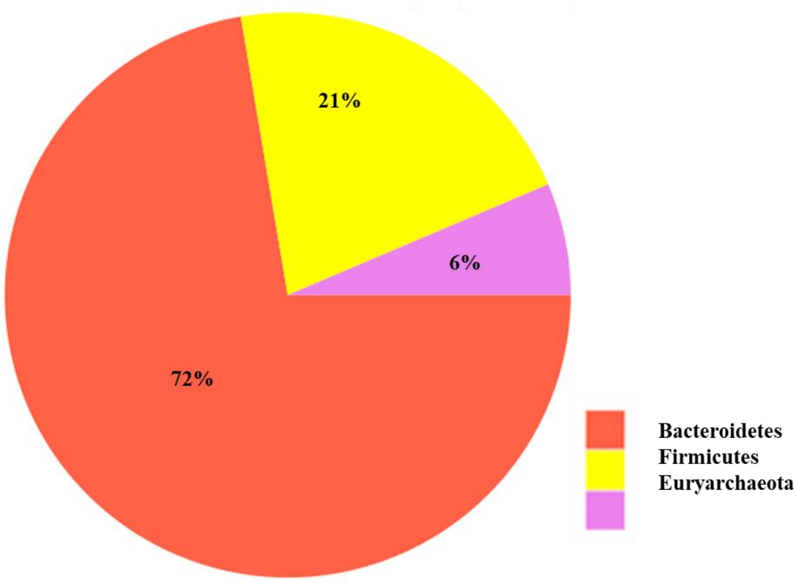

Figure 4-1 A pie chart showing distribution at phylum level for all samples

These bacterial phyla accounts for 93% of the community taxonomic distribution, *Euryarcheota* represented 6% while other phyla including those unassigned composed of the remaining 1% of the community. More than 500 genera were identified, however, *Prevotella*, *Bacteroides*, *Eubacterium* and *Clostridium* formed the largest group (Figure 4-2a and b). *Clostridium* and *Eubacterium* accounted for 70% and 30% of the phylum *Firmicutes* (Figure 4-2a) while *Prevotella* and *Bacteroides* accounted for 67% and 33% of the phylum *Bacteroidetes* (Figure 4-2b). *Prevotella* was most abundant in treatment 1(control) compared to the other treatment as shown in Figure 4-3.

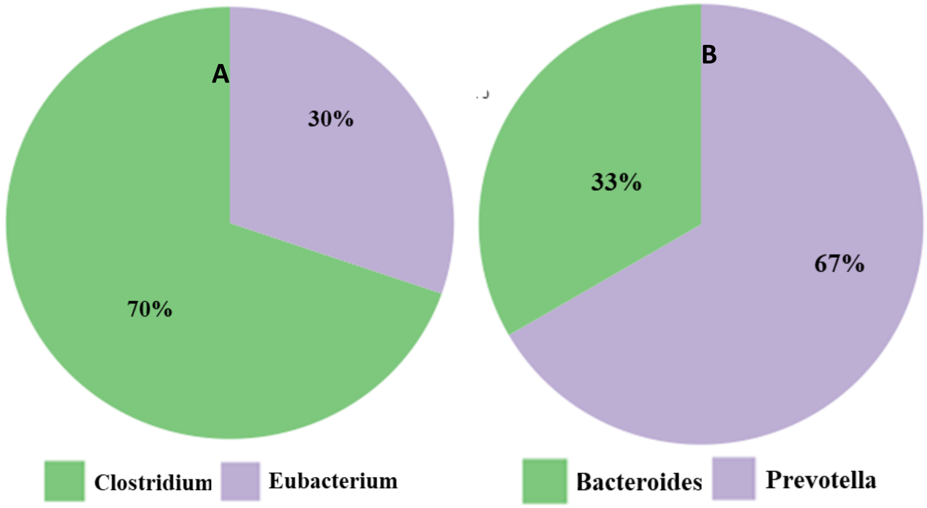

Figure 4-2a and b: The distribution at genus level for phylum *Firmicutes* and *Bacteroidetes* for all samples

4.1.2 Archaea

The taxonomic distribution of archaea at the phylum level is represented by *Euryarcheota* at 6% of the total microbial community across all samples (Figure 4-1). At the family level, *Methanobacteriaceae* is the predominant (Appendix Table 2). Forty-one genera (Appendix Table 2) where identified, however *Methanobrevibacter* (Figure 4-3), where found in higher abundance. *Methanobrevibacter* was most abundant in treatment 3 and 4 (Figure 4-3).

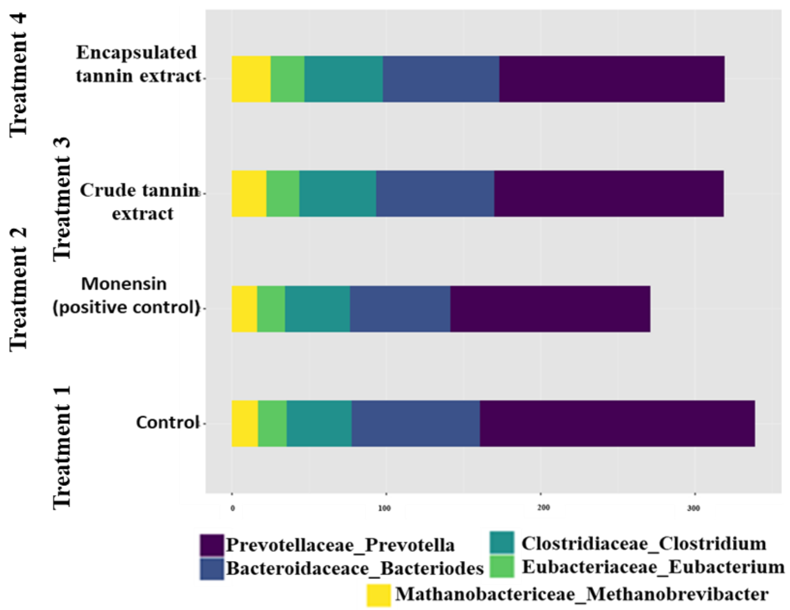

Figure 4-3: A stacked bar-chart of abundance across all animals and treatment

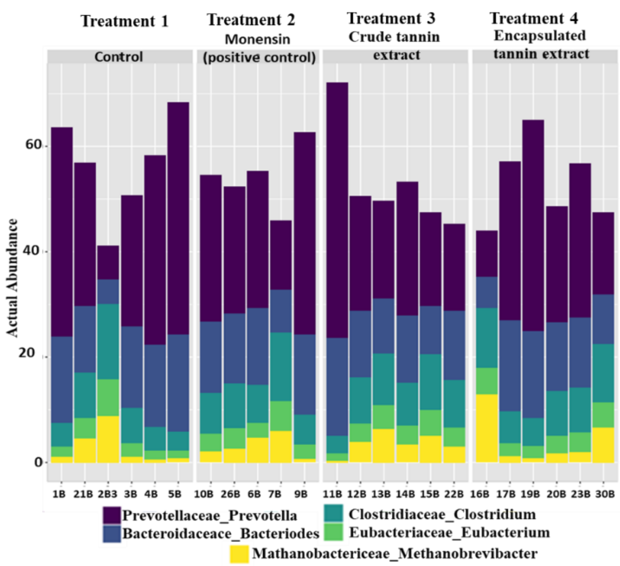

Figure 4-4: A stacked bar-chart illustration of abundance across all animals and treatment

4.2 Diversity indices

The alpha diversity indices- Shannon, showed that there was no significant difference (P>0.05) in the four dietary treatment both at the phylum and genus level as confirmed by Kruskal-Wallis test (Figure 4-5a and b). To further confirm for the richness of the samples, Simpson and Chao1 indices (Figure 4-6a and b) performed showed no difference among treatments with P values of 0.40 and 0.93 respectively.

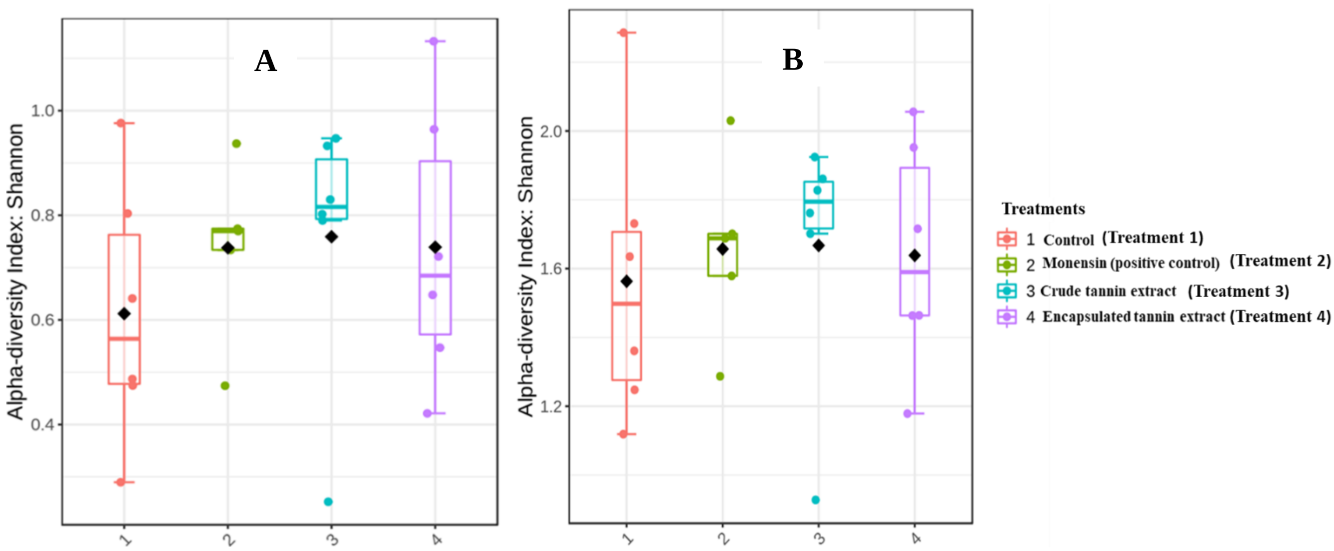

Figure 4-5a and b: Box plots illustrating Shannon diversity at phylum and genus level for all four treatments

The beta diversity indices - Bray-Curtis and Jaccard showed no significant difference (P>0.05) among the dietary treatment at phylum and genus level using the PERMANOVA test. Figure 4-7 provides Principal Coordinate Analyses results that showed no evidence of clustering against any particular treatment which is consistent with obtained P values.

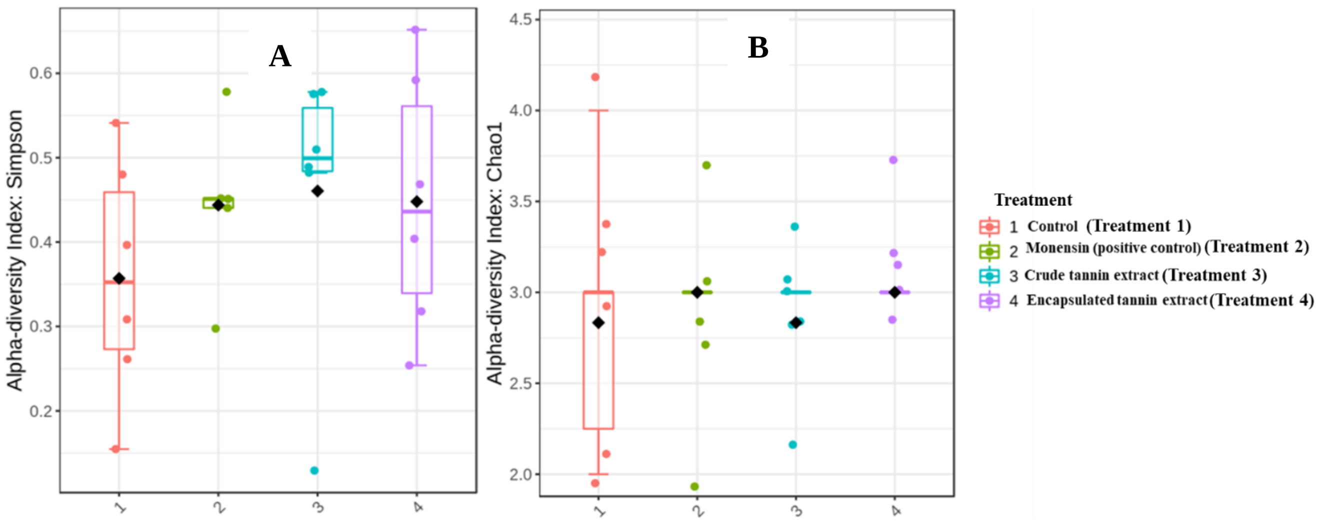

Figure 4-6a and b: Box plots illustrating Simpson and Chao1 diversity at phylum level for all four treatments

