In vitro fermentation and growth performance of Merino lambs fed on umbrella thorn (*Vachellia tortilis*) leaf meal and sunflower oil.

By

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Declaration

I, Mahlogonolo Daniel Serumula, declare that **in vitro fermentation and growth performance of Merino lambs fed on umbrella thorn (*Vachellia tortilis*) leaf meal and sunflower oil** is my own work, except where indicated through referencing. This thesis has not been submitted for a degree in any other institution for higher learning other than University of KwaZulu-Natal.

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List of Abbreviations

Abbreviations	Definitions
ADF	Acid detergent fibre
ADG	Average daily gain
ADL	Acid detergent lignin
AOAC	Association of Official Analytical Chemists
ATP	Adenosine triphosphate
CFC	Chlorofluorocarbon
CH_4	Methane
Co-EDTA	cobalt Ethylenediaminetetraacetic acid
DCFB	Densified complete feed blocks
DEI	Digestible energy intake
DM	Dry matter
DMI	Dry matter intake
EDTA	Ethylenediaminetetraacetic acid
FCR	Feed conversion ratio
FID	Flame ionization detector
GEI	Gross energy intake
GHG	Greenhouse gas
GLM	General linear model
H_2	Hydrogen
HG	Hindgut
IPCC	Intergovernmental panel on climate change
IVDMD	In vitro dry matter digestibility
MEI	Metabolizable energy intake
MRT	Mean retention time
N	Nitrogen
NDF	Neutral detergent fibre
NPN	Non-protein nitrogen
PEG	Polyethylene glycol

PSM	Plant secondary metabolites
PUFA	Polyunsaturated fatty acid
PVP	Polyvinylpyrrolidone
RFI	Residual feed intake
SCFAs	Short-chain fatty acids
SPM	Secondary plant metabolite
TMRT	Total mean retention time
UTH	Urea-treated hay

General Abstract

Forage legumes and vegetable oils are supplemented in ruminant diets to improve nutrient quality (energy density and crude protein content) and mitigating rumen gaseous emissions. The effects of both forage legumes and vegetable oils would depend on source, inclusion level and animal species. The broad objective of the study was to determine the effect of *Vachellia tortilis* leaf meal and sunflower oil on *in vitro* short-chain fatty acids (SCFAs) production, proportion of methane and growth performance of Merino lambs. The specific objectives of this study were to determine (1) the effect of *Vachellia tortilis* leaf meal and sunflower oil on *in vitro* total SCFA production, individual SCFAs composition, proportion of methane, carbon dioxide, and IVDMD; (2) the effect of *Vachellia tortilis* leaf meal and sunflower oil on growth performance of Merino lambs and (3) the effect of *Vachellia tortilis* leaf meal and sunflower oil on fractional outflow rate of particulate and liquid fractions of digesta in sheep. Five dietary treatments used were: the control diet (CT), *Vachellia tortilis* (VT) leaf meal diet (121.5 g/kg DM), sunflower oil (SFO) diet (40.8 g/kg DM), combination of *Vachellia tortilis* leaf meal and sunflower oil (VSFO) diet (63.4 g/kg + 19.5 g/kg DM) and maize grain – lucerne (ML) (300 g/kg + 180 g/kg DM) diet. Fresh samples were collected, dried in oven, ground and analysed for nutrient composition. Twenty-two duran bottles were incubated, including two blanks for 48 hours. Total SCFAs, acetate and propionate, acetate to propionate ratio, proportion of methane and carbon dioxide were not affected by the inclusion of *Vachellia tortilis* leaf meal and sunflower oil. Butyrate production and proportion of carbon dioxide were highest in VSFO diet at 16 hours compared to the control. *In vitro* dry matter digestibility (IVDMD) was higher in VT and VSFO diets compared to the control. The condensed tannin, ether extract and sunflower cake content did not influence production of total SCFAs, individual SCFA, proportion of methane and carbon dioxide. The high ether extract content in SFO diet negatively affected IVDMD. It was evident that inclusion of *Vachellia tortilis* leaf meal and sunflower oil did not affect production of total SCFAs, acetate to propionate ratio and proportion of methane. The proportion of methane was calculated based on stoichiometric method as observed and the Moss *et al*. (2000) equation. Both methods displayed a linear relationship with similar results. Ten mixed sex Merino lambs (n= 6) were fed on similar dietary treatments as *in vitro* fermentation study. An incomplete Latin square design was used where each treatment was represented by a random pair of lambs housed in individual pens for three periods (126 days). Lambs were offered 480 g/kg DM daily of dietary treatments with *ad libitum* accesses to urea-treated hay (*Themeda trianda*). For passage rate trial, five lambs were

selected with one from each dietary treatment. The crude protein content was higher in VT and VSFO diets, due to inclusion of *Vachellia tortilis* leaf meal. Dry matter intake was lower in maize-lucerne diet compared to other diets including the control. Total dry matter intake (TDMI), average daily gain (ADG), feed conversion ratio (FCR) and neutral detergent fibre digestibility (NDFD) were similar across all dietary treatments. The inclusion of sunflower oil in dietary treatments negatively affected apparent digestibility, which was due to high ether extract content. Fractional passage rate, total mean retention time (TMRT) in both reticulorumen (RR) and hindgut (HG) of particulate and liquid fraction of digesta were similar across all dietary treatments. The improvement of roughage with a non-protein nitrogen source (NPN) provided a nutrient balance for lambs. The ether extract content in sunflower oil diets were above the recommended levels, thus possibly explaining the poor digestibility. In conclusion, the inclusion of *Vachellia tortilis* leaf meal and sunflower oil did not affect growth performance. The combination of *Vachellia tortilis* leaf meal and sunflower oil has potential to improve average daily gain.

Key words: ether extract, fatty acids, methane, sunflower oil, tannins, weight gain

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Dedication

This thesis is dedicated to my family, both my parents (Philip and Gloria Serumula) for their support and encouragement. Also, my siblings (Mahlatse, Malegobe and Tshepang Serumula) for believing in my desire to further my studies.

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CHAPTER 1

General introduction

1.1 Background

Microbial fermentation allows ruminants to utilise poor-quality roughages yet this results in energy loss (10 – 12 %) and emissions of some greenhouse gases like methane, ammonia, sulphides and carbon dioxide (Morgavi *et al*., 2010; Eckard *et al*., 2010; Pérez-Barbería, 2017). Methanogenic *Archaea* found in the rumen utilise hydrogen and carbon dioxide obtained from a free rumen environment or through a symbiotic relationship with other rumen microbes in methane formation (Wolin *et al*., 1997; Janssen, 2010). During rumen fermentation, short-chain fatty acids (SCFAs) are produced yielding energy (ATP) for synthesis of microbial matter, particularly microbial protein synthesis (den Besten *et al*., 2013). Population growth put pressure on livestock production to increase output of animal products. Consumer meat consumption will rise due to increasing global population from 229 - 465 million tonnes by 2050 with rising household incomes. This will negatively affect the environment with high manure production emitting nitrous oxide (N_2O) beside enteric methane emissions (Snyder *et al*., 2014).

Smith *et al*. (2007) reported approximately 90 % nitrous oxide emissions resulting from denitrification and nitrification through microbial processes in the soil. Agricultural operations and intensive production systems (feedlots) produce high amounts of GHG due to high manure production, poor storage and management (Bunglavan *et al*., 2010). Mitigation strategies to reduce methane emissions include; animal manipulation (animal breeding & management systems), diet manipulation (forage quality, plant secondary metabolites) and rumen manipulation (defaunation and vaccination) (Bunglavan *et al*., 2010; den Besten *et al*., 2013; Eckard *et al*., 2010). Different mitigation strategies have been reported through *in vitro* and *in vivo* procedures, focusing on improving feed efficiency, nutrient utilization and digestibility (Theart, 2015; Yáñez-Ruiz *et al*., 2016).