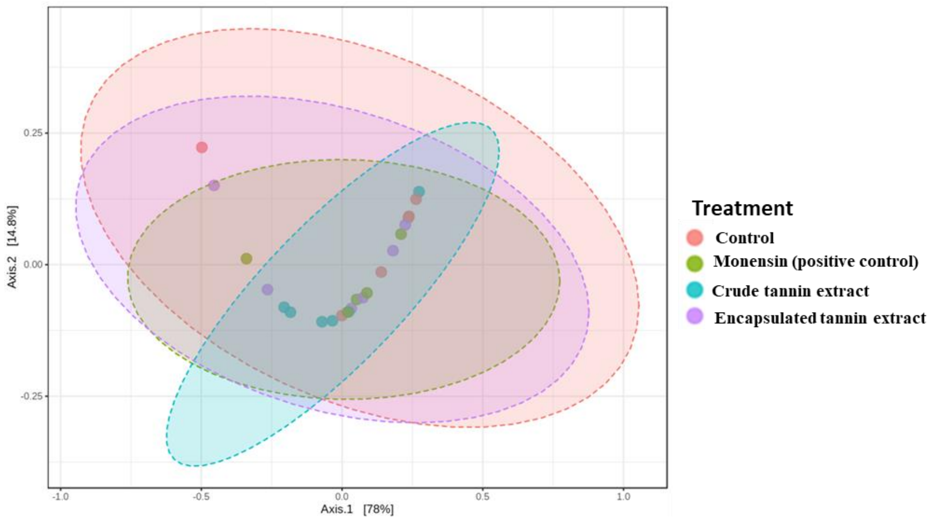

Figure 4-7: PCoA plot illustrating Jaccard index at genus level for all four treatments

Chapter 5

5 Discussion

5.1 Effect of diet on rumen microbial diversity

5.1.1 Bacterial microbiome

Firmicutes and *Bacteroidetes* were the two dominant phyla observed across all samples in this study. Similar results have been reported on the rumen microbiota (Parmar et al., 2014, Nathani et al., 2015, Knoell, 2016). *Bacteroidetes* were the most abundant across the treatments followed by *Firmicutes*. The abundance could be seen at the genus level too, where *Prevotella* and *Bacteroides* dominated. The genus *Prevotella* has been reported in the findings of several researchers (Whitford et al., 1998, Koike et al., 2003, Stevenson and Weimer, 2007, Danielsson et al., 2017, Knoell, 2021) as the most abundant genus of the bacterial community in the rumen from their 16S rRNA data. It has been associated with the breakdown of polysaccharide in the rumen (Knoell, 2021) *Prevotella* is also said to be involved in the production of VFAs such as propionic acid used as an additional energy source by the host (Strobel, 1992, Nathani et al., 2015). Also, at genus level, *Eubacterium* and *Clostridium* were the most represented for the *Firmicutes* genes Figure 4-2. These are potent cellulose and pectin degraders and are therefore classified as important microbes in the utilization of dietary fiber in the rumen (Kong et al., 2010).

Prevotella which belongs to the family *Prevotellaceae* has been reported to be dominant bacteria in the rumen under varying dietary conditions accounting for up to 70% of total bacterial population (Knoell, 2016). The *Prevotella* are gram-negative and thrive well under anaerobic conditions in the rumen and are known to degrade and utilize pectin xylan and starch. In separate studies by Kittelmann et al. (2014) and Danielsson (2016) where microbiota of sheep was explored, it was found that in different *Prevotella* OTUs there were some that were associated with high methane phenotype and others were correlated with low methane emissions. Henderson et al. (2016) also reported that Prevotella is rarely affected by dietary changes in ruminant. This may be the reason why significant differences in the abundance of *Prevotella* was not found across the treatments. In this study, the alpha diversity metrics revealed no significant differences in both richness and evenness across treatments (Figure 4-5 to Figure 4-6). The diets did not change in terms of their composition. It was therefore highly unlikely that the tannin additives would cause a significant shift in diversity of microbial population.

5.1.2 Archaeal community

The archaea community was the main focus of this research as they are responsible for methane production at the terminal step of the fermentation process in ruminants. It is this group of the rumen microbes that is targetted when utilizing dietary intervention (either directly or indirectly) in the reduction of methane.

It has been found that *Methanobacteriales*, *Methanomassiliicoccales*, *Methanococcales*, *Methanomicrobiales*, and *Methanosarcinales*, are the order of archaeal community in the rumen (Janssen and Kirs, 2008, Zhu et al., 2017). *Methanobacteriales* to which the genus *Methanobrevibacter* belong is the largest population of methanogens in the rumen and the abundance varies from 30 to 99% (Knoell, 2021), this genus is the most frequently identified archaea present in the rumen (Morgavi et al., 2010a, Nagaraja, 2016, Tapio et al., 2017).

The phylum Euryarcheota dominated the archaea domain, and the largest population of microbes under this phylum was represented by the Methanobrevibacter at the genus level. The shift observed in the archaea community showed an increase in the genus (though not significant statictically) *Methanobrevibacter* across treatment 3 (crude tannin) and 4 (encapsulated tannin) (Figure 4-3). *Methanobrevibacter* is usually present in the rumen of ruminants that are on different kind of diet, this group of microbes convert CO_2, H_2O and CHO_2 into methane (Leahy et al., 2010). The parent experiment of this research conducted earlier revealed that methane emission was reduced in the tannin containing diet compared to the control (Ibrahim and Hassen, 2022). This shows that reduction of methane by tannin was not through the direct inhibition of methanogens, as the result obtained in current study showed an increased population of the methanogens in both tannin supplemented diets. This is not surprising as four mode of action through which tannins bring about a reduction in methane have been proposed, although according to Aboagye and Beauchemin (2019) this methods is inconclusive. According to Beauchemin et al. (2007) condensed tannins extracted from quebracho trees had no effect on methane production when included at 1% and 2% of the DM diet in production heifers. It was suggested that the lack of effect of tannin on methane emission in the report of Focant et al. (2019) was due to the fact that there was no direct interference of tannins on the methanogenic archaea as well as the protozoa population.

The parent experiment of this study reported an increased feed intake and weight gain in the tannin based diet, although suggested the decrease in methane emission was likely due to the suppressed activity of fiber degradation (Ibrahim and Hassen, 2022) or due to the ability of tannin to serve as a H_2 sink as reported by many researchers (Pereira et al., 2022, Vargas-Ortiz et al., 2022). Min et al. (2019) also, reported peanut skin (tannin-rich diets) as a H_2 sink when supplemented in the diet of beef cattle which resulted in reduction. The effect of tannin on DMI and weight gain has been reported as inconsistent. When a pure culture of tannin tolerant/degrading bacteria *Streptococcus caprinus* was introduced to sheep placed on an Acacia diet, an enhanced DMI and nitrogen balance was reported (Goel et al., 2005). A recent study by Stewart et al. (2019) compared condensed tannin (CT)-containing hay, hydrolysable tannin (HT)-containing hay and no-tannin containing hay in diet of heifers (DM basis). A decrease in methane emission (of 25%) was observed in the HT-containing hay compared to the CT and non-tannin hay. The result obtained indicated that a much lower DMI was recorded in the heifers placed on the HT-containing hay. It can thus be inferred that, tannins play a role in the DMI of the animals depending on the concentration or type, it can either increase or decrease DMI which can impact methane emission. The effect of tannins as methane mitigating agent can be highly inconsistent at low concentrations (20 /kg DM), which is in part due to the ability of tannin to bind to dietary nutrients (Aboagye and Beauchemin, 2019, Jayanegara et al., 2012). At low tannin concentrations, there are fewer number of free tannins available to directly inhibit methanogens as other dietary components such as proteins, minerals and fiber bind to free tannins easily. It has been shown that these interactions can lead to a loss of about 78% of free CT in the rumen of goats and sheep (Perez-Maldonado and Norton, 1996, McSweeney et al., 2001). Therefore, extracts and forages with low tannin concentration used as dietary supplementation may produce inconsistent result of methane reduction. The relationship between the concentration of tannin and dry matter intake is further confounded by forage digestibility and many other factors that affect intake (Waghorn et al., 2002). This relationship also depends on the ruminant species.

The abundance of *Methanobrevibacter* in this study is consistent with the findings of other researchers which was regardless of the animal breed or primer set used (Hook et al., 2010, Zhou et al., 2011a, Zhou et al., 2011b, Mohammed et al., 2011). It is the predominant methanogenic archaea in the rumen and it synthesizes methane by reducing CO_2 with H_2 through the hydrogenotropic pathway (Hungate, 1966). Aside from the genus *Methanobrevibacter*, *Methanosarcina* were the most abundant but it was less than 0.3% across the treatment so it can be concluded that they played a less significant role in contributing to the production of methane due to their low abundance. *Methanosarcina* produces methane by using acetate as substrate through acetoclastic pathway (Liu and Whitman, 2008, Mohammed et al., 2011), and it has been reported to produce methane using methanol and methylamine especially if the animal diet contains materials that promotes the production of these compounds (Mohammed et al., 2011).

5.2 Microbial defence against tannins

The result of this experiment showed that there was no impact of dietary tannin on the microbial community especially methanogens directly associated with methane production or methane reduction, and this is in line with the findings of several researchers (Śliwiński et al., 2002, Pesta et al., 2015, Knoell, 2021).

Goel et al. (2005) among other researchers explained that rumen microbes have developed a variety of strategies to help them overcome effects of tannins in feeds and this have been broadly classified as 'active approach and elaboration of alternative biologically inexpensive targets for tannins'. Apart from the first line of defence, which is the production of proline-rich saliva commonly found in goat and some breeds of wild sheep, production of Extracellular Polysaccharide (ESP) by the microbes is one of such mechanisms to reduce effect of tannins (Degeest and De Vuyst, 1999). In other words, any of these strategies developed by the microbes to subdue the inhibitory effects of tannins, could have been a reason for the lack of effect observed on the microbial population. Unfortunately, other than the standard rumen fermentation parameters, the concentration of ESP in the rumen was not quantified in the parent experiment.

Chapter 6

6 Conclusion and Recommendation

Ruminants are the major contributors of methane with an estimate of approx. 80% of the total livestock emission. Methane is one of the GHGs that is a global concern due to its global warming potential. Methane emission from ruminant comes from both the enteric fermentation and from the manure of the animal. Various methane mitigation strategies are being utilized to reduce the production of methane in ruminant animals, this includes dietary supplementation using PSM such as tannins. The dietary interventions act by having direct or indirect effect of the rumen microbes. The aim of this study was to determine the changes in the microbial ecosystem in the rumen when South Africa Mutton Merino sheep diet was supplemented with tannins to reduce methane production. This study showed that tannins either crude or encapsulated had no effect on the methane producing microbes as well as the overall microbial community in the rumen of South Africa Mutton Merino weaned. This suggests that factors that may be responsible for increase in methanogens as observed in the tannin-based diet could be that the inclusion level of the acacia tannin extract was too low. It could also be that sample size was too small which did not allow to see wide variation in the result.

There is still lack of consistency on the effect of condensed tannin sources on methane reduction in ruminant as some of the mitigating effect observed may be as a result of decrease in dry matter intake or diet digestibility (Aboagye and Beauchemin, 2019). It is therefore evident that impacts of tannin on mitigating methane may depend on several factors including the concentration of tannin present in the forage or extract (Aboagye and Beauchemin, 2019) as well as binding of H_2 with tannin (Besharati et al., 2022).

Both fungi and protozoa are not recorded because their abundance were either very low or completely absent. This is so because MG-RAST significantly underrepresented the eukaryotic component in the samples (Lindgreen et al., 2016, Wilke et al., 2017).

In the future larger sample size is recommended to enable wider variation to be observed. Also, higher concentration of tannin should be used in supplementing the diet of the animals so as to see more effect of the methanogens. Collection of rumen fluid should be explored at multiple time point throughout the growth period. Another pipeline is highly recommended in other to have detailed information on eukaryotic components (fungi and protozoa) as MG-RAST is not recommended. Interdisciplinary collaboration between bioinformatics, nutrition and rumen microbiology are highly encouraged. These will allow a better understanding of the effect of tannin on the rumen microbiome.

References

ABOAGYE, I. A. & BEAUCHEMIN, K. A. 2019. Potential of Molecular Weight and Structure of Tannins to Reduce Methane Emissions from Ruminants: A Review. *Animals,* 9**,** 856.

ACKERMANN, H.-W. 2007. 5500 Phages examined in the electron microscope. *Archives of virology,* 152**,** 227-243.

ADEJORO, F. A. 2019. *The use of condensed tannins and nitrate to reduce enteric methane emission and enhance utilization of high-forage diets in sheep.* University of Pretoria.

ADEJORO, F. A., HASSEN, A. & AKANMU, A. M. 2019. Effect of lipid-encapsulated acacia tannin extract on feed intake, nutrient digestibility and methane emission in sheep. *Animals,* 9**,** 863.

ANIMUT, G., PUCHALA, R., GOETSCH, A., PATRA, A., SAHLU, T., VAREL, V. & WELLS, J. 2008. Methane emission by goats consuming different sources of condensed tannins. *Animal Feed Science and Technology,* 144**,** 228-241.

ARONESTY, E. 2013. Comparison of sequencing utility programs. *The open bioinformatics journal,* 7.

ARTHUR, P., ARCHER, J., JOHNSTON, D., HERD, R., RICHARDSON, E. & PARNELL, P. 2001. Genetic and phenotypic variance and covariance components for feed intake, feed efficiency, and other postweaning traits in Angus cattle. *Journal of animal science,* 79**,** 2805-2811.