Forage legumes are provided as supplements during cold-dry seasons, due to their high crude protein content and secondary metabolites (Waghorn, 2008; Degen *et al*., 2010; Kronberg *et al*., 2018). Anti-nutritional factors caused by forage legumes result from their polyphenolic

compounds like tannins, saponins and essential oils. Proanthocyanidins (condensed tannins) have a unique ability to reduce enteric emissions depending on source, concentration, diets and animal species (Theart, 2015). They are beneficial against bloat and as anthelmintic to improve livestock health.

Polyphenolic compounds and other secondary plant metabolites are produced from plants as survival mechanism against herbivory; toxicity is another reason for their limited use in animal production (Wang *et al*., 2000; Śliwiński *et al*., 2002). Tannin-binding complexes improve nutrient utilisation, shifting protein digestion to small intestines (Tamir and Asefa, 2009; Degen *et al*., 2010). Vegetable oils are effective against ruminant methane production, yet their inclusion levels above 5 % DM negatively affects feed intake and digestibility (Shingfield *et al*., 2008; Dey *et al*., 2018). The inclusion of unsaturated fatty acids reduces hydrogen concentration in the rumen through biohydrogenation; their antimicrobial properties influence activity of rumen microbes (Shingfield *et al*., 2008). Greenhouse gas emissions contribute to global warming and changes in climate conditions will threaten human livelihood and food security.

1.2 Justification

Livestock production contributes to global warming through enteric methane emissions. Supplementation with forage legumes that are rich in polyphenolic compounds is effective in reducing methane production. In addition, forage legumes improve performance of ruminants in the tropical regions when supplemented with poor-quality roughages due to their high crude protein content (Lima *et al*., 2019). The inclusion of fats increases energy density of rations and quality of animal products. They have a high unsaturated fatty acid composition effective against rumen methane production through hydrolysis and antimicrobial properties. The aim is to reduce proportion of methane produced in the rumen and improve growth performance of lambs.

1.3 Objectives

The broad objective of the study was to assess the effect of *Vachellia tortilis* leaf meal and sunflower oil on *in vitro* fermentation and growth performance of Merino lambs.

The specific objectives were to:

- Determine the effect of *Vachellia tortilis* leaf meal and sunflower oil on *in vitro* total SCFAs, individual SCFA and proportion of methane.
- Determine the effect of *Vachellia tortilis* leaf meal and sunflower oil on growth performance of lambs.
- Determine the effect of *Vachellia tortilis* leaf meal and sunflower oil on fractional passage rate of particulate and liquid fraction of digesta.

1.4 Hypothesis

The hypothesis tested were:

1. The inclusion of *Vachellia tortilis* leaf meal and sunflower oil will reduce total SCFAs, acetate to propionate ratio and proportion of methane.

2. The inclusion of *Vachellia tortilis* leaf meal and sunflower oil will improve growth performance and fractional passage rate of digesta.

CHAPTER 2

Review of literature

2.1 INTRODUCTION

Feeds consumed by ruminants usually undergo fermentation effected by microbes (bacteria, protozoa and fungi) in an anaerobic environment literally deprived of oxygen. End products of fermentation are short chain fatty acids (typified by acetic acid, propionic acid and butyric acid). Some carbon dioxide in the presence of hydrogen is used in the synthesis of methane, which is one of the three main agricultural products (CO_2; CH_4; NO_2) threatening the environment and is of global concern in view rising ocean levels and changing climatic condition. This study looks at two strategies using *Vachellia tortilis* and sunflower oil as ways of reducing methane emissions.