ASHBURNER, M., BALL, C. A., BLAKE, J. A., BOTSTEIN, D., BUTLER, H., CHERRY, J. M., DAVIS, A. P., DOLINSKI, K., DWIGHT, S. S., EPPIG, J. T., HARRIS, M. A., HILL, D. P., ISSEL-TARVER, L., KASARSKIS, A., LEWIS, S., MATESE, J. C., RICHARDSON, J. E., RINGWALD, M., RUBIN, G. M. & SHERLOCK, G. 2000. Gene Ontology: tool for the unification of biology. *Nature Genetics,* 25**,** 25-29.

BACH, S., MCALLISTER, T., VEIRA, D., GANNON, V. & HOLLEY, R. 2002. Transmission and control of Escherichia coli O157: H7—a review. *Canadian journal of animal science,* 82**,** 475-490.

BAKER, S. K., GNANASAMPANTHAN, G., PURSER, D. B. & HOSKINSON, R. M. 2004. Immunogenic preparation and method for improving the productivity of ruminant animals. Google Patents.

BARUAH, L., MALIK, P. K., KOLTE, A. P., GOYAL, P., DHALI, A. & BHATTA, R. 2019. Rumen methane amelioration in sheep using two selected tanniferous phyto-leaves. *Carbon Management,* 10**,** 299-308.

BATEMAN, A., COIN, L., DURBIN, R., FINN, R. D., HOLLICH, V., GRIFFITHS-JONES, S., KHANNA, A., MARSHALL, M., MOXON, S. & SONNHAMMER, E. L. 2004. The Pfam protein families database. *Nucleic acids research,* 32**,** D138-D141.

BAYAT, A. R., TAPIO, I., VILKKI, J., SHINGFIELD, K. & LESKINEN, H. 2018. Plant oil supplements reduce methane emissions and improve milk fatty acid composition in dairy cows fed grass silage-based diets without affecting milk yield. *Journal of Dairy Science,* 101**,** 1136-1151.

BEAUCHEMIN, K., KREUZER, M., O'MARA, F. & MCALLISTER, T. 2008. Nutritional management for enteric methane abatement: a review. *Australian Journal of Experimental Agriculture,* 48**,** 21-27.

BEAUCHEMIN, K., MCGINN, S., BENCHAAR, C. & HOLTSHAUSEN, L. 2009. Crushed sunflower, flax, or canola seeds in lactating dairy cow diets: Effects on methane production, rumen fermentation, and milk production. *Journal of dairy science,* 92**,** 2118-2127.

BEAUCHEMIN, K., MCGINN, S., MARTINEZ, T. & MCALLISTER, T. 2007. Use of condensed tannin extract from quebracho trees to reduce methane emissions from cattle. *Journal of Animal Science,* 85**,** 1990-1996.

BEAUCHEMIN, K. A., UNGERFELD, E. M., ECKARD, R. J. & WANG, M. 2020. Review: Fifty years of research on rumen methanogenesis: lessons learned and future challenges for mitigation. *Animal,* 14**,** s2-s16.

BECKER, P. M., VAN WIKSELAAR, P. G., FRANSSEN, M. C., DE VOS, R. C., HALL, R. D. & BEEKWILDER, J. 2014. Evidence for a hydrogen-sink mechanism of (+) catechin-mediated emission reduction of the ruminant greenhouse gas methane. *Metabolomics,* 10**,** 179-189.

BELANCHE, A., DE LA FUENTE, G. & NEWBOLD, C. J. 2014. Study of methanogen communities associated with different rumen protozoal populations. *FEMS Microbiology Ecology,* 90**,** 663-677.

BENCHAAR, C., CALSAMIGLIA, S., CHAVES, A., FRASER, G., COLOMBATTO, D., MCALLISTER, T. & BEAUCHEMIN, K. 2008. A review of plant-derived essential oils in ruminant nutrition and production. *Animal Feed Science and Technology,* 145**,** 209-228.

BERARD, N. C., WANG, Y., WITTENBERG, K., KRAUSE, D., COULMAN, B., MCALLISTER, T. & OMINSKI, K. 2011. Condensed tannin concentrations found in vegetative and mature forage legumes grown in western Canada. *Canadian Journal of Plant Science,* 91**,** 669-675.

BESHARATI, M., MAGGIOLINO, A., PALANGI, V., KAYA, A., JABBAR, M., ESECELI, H., DE PALO, P. & LORENZO, J. M. 2022. Tannin in Ruminant Nutrition: Review. *Molecules,* 27**,** 8273.

BHATTA, R., BARUAH, L., SARAVANAN, M., SURESH, K. & SAMPATH, K. 2013. Effect of medicinal and aromatic plants on rumen fermentation, protozoa population and methanogenesis in vitro. *Journal of Animal Physiology and Animal Nutrition,* 97**,** 446-456.

BHATTA, R., UYENO, Y., TAJIMA, K., TAKENAKA, A., YABUMOTO, Y., NONAKA, I., ENISHI, O. & KURIHARA, M. 2009. Difference in the nature of tannins on in vitro ruminal methane and volatile fatty acid production and on methanogenic archaea and protozoal populations. *Journal of Dairy Science,* 92**,** 5512-5522.

BOADI, D., BENCHAAR, C., CHIQUETTE, J. & MASSÉ, D. 2004. Mitigation strategies to reduce enteric methane emissions from dairy cows: Update review. *Canadian Journal of Animal Science,* 84**,** 319-335.

BODAS, R., PRIETO, N., GARCÍA-GONZÁLEZ, R., ANDRÉS, S., GIRÁLDEZ, F. J. & LÓPEZ, S. 2012. Manipulation of rumen fermentation and methane production with plant secondary metabolites. *Animal Feed Science and Technology,* 176**,** 78-93.

BOLYEN, E., RIDEOUT, J. R., DILLON, M. R., BOKULICH, N. A., ABNET, C. C., AL-GHALITH, G. A., ALEXANDER, H., ALM, E. J., ARUMUGAM, M. & ASNICAR, F. 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature biotechnology,* 37**,** 852-857.

BRITO, L. F., BEDERE, N., DOUHARD, F., OLIVEIRA, H. R., ARNAL, M., PEÑAGARICANO, F., SCHINCKEL, A. P., BAES, C. F. & MIGLIOR, F. 2021. Review: Genetic selection of high-yielding dairy cattle toward sustainable farming systems in a rapidly changing world. *Animal,* 15**,** 100292.

BROMBERG, J. S., FRICKE, W. F., BRINKMAN, C. C., SIMON, T. & MONGODIN, E. F. 2015. Microbiota—implications for immunity and transplantation. *Nature Reviews Nephrology,* 11**,** 342.

BROOKER, J., O'DONOVAN, L., SKENE, I., CLARKE, K., BLACKALL, L. & MUSLERA, P. 1994. Streptococcus caprinus sp. nov., a tannin-resistant ruminal bacterium from feral goats. *Letters in Applied Microbiology,* 18**,** 313-318.

BROUDISCOU, L., OFFNER, A. & SAUVANT, D. 2014. Effects of inoculum source, pH, redox potential and headspace di-hydrogen on rumen in vitro fermentation yields. *Animal,* 8**,** 931-937.

BUAN, N. R. 2018. Methanogens: pushing the boundaries of biology. *Emerging Topics in Life Sciences,* 2**,** 629-646.

CARULLA, J., KREUZER, M., MACHMÜLLER, A. & HESS, H. 2005. Supplementation of Acacia mearnsii tannins decreases methanogenesis and urinary nitrogen in forage-fed sheep. *Australian journal of agricultural research,* 56**,** 961-970.

CASEY, N. H. 2021. A profile of South African sustainable animal production and greenhouse gas emissions. *Animal Frontiers,* 11**,** 7-16.

CASPI, R., ALTMAN, T., DALE, J. M., DREHER, K., FULCHER, C. A., GILHAM, F., KAIPA, P., KARTHIKEYAN, A. S., KOTHARI, A. & KRUMMENACKER, M. 2010. The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases. *Nucleic acids research,* 38**,** D473-D479.

CASTILLO-GONZÁLEZ, A., BURROLA-BARRAZA, M., DOMÍNGUEZ-VIVEROS, J. & CHÁVEZ-MARTÍNEZ, A. 2014. Rumen microorganisms and fermentation. *Archivos de Medicina Veterinaria,* 46**,** 349-361.

CHAKRAVORTY, S., HELB, D., BURDAY, M., CONNELL, N. & ALLAND, D. 2007. A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. *Journal of microbiological methods,* 69**,** 330-339.

CHAO, A. 1984. Nonparametric estimation of the number of classes in a population. *Scandinavian Journal of statistics***,** 265-270.

CHEN, H., WANG, C., HUASAI, S. & CHEN, A. 2021a. Effects of dietary forage to concentrate ratio on nutrient digestibility, ruminal fermentation and rumen bacterial composition in Angus cows. *Scientific reports,* 11**,** 17023-17023.

CHEN, L., BAO, X., GUO, G., HUO, W., XU, Q., WANG, C., LI, Q. & LIU, Q. 2021b. Effects of hydrolysable tannin with or without condensed tannin on alfalfa silage fermentation characteristics and in vitro ruminal methane production, fermentation patterns, and microbiota. *Animals,* 11**,** 1967.

CHIQUETTE, J., CHENG, K.-J., RODE, L. & MILLIGAN, L. 1989. Effect of tannin content in two isosynthetic strains of birdsfoot trefoil (Lotus corniculatus L.) on feed digestibility and rumen fluid composition in sheep. *Canadian Journal of Animal Science,* 69**,** 1031-1039.

CHONG, C. W., PEARCE, D., CONVEY, P., YEW, W. C. & TAN, I. 2012. Patterns in the distribution of soil bacterial 16S rRNA gene sequences from different regions of Antarctica. *Geoderma,* 181**,** 45-55.

CIESLAK, A., ZMORA, P., PERS-KAMCZYC, E. & SZUMACHER-STRABEL, M. 2012. Effects of tannins source (Vaccinium vitis idaea L.) on rumen microbial fermentation in vivo. *Animal feed science and technology,* 176**,** 102-106.

CLARK, D. P. & PAZDERNIK, N. J. 2013. Chapter 9 - Genomics & Systems Biology. *In:* CLARK, D. P. & PAZDERNIK, N. J. (eds.) *Molecular Biology (Second Edition).* Boston: Academic Press.

CZERKAWSKI, J., BLAXTER, K. & WAINMAN, F. 1966. The metabolism of oleic, linoleic and linolenic acids by sheep with reference to their effects on methane production. *British Journal of Nutrition,* 20**,** 349-362.

DANIELSSON, R. 2016. *Methane production in dairy cows: Impact of feed and rumen microbiota*, Department of Animal Nutrition and Management, Swedish University of ….

DANIELSSON, R., DICKSVED, J., SUN, L., GONDA, H., MÜLLER, B., SCHNÜRER, A. & BERTILSSON, J. 2017. Methane production in dairy cows correlates with rumen methanogenic and bacterial community structure. *Frontiers in microbiology,* 8**,** 226.

DARLING, A. E., JOSPIN, G., LOWE, E., MATSEN IV, F. A., BIK, H. M. & EISEN, J. A. 2014. PhyloSift: phylogenetic analysis of genomes and metagenomes. *PeerJ,* 2**,** e243.

DE FILIPPIS, F., LAIOLA, M., BLAIOTTA, G. & ERCOLINI, D. 2017. Different Amplicon Targets for Sequencing-Based Studies of Fungal Diversity. *Applied and environmental microbiology,* 83**,** e00905-17.

DEGEEST, B. & DE VUYST, L. 1999. Indication that the nitrogen source influences both amount and size of exopolysaccharides produced by Streptococcus thermophilus LY03 and modelling of the bacterial growth and exopolysaccharide production in a complex medium. *Applied and Environmental Microbiology,* 65**,** 2863-2870.

DEGNAN, P. H. & OCHMAN, H. 2012. Illumina-based analysis of microbial community diversity. *The ISME journal,* 6**,** 183-194.

DENMAN, S., NICHOLSON, M. J., BROOKMAN, J. L., THEODOROU, M. K. & MCSWEENEY, C. S. 2008. Detection and monitoring of anaerobic rumen fungi using an ARISA method. *Letters in applied microbiology,* 47**,** 492-499.

DERAMUS, H. A., CLEMENT, T. C., GIAMPOLA, D. D. & DICKISON, P. C. 2003. Methane emissions of beef cattle on forages: efficiency of grazing management systems. *Journal of environmental quality,* 32**,** 269-277.

DETHLEFSEN, L., HUSE, S., SOGIN, M. L. & RELMAN, D. A. 2008. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. *PLoS biol,* 6**,** e280.

DHANASEKARAN, D. K., DIAS-SILVA, T. P., ABDALLA FILHO, A. L., SAKITA, G. Z., ABDALLA, A. L., LOUVANDINI, H. & ELGHANDOUR, M. M. 2019. Plants extract and bioactive compounds on rumen methanogenesis. *Agroforestry Systems***,** 1-13.

DIAZ CARRASCO, J. M., CABRAL, C., REDONDO, L. M., PIN VISO, N. D., COLOMBATTO, D., FARBER, M. D. & FERNANDEZ MIYAKAWA, M. E. 2017. Impact of chestnut and quebracho tannins on rumen microbiota of bovines. *BioMed Research International,* 2017.

DOHME, F., MACHMÜLLER, A., ESTERMANN, B., PFISTER, P., WASSERFALLEN, A. & KREUZER, M. 1999. The role of the rumen ciliate protozoa for methane suppression caused by coconut oil. *Letters in applied Microbiology,* 29**,** 187-192.

DORMAN, H. D. & DEANS, S. G. 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *Journal of applied microbiology,* 88**,** 308-316.

DROUILLARD, J. S. 2018. Current situation and future trends for beef production in the United States of America—A review. *Asian-Australasian Journal of Animal Sciences,* 31**,** 1007.

DU TOIT, C. J. L. 2017. *Mitigation of enteric methane emissions from ruminants in subtropical production systems.* University of Pretoria.

DU TOIT, C. J. L., VAN NIEKERK, W. A. & MEISSNER, H. 2013. Direct greenhouse gas emissions of the South African small stock sectors. *South African Journal of Animal Science,* 43**,** 340-361.

EDWARDS, A., GIBBS, R. A., NGUYEN, P. N., ANSORGE, W. & CASKEY, C. T. 1989. Automated DNA sequencing methods for detection and analysis of mutations: applications to the Lesch-Nyhan syndrome. *Trans Assoc Am Physicians,* 102**,** 185-94.

ELLIS, J. E., WILLIAMS, A. G. & LLOYD, D. 1990. Formate and glucose stimulation of methane and hydrogen production in rumen liquor. *Current Microbiology,* 20**,** 251-254.

EVANS, J. D. & MARTIN, S. A. 2000. Effects of thymol on ruminal microorganisms. *Current Microbiology,* 41**,** 336-340.

FACIOLA, A. P. 2004. Effects of Lauric Acid on Fermentation Patterns, Ruminal Protozoa, and Performance of Dairy Cows.

FANIYI, T. O., ADEWUMI, M. K., PRATES, Ê. R. & AYANGBENRO, A. 2016. Effect of herbs and spices (plant extracts) on rumen microbial activities: a review. *PUBVET,* 10**,** 477-486.

FERRY, J. G. 1992. Methane from acetate. *Journal of bacteriology,* 174**,** 5489-5495.

FERRY, J. G. 2011. Fundamentals of methanogenic pathways that are key to the biomethanation of complex biomass. *Current opinion in biotechnology,* 22**,** 351-357.

FERRY, J. G. 2012. *Methanogenesis: ecology, physiology, biochemistry & genetics*, Springer Science & Business Media.

FIELD, J. & LETTINGA, G. 1992. Toxicity of tannic compounds to microorganisms. *Plant polyphenols.* Springer.

FIELD, J. A., KORTEKAAS, S. & LETTINGA, G. 1989. The tannin theory of methanogenic toxicity. *Biological Wastes,* 29**,** 241-262.

FIEVEZ, V., DOHME, F., DANNEELS, M., RAES, K. & DEMEYER, D. 2003. Fish oils as potent rumen methane inhibitors and associated effects on rumen fermentation in vitro and in vivo. *Animal Feed Science and Technology,* 104**,** 41-58.

FINLAY, B. & FENCHEL, T. 1993. Review article Methanogens and Other Bacteria as Symbionts of Free-Living Anaerobic Ciliates. *Symbiosis*.

FLINT, H. J., SCOTT, K. P., DUNCAN, S. H., LOUIS, P. & FORANO, E. 2012. Microbial degradation of complex carbohydrates in the gut. *Gut microbes,* 3**,** 289-306.

FOCANT, M., FROIDMONT, E., ARCHAMBEAU, Q., VAN, Q. D. & LARONDELLE, Y. 2019. The effect of oak tannin (Quercus robur) and hops (Humulus lupulus) on dietary nitrogen efficiency, methane emission, and milk fatty acid composition of dairy cows fed a low-protein diet including linseed. *Journal of dairy science,* 102**,** 1144-1159.

FONTY, G., SENAUD, J., JOUANY, J.-P. & GOUET, P. 1988. Establishment of ciliate protozoa in the rumen of conventional and conventionalized lambs: influence of diet and management conditions. *Canadian journal of microbiology,* 34**,** 235-241.

FRUTOS, P., HERVÁS, G., GIRÁLDEZ, F. J. & MANTECÓN, Á. R. 2004. Tannins and ruminant nutrition.

FU, L., NIU, B., ZHU, Z., WU, S. & LI, W. 2012. CD-HIT: accelerated for clustering the next-generation sequencing data. *Bioinformatics,* 28**,** 3150-3152.

GHOSH, A., MEHTA, A. & KHAN, A. M. 2019. Metagenomic Analysis and its Applications. *In:* RANGANATHAN, S., GRIBSKOV, M., NAKAI, K. & SCHÖNBACH, C. (eds.) *Encyclopedia of Bioinformatics and Computational Biology.* Oxford: Academic Press.

GOEL, G., PUNIYA, A., AGUILAR, C. & SINGH, K. 2005. Interaction of gut microflora with tannins in feeds. *Naturwissenschaften,* 92**,** 497-503.

GOMEZ-ALVAREZ, V., TEAL, T. K. & SCHMIDT, T. M. 2009. Systematic artifacts in metagenomes from complex microbial communities. *The ISME Journal,* 3**,** 1314-1317.

GOODRICH, R., GARRETT, J., GAST, D., KIRICK, M., LARSON, D. & MEISKE, J. 1984. Influence of monensin on the performance of cattle. *Journal of animal science,* 58**,** 1484-1498.

GORELICK, R. 2006. Combining richness and abundance into a single diversity index using matrix analogues of Shannon's and Simpson's indices. *Ecography,* 29**,** 525-530.

GUAN, H., WITTENBERG, K., OMINSKI, K. & KRAUSE, D. 2006. Potential use of ionophores for mitigation of enteric methane.

GUILLERMO, T., ANDREA, L., JUAN D, L., XOCHITL, H.-V., BILLY M, H. & TODD, C. 2015. Food-producing animals and their health in relation to human health. *Microbial ecology in health and disease,* 26**,** 25876.

GWORGWOR, Z., MBAHI, T. & YAKUBU, B. 2006. Environmental implications of methane production by ruminants: a review. *Journal of Sustainable Development in Agriculture and Environment,* 2**,** 1-14.

HAFT, D. H., SELENGUT, J. D. & WHITE, O. 2003. The TIGRFAMs database of protein families. *Nucleic acids research,* 31**,** 371-373.

HANDELSMAN, J. 2004. Metagenomics: application of genomics to uncultured microorganisms. *Microbiology and molecular biology reviews,* 68**,** 669-685.

HAQUE, M. N. 2018. Dietary manipulation: a sustainable way to mitigate methane emissions from ruminants. *Journal of animal science and technology,* 60**,** 1-10.

HARDISON, R. C. 2003. Comparative genomics. *PLoS biology,* 1**,** e58.

HEDDERICH, R. & WHITMAN, W. B. 2006. Physiology and biochemistry of the methane-producing Archaea. *The prokaryotes,* 2**,** 1050-1079.

HEGARTY, R. 1999. Reducing rumen methane emissions through elimination of rumen protozoa. *Australian Journal of Agricultural Research,* 50**,** 1321-1328.

HEGARTY, R. & GERDES, R. 1999. Hydrogen production and transfer in the rumen. *Recent Advances in Animal Nutrition in Australia,* 12**,** 37-44.

HEGARTY, R., GOOPY, J. P., HERD, R. & MCCORKELL, B. 2007. Cattle selected for lower residual feed intake have reduced daily methane production. *Journal of animal science,* 85**,** 1479-1486.

HENDERSON, G., COX, F., GANESH, S., JONKER, A., YOUNG, W., ABECIA, L., ANGARITA, E., ARAVENA, P., ARENAS, G. N. & ARIZA, C. 2016. Erratum: rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. *Scientific reports,* 6**,** 19175.

HESS, H.-D., KREUZER, M., DıAZ, T., LASCANO, C. E., CARULLA, J. E., SOLIVA, C. R. & MACHMÜLLER, A. 2003. Saponin rich tropical fruits affect fermentation and methanogenesis in faunated and defaunated rumen fluid. *Animal feed science and technology,* 109**,** 79-94.

HOBSON, P. N. & STEWART, C. S. 2012. *The rumen microbial ecosystem*, Springer Science & Business Media.

HOGAN, K. B. 1993. Anthropogenic methane emissions in the United States, estimates for 1990.

HOLLAND, R., LOVEDAY, D. & FERGUSON, K. 2014. How much meat to expect from a beef carcass. *University of Tennessee Institute of Agriculture Extension Publication*.

HONAN, M., FENG, X., TRICARICO, J. & KEBREAB, E. 2021. Feed additives as a strategic approach to reduce enteric methane production in cattle: modes of action, effectiveness and safety. *Animal Production Science*.

HOOK, S. E., WRIGHT, A.-D. G. & MCBRIDE, B. W. 2010. Methanogens: methane producers of the rumen and mitigation strategies. *Archaea,* 2010.

HRISTOV, A. N., IVAN, M., NEILL, L. & MCALLISTER, T. 2003. Evaluation of several potential bioactive agents for reducing protozoal activity in vitro. *Animal Feed Science and Technology,* 105**,** 163-184.

HUNGATE, R. E. 1966. *The rumen and its microbes,* New York and London, Academic Press.

HUNTER, S., APWEILER, R., ATTWOOD, T. K., BAIROCH, A., BATEMAN, A., BINNS, D., BORK, P., DAS, U., DAUGHERTY, L. & DUQUENNE, L. 2009. InterPro: the integrative protein signature database. *Nucleic acids research,* 37**,** D211-D215.

IBRAHIM, S. L. & HASSEN, A. 2022. Effect of non-encapsulated and encapsulated mimosa (Acacia mearnsii) tannins on growth performance, nutrient digestibility, methane and rumen fermentation of South African mutton Merino ram lambs. *Animal Feed Science and Technology,* 294**,** 115502.

IQBAL, M. F., CHENG, Y.-F., ZHU, W.-Y. & ZESHAN, B. 2008. Mitigation of ruminant methane production: current strategies, constraints and future options. *World Journal of Microbiology and Biotechnology,* 24**,** 2747-2755.

ISHLER, V. & VARGA, G. 2001. Carbohydrate nutrition for lactating dairy cattle. *Pennsylvania State University, Code#: DAS***,** 01-29.

IVAN, M., VEIRA, D. & KELLEHER, C. 1986. The alleviation of chronic copper toxicity in sheep by ciliate protozoa. *British Journal of Nutrition,* 55**,** 361-367.

JANSSEN, P. H. 2010. Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. *Animal Feed Science and Technology,* 160**,** 1-22.

JANSSEN, P. H. & KIRS, M. 2008. Structure of the Archaeal Community of the Rumen. *Applied and Environmental Microbiology,* 74**,** 3619.

JAYANEGARA, A., LEIBER, F. & KREUZER, M. 2012. Meta-analysis of the relationship between dietary tannin level and methane formation in ruminants from in vivo and in vitro experiments. *Journal of animal physiology and animal nutrition,* 96**,** 365-375.

JENSEN, L. J., JULIEN, P., KUHN, M., VON MERING, C., MULLER, J., DOERKS, T. & BORK, P. 2007. eggNOG: automated construction and annotation of orthologous groups of genes. *Nucleic Acids Research,* 36**,** D250-D254.

JERÓNIMO, E., PINHEIRO, C., LAMY, E., DENTINHO, M. T., SALES-BAPTISTA, E., LOPES, O. & SILVA, F. 2016. Tannins in ruminant nutrition: Impact on animal performance and quality of edible products.

JOHNSON, D., ABO-OMAR, J., SAA, C. & CARMEAN, B. 1994. Persistence of methane suppression by propionate enhancers in cattle diets. *PUBLICATION-EUROPEAN ASSOCIATION FOR ANIMAL PRODUCTION,* 76**,** 339-339.

JOHNSON, D. & JOHNSON, K. Recent developments in understanding enteric methane production by ruminants: Implications for mitigation. International Non-CO2 GHG Mitigation Workshop. Washington, DC, 2002.

JONES, G., MCALLISTER, T., MUIR, A. & CHENG, K.-J. 1994. Effects of sainfoin (Onobrychis viciifolia Scop.) condensed tannins on growth and proteolysis by four strains of ruminal bacteria. *Applied and environmental microbiology,* 60**,** 1374-1378.

JORDAN, E., LOVETT, D., MONAHAN, F., CALLAN, J., FLYNN, B. & O'MARA, F. 2006. Effect of refined coconut oil or copra meal on methane output and on intake and performance of beef heifers. *Journal of Animal Science,* 84**,** 162-170.

JUNIOR, F. P., CASSIANO, E., MARTINS, M., ROMERO, L., ZAPATA, D., PINEDO, L., MARINO, C. & RODRIGUES, P. 2017. Effect of tannins-rich extract from Acacia mearnsii or monensin as feed additives on ruminal fermentation efficiency in cattle. *Livestock Science,* 203**,** 21-29.

JUOTTONEN, H., GALAND, P. E. & YRJÄLÄ, K. 2006. Detection of methanogenic Archaea in peat: comparison of PCR primers targeting the mcrA gene. *Research in Microbiology,* 157**,** 914-921.

KAMRA, D., AGARWAL, N. & CHAUDHARY, L. Inhibition of ruminal methanogenesis by tropical plants containing secondary compounds. International Congress Series, 2006. Elsevier, 156-163.