2.1.1 Umbrella thorn Acacia (Vachellia tortilis)

The forage tree is widely known as *Acacia tortilis*, current attributed to the genus *Vachellia*. It is known as the umbrella thorn acacia. *Vachellia tortilis* tree can usually grow to about 4 to 8m high. Its dense crawn, umbrella-like and flat-topped; is the reason it is called umbrella tree in some areas. Flowers are white, cream or yellow and highly aromatic. *Vachellia tortilis* is a drought tolerant species due to its deep taproot system. The forage tree is from a group of leguminous trees used as protein sources during dry, winter seasons (Thabethe *et al*., 2019). Common species available in Southern Africa include *Vachellia tortilis, Vachellia nilotica, Vachellia robusta, Vachellia nigrescens and Vachellia xanthophloea*. In communal areas, the leguminous trees are used to supplement natural pastures with an inherently high fibre content. Leaves are used as feed source; while branches, tree trunk are used for fence and firewood (Khanyile *et al*., 2014). Forage trees play an important role in both farming systems and in livestock production. They are generally rich in protein and minerals. They are abundant in arid and semi-arid regions of Africa and Middle-East, improving growth performance of small ruminants when most feed resources depreciate in nutrient quality and quantity (Theart, 2015).

Some forage species like *Dichrostachys cinereal*, *Kochia indica*, and *Moringa oleifera* are common browse forage legumes and shrubs fed to ruminants (Khanyile *et al*., 2014; Al-masri, 2003). Forage legumes are mainly utilised by small ruminants and cattle in free-ranging

production systems across sub-Saharan Africa. Polyphenolic compounds in *Vachellia tortilis* range between 55 and 110 g/kg DM (Gxasheka *et al.*, 2015). *Vachellia tortilis* leaf meal has a crude protein content of 180 g/kg DM. The nutrient content of its leaves is affected by growth stage, soil properties and season, while polyphenolic concentration vary based on different conditions in the particular environment along with browse-herbivore selection (Nyamukanza & Scogings, 2008) (Table 2.1). Strategies have been proposed to reduce the negative effects of polyphenolic compounds in ruminants like polyvinyl pyrrolidone (PVP) and polyethylene glycol (PEG). These compounds bind to plant secondary compounds to reduce their negative effects on intake, nutrient utilisation and digestibility (Makkar, 2003; Birteeb *et al*., 2011).

Table2. 1 Chemical composition (g/kg DM) of Vachellia tortilis leaf meal

Composition	**Mean**	**Sources**
Organic matter	940.0	Abdulrazak *et al*. (2000)
Crude protein	189.0	Abdulrazak *et al*. (2000)
Dry matter	947.7	Mokoboki *et al*. (2005)
Neutral detergent fibre	494.0	Khanyile *et al*. (2014)
Acid detergent fibre	298.0	Khanyile *et al*. (2014)
Ether extracts	40.1	Khanyile *et al*. (2014)
Total phenolic	89.7	Mokoboki *et al*. (2005)
Simple phenolic	13.9	Mokoboki *et al*. (2005)
Extracted phenolic	241.0	Dube *et al*. (2001)
Extracted condensed tannins	100.0	Abdulrazak *et al*. (2000)
Total condensed tannins	77.8	Rubanza *et al*. (2005)
Protein-bound condensed tannins	37.5	Rubanza *et al*. (2005)
Condensed tannins in acid detergent fibre	16.3	Mokoboki *et al*. (2005)
Condensed tannins in neutral detergent fibre	19.8	Mokoboki *et al*. (2005)
Energy digestibility	0.62	Heuzé and Trana, (2011)

2.1.2 Sunflower plants

Sunflower plants are from genus *Helianthus* of herbaceous plants of the aster family. Sunflowers are native primarily to North and South America. The common *Helianthus annuus* is an annual herb with a rough hairy stem 1 - 4.5 m high and broad, coarsely toothed, rough leaves 7.5 – 30 cm long arranged in spirals. The disk flowers are brown, yellow, or purple, while the petal-like ray flowers are yellow. The fruit is a single-seeded achene. The common sunflower is valuable for economic and ornamental purposes. The leaves are used as fodder and the seeds contain oil and are used as food. The conventional procedure for sunflower oil extraction involves seed preparation, mechanical extraction (continuous pressing to produce oilcake with 16-24 % of oil) and final stage of solvent extraction from ground oilcakes (Baümler *et al*., 2016). Sunflower oil is considered as a renewable and domestic fuel resource, biodiesel use to reduce emission of environmental pollutants (Roy *et al*., 2016). In livestock production, sunflower plants (oil and seeds) are used to improve fatty acid composition in animal products and reducing rumen enteric emissions.