KAMRA, D. N. 2005. Rumen microbial ecosystem. *Current Science,* 89**,** 124-135.

KANEHISA, M., GOTO, S., FURUMICHI, M., TANABE, M. & HIRAKAWA, M. 2010. KEGG for representation and analysis of molecular networks involving diseases and drugs. *Nucleic acids research,* 38**,** D355-D360.

KAYANI, M. U. R., HUANG, W., FENG, R. & CHEN, L. 2021. Genome-resolved metagenomics using environmental and clinical samples. *Briefings in Bioinformatics,* 22.

KENT, W. J. 2002. BLAT—the BLAST-like alignment tool. *Genome research,* 12**,** 656-664.

KEREPESI, C., BÁNKY, D. & GROLMUSZ, V. 2014. AmphoraNet: The webserver implementation of the AMPHORA2 metagenomic workflow suite. *Gene,* 533**,** 538-540.

KIBEGWA, F. M., BETT, R. C., GACHUIRI, C. K., STOMEO, F. & MUJIBI, F. D. 2020. A Comparison of Two DNA Metagenomic Bioinformatic Pipelines while evaluating the Microbial Diversity in feces of Tanzanian small holder dairy cattle. *BioMed research international,* 2020.

KIM, B. H. & GADD, G. M. 2019. *Prokaryotic Metabolism and Physiology*, Cambridge University Press.

KINSMAN, R., SAUER, F., JACKSON, H., PATNI, N., MASSE, D., WOLYNETZ, M. & MUNROE, J. 1997. Methane and carbon dioxide emissions from lactating Holsteins. 1997. *Dairy Research Report, Centre for Food and Animal Research, Agriculture and Agri-Food Canada*.

KITTELMANN, S., PINARES-PATINO, C. S., SEEDORF, H., KIRK, M. R., GANESH, S., MCEWAN, J. C. & JANSSEN, P. H. 2014. Two different bacterial community types are linked with the low-methane emission trait in sheep. *PloS one,* 9**,** e103171.

KLIEVE, A., HECK, G., PRANCE, M. & SHU, Q. 1999. Genetic homogeneity and phage susceptibility of ruminal strains of Streptococcus bovis isolated in Australia. *Letters in Applied Microbiology,* 29**,** 108-112.

KNOELL, A. L. 2016. The Effect of Diet on the Bovine Rumen Microbial Community Structure and Composition and Its Effects on Methane Production in Growing and Finishing Cattle.

KNOELL, A. L. 2021. *Understanding Rumen Microbial Community Structure and Function Towards Decreasing Methane Emissions.* The University of Nebraska-Lincoln.

KOIKE, S., YOSHITANI, S., KOBAYASHI, Y. & TANAKA, K. 2003. Phylogenetic analysis of fiber-associated rumen bacterial community and PCR detection of uncultured bacteria. *FEMS microbiology letters,* 229**,** 23-30.

KONG, Y., TEATHER, R. & FORSTER, R. 2010. Composition, spatial distribution, and diversity of the bacterial communities in the rumen of cows fed different forages. *FEMS microbiology ecology,* 74**,** 612-622.

KRAUSE, D., NAGARAJA, T., WRIGHT, A. & CALLAWAY, T. 2013. Board-invited review: rumen microbiology: leading the way in microbial ecology. *Journal of animal science,* 91**,** 331-341.

KREUZER, M., KIRCHGESSNER, M. & MÜLLER, H. 1986. Effect of defaunation on the loss of energy in wethers fed different quantities of cellulose and normal or steamflaked maize starch. *Animal Feed Science and Technology,* 16**,** 233-241.

KUMAR, S., PUNIYA, A. K., PUNIYA, M., DAGAR, S. S., SIROHI, S. K., SINGH, K. & GRIFFITH, G. W. 2009. Factors affecting rumen methanogens and methane mitigation strategies. *World Journal of Microbiology and Biotechnology,* 25**,** 1557-1566.

KUMAR, V., TANDON, M. & VERMA, M. 2008. Environment friendly dairy farming: Nutritional Techniques for Mitigating Methane Production from Ruminants. *Dairy Planner,* 4**,** 12-14.

KUNIN, V., COPELAND, A., LAPIDUS, A., MAVROMATIS, K. & HUGENHOLTZ, P. 2008. A bioinformatician's guide to metagenomics. *Microbiology and molecular biology reviews,* 72**,** 557-578.

LAMY, E., RAWEL, H., SCHWEIGERT, F. J., CAPELA E SILVA, F., FERREIRA, A., COSTA, A. R., ANTUNES, C., ALMEIDA, A. M., COELHO, A. V. & SALES-BAPTISTA, E. 2011. The effect of tannins on Mediterranean ruminant ingestive behavior: The role of the oral cavity. *Molecules,* 16**,** 2766-2784.

LANGILLE, M. G., ZANEVELD, J., CAPORASO, J. G., MCDONALD, D., KNIGHTS, D., REYES, J. A., CLEMENTE, J. C., BURKEPILE, D. E., THURBER, R. L. V. & KNIGHT, R. 2013. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nature biotechnology,* 31**,** 814-821.

LANGMEAD, B. & SALZBERG, S. L. 2012. Fast gapped-read alignment with Bowtie 2. *Nature Methods,* 9**,** 357-359.

LEAHY, S. C., KELLY, W. J., ALTERMANN, E., RONIMUS, R. S., YEOMAN, C. J., PACHECO, D. M., LI, D., KONG, Z., MCTAVISH, S. & SANG, C. 2010. The genome sequence of the rumen methanogen Methanobrevibacter ruminantium reveals new possibilities for controlling ruminant methane emissions. *PloS one,* 5**,** e8926.

LEE, S., HA, J. & CHENG, K. 2001. Effects of LCFA on the gas production, cellulose digestion and cellulase activities by the rumen anaerobic fungus, Neocallimastix frontalis RE1. *Asian-Australasian Journal of Animal Sciences,* 14**,** 1110-1117.

LI, Y., MENG, Z., XU, Y., SHI, Q., MA, Y., AUNG, M., CHENG, Y. & ZHU, W. 2021. Interactions between anaerobic fungi and methanogens in the rumen and their biotechnological potential in biogas production from lignocellulosic materials. *Microorganisms,* 9**,** 190.

LINDGREEN, S., ADAIR, K. L. & GARDNER, P. P. 2016. An evaluation of the accuracy and speed of metagenome analysis tools. *Scientific reports,* 6**,** 19233-19233.

LISCHER, H. E. L. & SHIMIZU, K. K. 2017. Reference-guided de novo assembly approach improves genome reconstruction for related species. *BMC Bioinformatics,* 18**,** 474.

LIU, K., ZHANG, Y., YU, Z., XU, Q., ZHENG, N., ZHAO, S., HUANG, G. & WANG, J. 2021. Ruminal microbiota–host interaction and its effect on nutrient metabolism. *Animal Nutrition,* 7**,** 49-55.

LIU, Y. & WHITMAN, W. B. 2008. Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. *Annals of the New York Academy of Sciences,* 1125**,** 171-189.

LÓPEZ-GARCÍA, A., SABORÍO-MONTERO, A., GUTIÉRREZ-RIVAS, M., ATXAERANDIO, R., GOIRI, I., GARCÍA-RODRÍGUEZ, A., JIMÉNEZ-MONTERO, J. A., GONZÁLEZ, C., TAMAMES, J. & PUENTE-SÁNCHEZ, F. 2022. Fungal and ciliate protozoa are the main rumen microbes associated with methane emissions in dairy cattle. *GigaScience,* 11.

LOZUPONE, C., HAMADY, M. & KNIGHT, R. 2006. UniFrac–an online tool for comparing microbial community diversity in a phylogenetic context. *BMC bioinformatics,* 7**,** 371.

LUTON, P. E., WAYNE, J. M., SHARP, R. J. & RILEY, P. W. 2002. The mcrA gene as an alternative to 16S rRNA in the phylogenetic analysis of methanogen populations in landfillbbThe GenBank accession numbers for the mcrA sequences reported in this paper are AF414034–AF414051 (see Fig. 2) and AF414007–AF414033 (environmental isolates in Fig. 3). *Microbiology,* 148**,** 3521-3530.

MAAS, P. J. 1987. *Testing the calculation of the nutritive value according to the NEL system of fresh grass with a high N content.* MSc thesis, Wageningen Agricultural University.

MACHMÜLLER, A., OSSOWSKI, D. & KREUZER, M. 2000. Comparative evaluation of the effects of coconut oil, oilseeds and crystalline fat on methane release, digestion and energy balance in lambs. *Animal Feed Science and Technology,* 85**,** 41-60.

MACHMÜLLER, A., OSSOWSKI, D., WANNER, M. & KREUZER, M. 1998. Potential of various fatty feeds to reduce methane release from rumen fermentation in vitro (Rusitec). *Animal feed science and technology,* 71**,** 117-130.

MACHMÜLLER, A., SOLIVA, C. R. & KREUZER, M. 2003. Methane-suppressing effect of myristic acid in sheep as affected by dietary calcium and forage proportion. *British Journal of Nutrition,* 90**,** 529-540.

MADHAVAN, A., SINDHU, R., PARAMESWARAN, B., SUKUMARAN, R. K. & PANDEY, A. 2017. Metagenome analysis: a powerful tool for enzyme bioprospecting. *Applied biochemistry and biotechnology,* 183**,** 636-651.

MAKKAR, H. 2003. Effects and fate of tannins in ruminant animals, adaptation to tannins, and strategies to overcome detrimental effects of feeding tannin-rich feeds. *Small ruminant research,* 49**,** 241-256.

MALIK, P., KOLTE, A., BARUAH, L., SARAVANAN, M., BAKSHI, B. & BHATTA, R. 2017a. Enteric methane mitigation in sheep through leaves of selected tanniniferous tropical tree species. *Livestock science,* 200**,** 29-34.

MALIK, P. K., KOLTE, A. P., BAKSHI, B., BARUAH, L., DHALI, A. & BHATTA, R. 2017b. Effect of tamarind seed husk supplementation on ruminal methanogenesis, methanogen diversity and fermentation characteristics. *Carbon Management,* 8**,** 319-329.

MALMUTHUGE, N. & GUAN, L. L. 2016. Gut microbiome and omics: a new definition to ruminant production and health. *Animal Frontiers,* 6**,** 8-12.

MARTIN, C., MORGAVI, D. & DOREAU, M. 2010. Methane mitigation in ruminants: from microbe to the farm scale. *Animal,* 4**,** 351-365.

MATTHEWS, C., CRISPIE, F., LEWIS, E., REID, M., O'TOOLE, P. W. & COTTER, P. D. 2019. The rumen microbiome: a crucial consideration when optimising milk and meat production and nitrogen utilisation efficiency. *Gut microbes,* 10**,** 115-132.

MCALLISTER, T. & NEWBOLD, C. 2008. Redirecting rumen fermentation to reduce methanogenesis. *Australian Journal of Experimental Agriculture,* 48**,** 7-13.

MCCABE, M. S., CORMICAN, P., KEOGH, K., O'CONNOR, A., O'HARA, E., PALLADINO, R. A., KENNY, D. A. & WATERS, S. M. 2015. Illumina MiSeq phylogenetic amplicon sequencing shows a large reduction of an uncharacterised Succinivibrionaceae and an increase of the Methanobrevibacter gottschalkii clade in feed restricted cattle. *PloS one,* 10**,** e0133234.

MCCAUGHEY, W., WITTENBERG, K. & CORRIGAN, D. 1997. Methane production by steers on pasture. *Canadian Journal of Animal Science,* 77**,** 519-524.

MCGINN, S., BEAUCHEMIN, K., COATES, T. & COLOMBATTO, D. 2004. Methane emissions from beef cattle: Effects of monensin, sunflower oil, enzymes, yeast, and fumaric acid. *Journal of animal science,* 82**,** 3346-3356.

MCSWEENEY, C., PALMER, B., BUNCH, R. & KRAUSE, D. 2001. Effect of the tropical forage calliandra on microbial protein synthesis and ecology in the rumen. *Journal of Applied Microbiology,* 90**,** 78-88.

MEISSNER, H., SCHOLTZ, M. & ENGELBRECHT, F. 2013a. Sustainability of the South African Livestock Sector towards 2050 Part 2: Challenges, changes and required implementations. *South African Journal of Animal Science,* 43**,** 289-319.

MEISSNER, H., SCHOLTZ, M. & PALMER, A. 2013b. Sustainability of the South African livestock sector towards 2050 Part 1: Worth and impact of the sector. *South African Journal of Animal Science,* 43**,** 282-297.

MEYER, F., PAARMANN, D., D'SOUZA, M., OLSON, R., GLASS, E. M., KUBAL, M., PACZIAN, T., RODRIGUEZ, A., STEVENS, R., WILKE, A., WILKENING, J. & EDWARDS, R. A. 2008. The metagenomics RAST server – a public resource for the automatic phylogenetic and functional analysis of metagenomes. *BMC Bioinformatics,* 9**,** 386.

MILLEN, D. D., ARRIGONI, M. D. B. & PACHECO, R. D. L. 2016. *Rumenology*, Springer.

MIN, B.-R., LEE, S., JUNG, H., MILLER, D. N. & CHEN, R. 2022. Enteric Methane Emissions and Animal Performance in Dairy and Beef Cattle Production: Strategies, Opportunities, and Impact of Reducing Emissions. *Animals,* 12**,** 948.

MIN, B., ATTWOOD, G., MCNABB, W., MOLAN, A. & BARRY, T. 2005. The effect of condensed tannins from Lotus corniculatus on the proteolytic activities and growth of rumen bacteria. *Animal Feed Science and Technology,* 121**,** 45-58.

MIN, B. R., CASTLEBERRY, L., ALLEN, H., PARKER, D., WALDRIP, H., BRAUER, D. & WILLIS, W. 2019. Associative effects of wet distiller's grains plus solubles and tannin-rich peanut skin supplementation on in vitro rumen fermentation, greenhouse gas emissions, and microbial changes1. *Journal of Animal Science,* 97**,** 4668-4681.

MIN, B. R., SOLAIMAN, S., WALDRIP, H. M., PARKER, D., TODD, R. W. & BRAUER, D. 2020. Dietary mitigation of enteric methane emissions from ruminants: A review of plant tannin mitigation options. *Animal Nutrition,* 6**,** 231-246.

MIRZAEI-AGHSAGHALI, A., MAHERI-SIS, N., MIRZA AGHAZADEH, A., EBRAHIMNEZHAD, Y., DASTOORI, M. & AGHAJANZADE-GOLSHANI, A. 2008. Estimation of methane production in sheep using nutrient composition of the diet. *J. Anim. Vet. Adv,* 7**,** 765-770.

MOE, P. & TYRRELL, H. 1979. Methane production in dairy cows. *Journal of Dairy Science,* 62**,** 1583-1586.

MOHAMMED, R., ZHOU, M., KOENIG, K., BEAUCHEMIN, K. & GUAN, L. 2011. Evaluation of rumen methanogen diversity in cattle fed diets containing dry corn distillers grains and condensed tannins using PCR-DGGE and qRT-PCR analyses. *Animal feed science and technology,* 166**,** 122-131.

MOLOSSI, L., HOSHIDE, A. K., PEDROSA, L. M., OLIVEIRA, A. S. D. & ABREU, D. C. D. 2020. Improve pasture or feed grain? Greenhouse gas emissions, profitability, and resource use for nelore beef cattle in Brazil's Cerrado and Amazon Biomes. *Animals,* 10**,** 1386.

MORGAVI, D., FORANO, E., MARTIN, C. & NEWBOLD, C. 2010a. Microbial ecosystem and methanogenesis in ruminants – CORRIGENDUM. *Animal : an international journal of animal bioscience,* 4**,** 1024-36.

MORGAVI, D., FORANO, E., MARTIN, C. & NEWBOLD, C. J. 2010b. Microbial ecosystem and methanogenesis in ruminants. *animal,* 4**,** 1024-1036.

MORVAN, B., RIEU-LESME, F., FONTY, G. & GOUET, P. 1996. In vitroInteractions between rumen H2-producing cellulolytic microorganisms and H2-utilizing acetogenic and sulfate-reducing bacteria. *Anaerobe,* 2**,** 175-180.

MOSS, A. R., JOUANY, J.-P. & NEWBOLD, J. Methane production by ruminants: its contribution to global warming. Annales de zootechnie, 2000. EDP Sciences, 231-253.

NAGARAJA, T. 2016. Microbiology of the rumen. *Rumenology.* Springer.

NATHANI, N. M., PATEL, A. K., MOOTAPALLY, C. S., REDDY, B., SHAH, S. V., LUNAGARIA, P. M., KOTHARI, R. K. & JOSHI, C. G. 2015. Effect of roughage on rumen microbiota composition in the efficient feed converter and sturdy Indian Jaffrabadi buffalo (Bubalus bubalis). *BMC genomics,* 16**,** 1-15.

NEWBOLD, C., LASSALAS, B. & JOUANY, J. 1995. The importance of methanogens associated with ciliate protozoa in ruminal methane production in vitro. *Letters in applied microbiology,* 21**,** 230-234.

NEWBOLD, C. J., DE LA FUENTE, G., BELANCHE, A., RAMOS-MORALES, E. & MCEWAN, N. R. 2015. The Role of Ciliate Protozoa in the Rumen. *Frontiers in microbiology,* 6**,** 1313-1313.

NGUYEN, N.-P., MIRARAB, S., LIU, B., POP, M. & WARNOW, T. 2014. TIPP: taxonomic identification and phylogenetic profiling. *Bioinformatics,* 30**,** 3548-3555.

O'HARA, E., NEVES, A. L., SONG, Y. & GUAN, L. L. 2020. The Role of the Gut Microbiome in Cattle Production and Health: Driver or Passenger? *Annual Review of Animal Biosciences,* 8**,** 199-220.

O'KELLY, J. & SPIERS, W. 1992. Effect of monensin on methane and heat productions of steers fed lucerne hay either ad libitum or at the rate of 250 g/hour. *Australian Journal of Agricultural Research,* 43**,** 1789-1793.

O'BANNON, J. 2021. Greenhouse Gas Technical Report. *In:* MIKHAIL, R. (ed.). 210 South Juniper Street, Suite 100, Escondido, California 92025.

ODENYO, A., MCSWEENEY, C., PALMER, B., NEGASSA, D. & OSUJI, P. 1999. In vitro screening of rumen fluid samples from indigenous African ruminants provides evidence for rumen fluid with superior capacities to digest tannin-rich fodders. *Australian Journal of Agricultural Research,* 50**,** 1147-1157.

ODENYO, A. & OSUJI, P. 1998. Tannin-tolerant ruminal bacteria from East African ruminants. *Canadian Journal of Microbiology,* 44**,** 905-909.

OMAR, J. A. 2004. Effect of different ionophore treatments on some rumen metabolic measures of steers. *Dirasat Agric Sci,* 31**,** 178-184.

ORPIN, C. & JOBLIN, K. 1997. The rumen anaerobic fungi. *The rumen microbial ecosystem.* Springer.

OSAWA, R., FUJISAWA, T. & SLY, L. I. 1995a. Streptococcus gallolyticus sp. nov.; gallate degrading organisms formerly assigned to Streptococcus bovis. *Systematic and applied microbiology,* 18**,** 74-78.

OSAWA, R., RAINEY, F., FUJISAWA, T., LANG, E., BUSSE, H., WALSH, T. & STACKEBRANDT, E. 1995b. Lonepinella koalarum gen. nov., sp. nov., a new tannin-protein complex degrading bacterium. *Systematic and Applied Microbiology,* 18**,** 368-373.

OWENS, F. N. & BASALAN, M. 2016. Ruminal fermentation. *Rumenology.* Springer.

PARMAR, N. R., SOLANKI, J. V., PATEL, A. B., SHAH, T. M., PATEL, A. K., PARNERKAR, S., JI, N. K. & JOSHI, C. G. 2014. Metagenome of Mehsani buffalo rumen microbiota: an assessment of variation in feed-dependent phylogenetic and functional classification. *Journal of molecular microbiology and biotechnology,* 24**,** 249-261.

PATRA, A., KAMRA, D. & AGARWAL, N. 2006. Effect of plant extracts on in vitro methanogenesis, enzyme activities and fermentation of feed in rumen liquor of buffalo. *Animal Feed Science and Technology,* 128**,** 276-291.
PATRA, A., PARK, T., KIM, M. & YU, Z. 2017a. Rumen methanogens and mitigation of methane emission by anti-methanogenic compounds and substances. *Journal of animal science and biotechnology,* 8**,** 1-18.
PATRA, A., PARK, T., KIM, M. & YU, Z. 2017b. Rumen methanogens and mitigation of methane emission by anti-methanogenic compounds and substances. *Journal of animal science and biotechnology,* 8**,** 13-13.
PATRA, A. K. & SAXENA, J. 2009. Dietary phytochemicals as rumen modifiers: a review of the effects on microbial populations. *Antonie van Leeuwenhoek,* 96**,** 363-375.
PATRA, A. K. & SAXENA, J. 2011. Exploitation of dietary tannins to improve rumen metabolism and ruminant nutrition. *Journal of the Science of Food and Agriculture,* 91**,** 24-37.
PAUL, S., KAMRA, D., SASTRY, V., SAHU, N. & KUMAR, A. 2003. Effect of phenolic monomers on biomass and hydrolytic enzyme activities of an anaerobic fungus isolated from wild nil gai (Baselophus tragocamelus). *Letters in applied microbiology,* 36**,** 377-381.
PEREIRA, A. M., DE LURDES NUNES ENES DAPKEVICIUS, M. & BORBA, A. E. 2022. Alternative pathways for hydrogen sink originated from the ruminal fermentation of carbohydrates: Which microorganisms are involved in lowering methane emission? *Animal Microbiome,* 4**,** 1-12.
PEREZ-MALDONADO, R. & NORTON, B. 1996. The effects of condensed tannins from Desmodium intortum and Calliandra calothyrsus on protein and carbohydrate digestion in sheep and goats. *British Journal of Nutrition,* 76**,** 515-533.
PESTA, A. C. 2015. Dietary strategies for mitigation of methane production by growing and finishing cattle.
PESTA, A. C., WATSON, A. K., BONDURANT BONDURANT, R. G., FERNANDO, S. C. & ERICKSON, G. E. 2015. Effects of Dietary Fat Source and Monensin on Methane Emissions, VFA Profile, and Performance of Finishing Steers.
PILUZZA, G., SULAS, L. & BULLITTA, S. 2014. Tannins in forage plants and their role in animal husbandry and environmental sustainability: a review. *Grass and Forage Science,* 69**,** 32-48.
PLUMMER, E., TWIN, J., BULACH, D. M., GARLAND, S. M. & TABRIZI, S. N. 2015. A comparison of three bioinformatics pipelines for the analysis of preterm gut microbiota using 16S rRNA gene sequencing data. *Journal of Proteomics & Bioinformatics,* 8**,** 283-291.
POULSEN, M., SCHWAB, C., JENSEN, B. B., ENGBERG, R. M., SPANG, A., CANIBE, N., HØJBERG, O., MILINOVICH, G., FRAGNER, L. & SCHLEPER, C. 2013. Methylotrophic methanogenic Thermoplasmata implicated in reduced methane emissions from bovine rumen. *Nature communications,* 4**,** 1-9.
PRIOLO, A., WAGHORN, G., LANZA, M., BIONDI, L. & PENNISI, P. 2000. Polyethylene glycol as a means for reducing the impact of condensed tannins in carob pulp: effects on lamb growth performance and meat quality. *Journal of Animal Science,* 78**,** 810-816.
PRUITT, K. D., TATUSOVA, T. & MAGLOTT, D. R. 2007. NCBI reference sequences (RefSeq): a curated non-redundant sequence database of genomes, transcripts and proteins. *Nucleic acids research,* 35**,** D61-D65.
PUNIYA, A. K., SINGH, R. & KAMRA, D. N. 2015. Rumen microbiology: from evolution to revolution.
QUINCE, C., WALKER, A. W., SIMPSON, J. T., LOMAN, N. J. & SEGATA, N. 2017. Shotgun metagenomics, from sampling to analysis. *Nature biotechnology,* 35**,** 833-844.
RANILLA, M. J., MORGAVI, D. & JOUANY, J. P. Effect of time after defaunation on methane production in vitro. 4. Joint INRA-RRI Symposium Gut Microbiology, 2004. EDP Sciences.
RAUSCH, P., RÜHLEMANN, M., HERMES, B. M., DOMS, S., DAGAN, T., DIERKING, K., DOMIN, H., FRAUNE, S., VON FRIELING, J. & HENTSCHEL, U. 2019. Comparative analysis of amplicon and metagenomic sequencing methods reveals key features in the evolution of animal metaorganisms. *Microbiome,* 7**,** 1-19.
REZAEIAN, M., BEAKES, G. W. & PARKER, D. S. 2004. Distribution and estimation of anaerobic zoosporic fungi along the digestive tracts of sheep. *Mycological research,* 108**,** 1227-1233.

ROSENTRETER, J. A., BORGES, A. V., DEEMER, B. R., HOLGERSON, M. A., LIU, S., SONG, C., MELACK, J., RAYMOND, P. A., DUARTE, C. M. & ALLEN, G. H. 2021. Half of global methane emissions come from highly variable aquatic ecosystem sources. *Nature Geoscience,* 14**,** 225-230.

RUFENER, W., NELSON, W. & WOLIN, M. 1963. Maintenance of the rumen microbial population in continuous culture. *Applied Microbiology,* 11**,** 196-201.

SALEM, H. B., NEFZAOUI, A., SALEM, L. B. & TISSERAND, J. 1997. Effect of Acacia cyanophylla Lindl. foliage supply on intake and digestion by sheep fed lucerne hay-based diets. *Animal feed science and technology,* 68**,** 101-113.

SALMON, G. R., MACLEOD, M., CLAXTON, J. R., PICA CIAMARRA, U., ROBINSON, T., DUNCAN, A. & PETERS, A. R. 2020. Exploring the landscape of livestock 'Facts'. *Global Food Security,* 25**,** 100329.

SAMINATHAN, M., SIEO, C. C., GAN, H. M., ABDULLAH, N., WONG, C. M. V. L. & HO, Y. W. 2016. Effects of condensed tannin fractions of different molecular weights on population and diversity of bovine rumen methanogenic archaea in vitro, as determined by high-throughput sequencing. *Animal Feed Science and Technology,* 216**,** 146-160.

SCHOLTZ, M., VAN RYSSEN, J. V., MEISSNER, H. & LAKER, M. C. 2013. A South African perspective on livestock production in relation to greenhouse gases and water usage. *South African Journal of Animal Science,* 43**,** 247-254.

SEJIAN, V. & NAQVI, S. 2012. Livestock and climate change: Mitigation strategies to reduce methane production. *Greenhouse Gases-Capturing, Utilization and Reduction***,** 255-276.

SHANMUGAPRIYA, P., RATHIKA, S. & RAMESH, T. 2019. Carbon sequestration in agriculture for challenging climate change-An overview.

SHARPTON, T. J. 2014. An introduction to the analysis of shotgun metagenomic data. *Frontiers in plant science,* 5**,** 209.

SHU, Q., GILL, H., HENNESSY, D., LENG, R., BIRD, S. & ROWE, J. 1999. Immunisation against lactic acidosis in cattle. *Research in Veterinary Science,* 67**,** 65-71.

SIROHI, S. K., SINGH, N., DAGAR, S. S. & PUNIYA, A. K. 2012. Molecular tools for deciphering the microbial community structure and diversity in rumen ecosystem. *Applied microbiology and biotechnology,* 95**,** 1135-1154.

SKENE, I. & BROOKER, J. D. 1995. Characterization of tannin acylhydrolase activity in the ruminal bacterium Selenomonas ruminantium. *Anaerobe,* 1**,** 321-327.

ŚLIWIŃSKI, B., KREUZER, M., WETTSTEIN, H.-R. & MACHMÜLLER, A. 2002. Rumen fermentation and nitrogen balance of lambs fed diets containing plant extracts rich in tannins and saponins, and associated emissions of nitrogen and methane. *Archives of Animal Nutrition,* 56**,** 379-392.

SLYTER, L. 1979. Monensin and dichloroacetamide influences on methane and volatile fatty acid production by rumen bacteria in vitro. *Applied and Environmental Microbiology,* 37**,** 283-288.

SMITH, A. H., ZOETENDAL, E. & MACKIE, R. I. 2005. Bacterial mechanisms to overcome inhibitory effects of dietary tannins. *Microbial ecology,* 50**,** 197-205.

STANTON, T. B. 2007. Prophage-like gene transfer agents—novel mechanisms of gene exchange for Methanococcus, Desulfovibrio, Brachyspira, and Rhodobacter species. *Anaerobe,* 13**,** 43-49.

STEVENSON, D. M. & WEIMER, P. J. 2007. Dominance of Prevotella and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-time PCR. *Applied microbiology and biotechnology,* 75**,** 165-174.

STEWART, E. K., BEAUCHEMIN, K. A., DAI, X., MACADAM, J. W., CHRISTENSEN, R. G. & VILLALBA, J. J. 2019. Effect of tannin-containing hays on enteric methane emissions and nitrogen partitioning in beef cattle. *Journal of Animal Science,* 97**,** 3286-3299.

STROBEL, H. J. 1992. Vitamin B12-dependent propionate production by the ruminal bacterium Prevotella ruminicola 23. *Applied and Environmental Microbiology,* 58**,** 2331-2333.

TAMMINGA, S., BANNINK, A., DIJKSTRA, J. & ZOM, R. 2007. Feeding strategies to reduce methane loss in cattle. Report 34. *Animal Science Group. http://edepot. wur. nl/28209*.

TAN, H., SIEO, C., ABDULLAH, N., LIANG, J., HUANG, X. & HO, Y. 2011. Effects of condensed tannins from Leucaena on methane production, rumen fermentation and populations of methanogens and protozoa in vitro. *Animal feed science and technology,* 169**,** 185-193.

TAPIO, I., SNELLING, T. J., STROZZI, F. & WALLACE, R. J. 2017. The ruminal microbiome associated with methane emissions from ruminant livestock. *Journal of animal science and biotechnology,* 8**,** 1-11.

TATUSOV, R. L., FEDOROVA, N. D., JACKSON, J. D., JACOBS, A. R., KIRYUTIN, B., KOONIN, E. V., KRYLOV, D. M., MAZUMDER, R., MEKHEDOV, S. L., NIKOLSKAYA, A. N., RAO, B. S., SMIRNOV, S., SVERDLOV, A. V., VASUDEVAN, S., WOLF, Y. I., YIN, J. J. & NATALE, D. A. 2003. The COG database: an updated version includes eukaryotes. *BMC Bioinformatics,* 4**,** 41.

TAVENDALE, M. H., MEAGHER, L. P., PACHECO, D., WALKER, N., ATTWOOD, G. T. & SIVAKUMARAN, S. 2005. Methane production from in vitro rumen incubations with Lotus pedunculatus and Medicago sativa, and effects of extractable condensed tannin fractions on methanogenesis. *Animal Feed Science and Technology,* 123**,** 403-419.

THOLEN, A., PESTER, M. & BRUNE, A. 2007. Simultaneous methanogenesis and oxygen reduction by Methanobrevibacter cuticularis at low oxygen fluxes. *FEMS Microbiology Ecology,* 62**,** 303-312.

TJAKRADIDJAJA, A., BROOKER, J. & BOTTEMA, C. 2000. Characterisation of tannin-resistant bacteria from the rumen fluid of feral goats and camels with restriction analysis of amplified 16S rDNA.

TUCKER, C. M., CADOTTE, M. W., CARVALHO, S. B., DAVIES, T. J., FERRIER, S., FRITZ, S. A., GRENYER, R., HELMUS, M. R., JIN, L. S., MOOERS, A. O., PAVOINE, S., PURSCHKE, O., REDDING, D. W., ROSAUER, D. F., WINTER, M. & MAZEL, F. 2017. A guide to phylogenetic metrics for conservation, community ecology and macroecology. *Biological reviews of the Cambridge Philosophical Society,* 92**,** 698-715.

ULYATT, M. J. & LASSEY, K. 2001. Methane emissions from pastoral systems: the situation in New Zealand. *Archivos Latinoamericanos de Producción Animal,* 9.

UNGERFELD, E. M. 2020a. Metabolic Hydrogen Flows in Rumen Fermentation: Principles and Possibilities of Interventions. *Frontiers in microbiology,* 11**,** 589-589.

UNGERFELD, E. M. 2020b. Metabolic hydrogen flows in rumen fermentation: principles and possibilities of interventions. *Frontiers in Microbiology***,** 589.

VAN DER WALT, A. J., VAN GOETHEM, M. W., RAMOND, J.-B., MAKHALANYANE, T. P., REVA, O. & COWAN, D. A. 2017. Assembling metagenomes, one community at a time. *BMC genomics,* 18**,** 521-521.

VAN NEVEL, C. & DEMEYER, D. 1979. EFFECT OF MONENSIN (1) ON SOME RUMEN FERMENTATION PARAMETERS.

VAN SOEST, P. J. 2018. *Nutritional ecology of the ruminant*, Cornell university press.

VARGA, G. A. & KOLVER, E. S. 1997. Microbial and animal limitations to fiber digestion and utilization. *The Journal of nutrition,* 127**,** 819S-823S.

VARGAS-ORTIZ, L., CHAVEZ-GARCIA, D., BARROS-RODRÍGUEZ, M., ANDRADE-YUCAILLA, V., LIMA-OROZCO, R., MACÍAS-RODRÍGUEZ, E., GUISHCA-CUNUHAY, C. & ZEIDAN MOHAMED SALEM, A. 2022. Rumen Function and In Vitro Gas Production of Diets Influenced by Two Levels of Tannin-Rich Forage. *Fermentation,* 8**,** 607.

WAGHORN, G., TAVENDALE, M. & WOODFIELD, D. Methanogenisis from forages fed to sheep. PROCEEDINGS OF THE CONFERENCE-NEW ZEALAND GRASSLAND ASSOCIATION, 2002. 167-172.

WALLACE, R. J. 2004. Antimicrobial properties of plant secondary metabolites. *Proceedings of the nutrition society,* 63**,** 621-629.

WELANDER, P. V. & METCALF, W. W. 2005. Loss of the mtr operon in Methanosarcina blocks growth on methanol, but not methanogenesis, and reveals an unknown methanogenic pathway. *Proceedings of the National Academy of Sciences,* 102**,** 10664-10669.

WELCH, J. G. 1986. Physical parameters of fiber affecting passage from the rumen. *J Dairy Sci,* 69**,** 2750-4.

WHEELER, D. L., BARRETT, T., BENSON, D. A., BRYANT, S. H., CANESE, K., CHETVERNIN, V., CHURCH, D. M., DICUCCIO, M., EDGAR, R., FEDERHEN, S., FEOLO, M., GEER, L. Y., HELMBERG, W., KAPUSTIN, Y., KHOVAYKO, O., LANDSMAN, D., LIPMAN, D. J., MADDEN, T. L., MAGLOTT, D. R., MILLER, V., OSTELL, J., PRUITT, K. D., SCHULER,

G. D., SHUMWAY, M., SEQUEIRA, E., SHERRY, S. T., SIROTKIN, K., SOUVOROV, A., STARCHENKO, G., TATUSOV, R. L., TATUSOVA, T. A., WAGNER, L. & YASCHENKO, E. 2007. Database resources of the National Center for Biotechnology Information. *Nucleic Acids Research,* 36**,** D13-D21.

WHITFORD, M. F., FORSTER, R. J., BEARD, C. E., GONG, J. & TEATHER, R. M. 1998. Phylogenetic analysis of rumen bacteria by comparative sequence analysis of cloned 16S rRNA genesß. *Anaerobe,* 4**,** 153-163.

WILKE, A., GERLACH, W., HARRISON, T., PACZIAN, T., TRIMBLE, W. L. & MEYER, F. 2017. MG-RAST manual for version 4, revision 3. *Lemont, IL: Argonne National Laboratory*.

WILLIAMS, A., WITHERS, S., NAYLOR, G. & JOBLIN, K. 1994. Effect of heterotrophic ruminal bacteria on xylan metabolism by the anaerobic fungus Piromyces communis. *Letters in applied microbiology,* 19**,** 105-109.

WILLIAMS, A. G. & COLEMAN, G. S. 2012. *The rumen protozoa*, Springer Science & Business Media.

WILLIAMS, C. L., THOMAS, B. J., MCEWAN, N. R., REES STEVENS, P., CREEVEY, C. J. & HUWS, S. A. 2020a. Rumen Protozoa Play a Significant Role in Fungal Predation and Plant Carbohydrate Breakdown. *Frontiers in Microbiology,* 11.

WILLIAMS, C. L., THOMAS, B. J., MCEWAN, N. R., REES STEVENS, P., CREEVEY, C. J. & HUWS, S. A. 2020b. Rumen protozoa play a significant role in fungal predation and plant carbohydrate breakdown. *Frontiers in microbiology,* 11**,** 720.

WILLIAMS, S., HANNAH, M., ECKARD, R., WALES, W. & MOATE, P. 2020c. Supplementing the diet of dairy cows with fat or tannin reduces methane yield, and additively when fed in combination. *animal,* 14**,** s464-s472.

WIRYAWAN, K. & TANGENDJAJA, B. Tannin degrading bacteria from Indonesian ruminants. ACIAR PROCEEDINGS, 1999. ACIAR; 1998, 123-126.

WOLIN, M., MILLER, T. & STEWART, C. 1997. Microbe-microbe interactions. *The rumen microbial ecosystem.* Springer.

WOLIN, M. J. 1979. The rumen fermentation: a model for microbial interactions in anaerobic ecosystems. *Advances in microbial ecology.* Springer.

WYLIE, K. M., TRUTY, R. M., SHARPTON, T. J., MIHINDUKULASURIYA, K. A., ZHOU, Y., GAO, H., SODERGREN, E., WEINSTOCK, G. M. & POLLARD, K. S. 2012. Novel bacterial taxa in the human microbiome. *PloS one,* 7**,** e35294.

XING, Z., ZHANG, Y., LI, M., GUO, C. & MI, S. 2020. RBUD: A New Functional Potential Analysis Approach for Whole Microbial Genome Shotgun Sequencing. *Microorganisms,* 8**,** 1563.

YAN, T., AGNEW, R., GORDON, F. & PORTER, M. 2000. Prediction of methane energy output in dairy and beef cattle offered grass silage-based diets. *Livestock Production Science,* 64**,** 253-263.

ZHAO, Y. & ZHAO, G. 2022. Decreasing ruminal methane production through enhancing the sulfate reduction pathway. *Animal Nutrition*.

ZHENG, H., LIU, J., YAO, J., YUAN, Q., YE, H., YE, J. & WU, Y. 2005. Effects of dietary sources of vegetable oils on performance of high-yielding lactating cows and conjugated linoleic acids in milk. *Journal of Dairy Science,* 88**,** 2037-2042.

ZHOU, M., CHUNG, Y. H., BEAUCHEMIN, K., HOLTSHAUSEN, L., OBA, M., MCALLISTER, T. & GUAN, L. 2011a. Relationship between rumen methanogens and methane production in dairy cows fed diets supplemented with a feed enzyme additive. *Journal of applied microbiology,* 111**,** 1148-1158.

ZHOU, M., O'HARA, E., TANG, S., CHEN, Y., WALPOLE, M. E., GÓRKA, P., PENNER, G. B. & GUAN, L. L. 2021. Accessing Dietary Effects on the Rumen Microbiome: Different Sequencing Methods Tell Different Stories. *Veterinary sciences,* 8**,** 138.

ZHOU, Y., MAO, H., JIANG, F., WANG, J., LIU, J. & MCSWEENEY, C. 2011b. Inhibition of rumen methanogenesis by tea saponins with reference to fermentation pattern and microbial communities in Hu sheep. *Animal Feed Science and Technology,* 166**,** 93-100.

ZHU, Z. 2016. *Dynamics of rumen bacterial and archaeal communities in dairy cows over different lactation cycle stages*, Department of Animal Science, Aarhus University.

ZHU, Z., LØVENDAHL, P., POULSEN, M., SAMANTHA, P. & NOEL, J. 2016. *Dynamics of rumen bacterial and archaeal communities in dairy cows over different lactation cycle stages.* Section for Immunology and Microbiology, Aarhus University.

ZHU, Z., NOEL, S. J., DIFFORD, G. F., AL-SOUD, W. A., BREJNROD, A., SØRENSEN, S. J., LASSEN, J., LØVENDAHL, P. & HØJBERG, O. 2017. Community structure of the metabolically active rumen bacterial and archaeal communities of dairy cows over the transition period. *PLoS One*, 12**,** e0187858.

Appendix

DNA extraction

A total of 250 µL of rumen sample was pipetted into the ZR BashingBead™ lysis tubes and 750 µL of ZymoBiomics lysis solution was added. The lysis tubes were capped tightly and then placed in a beadbug machine to be bead beat at the maximum speed for 9 to 12minutes. Thereafter, the ZR BashingBead™ lysis tubes were centrifuged for 1 minute at ≥ 10,000 x g. Upto 400 µL of the supernatant was then transferred to the Zymo-Spin™ III-F Filter placed in a collection tube and then centrifuged for 1 minutes at 8,000 x g. The Zymo-Spin™ III-F Filter was then discarded. For the binding step, 1,200 µL of ZymoBIOMICS™ DNA Binding Buffer was added to the filtrate in the collection tube from the step above and mixed thoroughly. A Zymo-Spin™ IICR Column was placed in a collection tube and 800 µL of the mixture above was transferred to the column and centrifuged at 10,000 x g for 1 minute. The flow through in the collection tube was discarded and the step repeated (800 µL of the mixture above was transferred to the column and centrifuged at 10,000 x g for 1 minute, the flow through was also discarded along with the collection tube). After the DNA binding step, 400 µL of ZymoBIOMICS™ DNA Wash Buffer 1 was added to the Zymo-Spin™ IICR Column placed in a new collection tube and then centrifuged at 10,000 x g for 1 minute. The flow through was discarded and the step repeated but with 700 µL ZymoBIOMICS™ DNA Wash Buffer 2. The flow through was discarded and the step repeated for a third time using 200 µL ZymoBIOMICS™ DNA Wash Buffer 2. The Zymo-Spin™ IICR Column is placed in a clean 1.5 ml microcentrifuge tube. Then 100 µL (minimum 50 µL) of DNase/RNase free water is added directly onto the matrix and was incubated for 1 minute before centrifuging it at 10,000 x g for 1 minute to elute the DNA. A Zymo-Spin™ III-HCR Filter was prepared by adding ZymoBIOMICS™ HRC Prep Solution to the filter in a new collection tube and then centrifuged at 8000 x g for 3 minutes. The flow through is discarded along with the collection tube and the Zymo-Spin™ III-HCR Filter placed in a clean 1.5 ml microcentrifuge tube. The eluted DNA from above is transferred onto the prepared Zymo-Spin™ III-HCR Filter and centrifuged at exactly 16,000 x g for 3 minutes. The filtered DNA sample collected in the 1.5 ml microcentrifuge tube was immediately stored at 4°C.

Appendix Table 1 Sample details

Sample (24)	Conc (ng/uL)	Volume (uL)	Purity (OD260/280)	Treatment
1B	120	>30	1.85	1
2B3	44.6	>30	1.9	1
3B	43.4	>30	1.97	1
4B	120	>30	1.92	1
5B	120	>30	1.89	1
21B	66.8	>30	1.91	1
6B	81.2	>30	1.87	2
7B	83.8	>30	1.89	2
9B	120	>30	1.85	2
10B	85.4	>30	1.88	2
26B	40.2	>30	1.87	2
11B	104	>30	1.88	3
12B	74	>30	1.88	3
13B	90.9	>30	1.87	3
14B	102	>30	1.94	3
15B	92.8	>30	1.82	3
22B	73.6	>30	1.87	3
16B	87	>30	1.87	4
17B	74.6	>30	1.87	4
19B	74.8	>30	2	4
20B	84.6	>30	1.87	4
23B	95	>30	1.9	4
30B	61.4	>30	1.89	4
8B*	84.4	>30	1.92	failed QC

Appendix Table 2 Family and genera of Achaea population belonging to the phylum Euryarcheota

family	genus	S1B_L2_	S2B3_L2_	S3B_L2_	S4B_L2_	S5B_L2_	S21B_L2_	S6B_L2_	S7B_L2_	S9B_L2_	S10B_L2_	S26B_L2_	S11B_L2_	S12B_L2_	S13B_L2_	S14B_L2_	S15B_L2_	S22B_L2_	S16B_L2_	S17B_L2_	S19B_L2_	S20B_L2_	S23B_L2_	S30B_L2_
Methanobacteriaceae	Methanobrevibacter	207003	1590048	154772	82530	132106	588298	869831	865142	106880	278868	330127	49577	594785	830740	579216	598958	374609	2469149	212532	130862	217938	282008	930079
Methanosarcinaceae	Methanosarcina	20103	31737	19010	15077	15987	17628	26808	23657	16855	18942	19378	14478	22887	20012	23023	17695	19756	40172	21832	16843	17823	17526	23787
Methanococcaceae	Methanococcus	13769	30068	11779	11155	9729	12858	20525	17281	10597	11192	10680	9094	14657	15938	16617	12791	12504	35996	12722	11149	11272	11530	17654
Methanobacteriaceae	Methanothermobacter	28354	204862	20128	11826	21119	73664	120687	107895	13493	32119	39828	6541	71051	101204	69179	71787	43557	331961	26591	16565	31045	36020	110503
Methanocorpusculaceae	Methanocorpusculum	5405	12022	5527	4196	3986	6401	7813	10401	5246	6215	6047	4352	7439	6950	8087	6842	7018	13954	6764	5162	5549	6721	8574
Methanobacteriaceae	Methanosphaera	13570	121158	11902	7449	10511	36605	53664	65708	9353	19347	26739	4334	39917	49681	34987	39830	24595	152537	15135	11173	19364	26418	54917
Methanocaldococcaceae	Methanocaldococcus	8743	44093	6851	5608	6240	16081	26130	22875	5641	8839	9801	3839	16657	22357	16582	15871	11321	68000	7836	5453	8474	8451	24461
Methanospirillaceae	Methanospirillum	3487	5021	3415	2660	3238	2741	4659	3821	3012	2768	2904	2238	3638	3504	4159	3004	3623	6581	3986	2929	2887	2798	3736
Thermococcaceae	Thermococcus	4065	7549	4303	3511	3163	3692	6083	5392	3121	3643	3482	2225	4957	4516	5372	4098	4597	10117	4534	2854	3972	3411	5746
Methanosarcinaceae	Methanococcoides	3019	5130	2621	2037	2522	2923	4032	3657	2466	2413	2400	2014	3249	3213	3527	2711	3255	6494	2862	2466	2440	2247	3695
Thermococcaceae	Pyrococcus	3508	8449	3529	2602	2613	3981	6590	5579	2789	3191	3396	1873	4947	4924	5028	4019	4316	11805	3901	2821	3532	3302	5667
Methanomicrobiaceae	Methanoculleus	2397	4111	2175	1896	1828	1886	2972	2832	2102	1971	2087	1736	2614	2413	2984	2039	2631	5160	2712	2038	1841	2105	2710
unclassified (derived fro	unclassified (derived from	2486	4456	2732	2145	1969	2268	3437	2993	2196	2271	2280	1709	2940	2779	3281	2461	2679	5760	2828	2199	2469	2509	3277
Methanomicrobiaceae	Methanoplanus	2080	2890	1827	1646	1857	1632	2420	2470	1719	1901	1833	1653	2506	2257	2081	1836	1944	3625	2028	1812	1502	1929	2445
unclassified (derived fro	Methanoregula	2117	4090	2197	1749	1707	2034	3031	2630	1826	1769	2008	1585	2654	2313	2831	2095	2458	6095	2472	1816	1990	1827	2661
Archaeoglobaceae	Archaeoglobus	2490	6254	2470	2034	2016	2682	4094	4258	1829	1970	2199	1455	3095	3477	3507	2720	2866	8904	2566	1778	2279	2148	3801
unclassified (derived fro	Methanosphaerula	1877	2653	1525	1408	1266	1409	2058	1715	1365	1668	1304	1210	1783	1638	1890	1567	1598	3509	1707	1158	1314	1626	1870
Methanosaetaceae	Methanosaeta	1948	3354	1834	1609	1424	1475	2410	2323	1189	1284	1468	1194	2092	1861	2155	1635	1842	4278	1665	1176	1715	1353	2122
Thermoplasmataceae	Thermoplasma	1299	2484	1280	854	923	1148	1812	1973	896	1122	1117	743	1468	1323	1474	1287	1462	3430	1309	1172	1243	994	1627
Methanosarcinaceae	Methanohalophilus	967	1537	952	824	703	697	1125	1082	806	804	847	701	881	787	1030	755	1050	1688	929	745	892	717	902
unclassified (derived fro	Aciduliprofundum	1084	2336	1275	819	871	1187	1850	1612	774	1076	1249	577	1681	1298	1409	1196	1859	3830	1171	778	1196	953	1520
Methanosarcinaceae	Methanohalobium	1255	1550	1597	762	614	670	1572	885	964	753	1011	559	887	802	1136	746	925	1952	906	1168	1229	541	1051
Methanothermaceae	Methanothermus	2085	12313	1441	1025	1749	4183	8025	5698	920	1470	2438	512	3915	5146	3866	3672	2514	19374	1662	1059	2351	1725	5651
Methanocellaceae	Methanocella	862	1463	945	687	600	770	1124	993	807	833	851	467	1073	951	1081	790	1049	1801	939	629	804	715	1059
Halobacteriaceae	Haloterrigena	412	502	427	401	352	341	538	426	362	365	406	459	484	381	520	355	419	794	558	434	459	488	481
Halobacteriaceae	Haloarcula	817	1263	635	581	520	754	1013	1009	649	582	787	411	752	811	927	704	702	1778	782	599	671	726	934
Halobacteriaceae	Halorhabdus	493	691	613	401	368	347	635	462	479	408	453	399	513	417	606	341	538	781	586	382	431	504	533
Methanopyraceae	Methanopyrus	977	4505	803	585	682	1704	2596	2527	545	978	1155	354	1855	2196	1745	1660	1352	6850	1098	633	953	983	2472
Halobacteriaceae	Natronomonas	529	846	459	420	349	417	529	538	387	372	425	309	511	505	586	403	446	1036	522	397	409	398	492
Picrophilaceae	Picrophilus	568	880	389	393	387	435	609	605	362	356	394	309	524	620	569	457	558	1072	435	347	450	281	477
Archaeoglobaceae	Ferroglobus	680	1032	520	389	350	389	655	717	341	397	468	276	525	532	708	468	594	1046	516	389	552	398	608
Ferroplasmaceae	Ferroplasma	400	647	336	274	330	333	487	412	419	252	376	264	384	346	484	400	430	659	408	333	363	399	475
Halobacteriaceae	Haloquadratum	386	840	378	344	238	411	633	634	373	331	423	257	546	456	546	481	441	1085	377	386	422	383	631
Halobacteriaceae	Halobacterium	402	559	390	303	251	316	446	471	284	307	306	220	408	323	494	357	414	909	437	262	359	280	482
Halobacteriaceae	Haloferax	227	372	224	215	191	204	316	315	273	258	287	198	294	242	324	231	319	463	246	225	239	197	307
Halobacteriaceae	Halogeometricum	359	512	358	288	271	286	420	381	375	318	288	197	430	304	489	289	406	634	366	269	345	234	396
Halobacteriaceae	Natrialba	220	410	292	228	211	253	384	308	216	196	202	185	239	287	356	221	317	416	388	264	291	253	331
Halobacteriaceae	Halomicrobium	314	427	353	253	180	227	415	271	177	208	225	162	288	251	362	251	303	526	394	195	256	266	249
Halobacteriaceae	Halorubrum	210	260	151	155	122	155	233	257	172	177	172	121	209	192	207	160	191	266	162	143	188	146	217
Halobacteriaceae	Halalkalicoccus	285	385	273	249	174	243	335	298	198	207	241	110	306	291	256	246	259	528	261	190	218	262	372
Methanococcaceae	Methanothermococcus	229	890	241	170	183	265	583	568	125	155	227	98	281	413	370	301	262	1153	203	122	184	155	398
		Treatment 1						Treatment 2					Treatment 3						Treatment 4					