2*.1.3 Sheep digestive system and rumen physiology*

Sheep belong to a group of herbivores called ruminants. Ruminants are able to digest a large portion of nutrient contained in fibrous plants material due to their unique digestive system, which integrates a large microbial population with the animal's biological system. The digestive tract is composed of the mouth, oesophagus, stomach, small and large intestines, and anus. The stomach of ruminants greatly differs in structure and function compared to monogastric animals. Sheep like other ruminants have three additional chambers (reticulum, rumen and omasum) that feed passes through before reaching 'true' stomach (abomasum). The rumen is a very large muscular pouch, which extends within the left side of the body cavity from the diaphragm to the pelvis. The rumen has a complex environment composed of microbes, feed at various stages of digestion, gases, and rumen fluid. The microbes (bacteria, protozoa and fungi) number in the billions and are the basis of the fermentation (digestion) process in the rumen (Wallace and Charles, 2013).

Ruminants utilise a wide range of plant feedstuff due to a diverse consortium of microbes in the rumen. Mixed ruminal microbes enable ruminants to utilise poor-quality roughages with high cellulose or hemicellulose content. During microbial fermentation, the rate of microbial

degradation is affected by nutrient composition in the diet consumed. Dietary components with inherently high fibre content favours cellulolytic (acetate-producing) bacteria. Readily fermentable carbohydrates favours amylolytic (propionate-producing) bacteria. This affects production of short-chain fatty acids (SCFAs) and methane yield. The degradation of feed particles in the rumen is coupled with production of different gases i.e. ammonia (NH_3), methane (CH_4), carbon dioxide (CO_2) and hydrogen (H_2) (Moate *et al*., 1997). Gaseous emissions from the rumen are some of the anthropogenic greenhouse gases (GHG) that contribute to climate change (Bell and Eckard, 2012). Methane is a potent GHG with a higher global warming potential (ca. 21 times) than carbon dioxide (Pérez-Barbería, 2017). Climate change is a change in regional or global climatic patterns due to increasing carbon dioxide, temperature, changes in precipitation, seasonal variation and extreme weather conditions (Henry *et al*., 2018).

The changes in climatic conditions have different effects across geographical regions. The impact of climate change on livestock production depends on location-specific conditions and economic vulnerability (Henry *et al*., 2018). In livestock production, proposed mitigation strategies against enteric emissions focus on improving residual feed intake, feed efficiency, management systems (removing unproductive animals) and breeding low-methane producers (Grainger and Beauchemin, 2011; Waghorn and Hegarty, 2011). In sub-Saharan regions, small ruminants utilise forage legumes to supplement poor-quality roughages. This has led to the incorporation of different forage trees and shrubs as leaf meals based on their nutrient quality (high crude protein and low fibre content). In North America and Europe, dietary supplements like monesin, probiotics, dietary oils and direct fed microbial are incorporated in ruminant rations in intensive production systems as measures of mitigation against methane production (Eckard *et al*., 2010). These are not affordable and readily available as opposed to leaf meals from multipurpose trees and shrubs to resource-limited farmers and nomadic pastoralists in sub-Saharan regions.

The production of methane is responsible for 10 – 12 % energy loss, this is influenced by dietary components and proportion of SCFAs; a higher fibre diet would result in energy loss compared to a low fibre diet due to a prolonged rate of degradation (Knapp *et al*., 2014). Pérez-Barbería (2017) reported different methane emission factors which are used to estimating methane yield across different ruminant species. Franz (2010) reported that cattle, sheep and goats have methane yields that range between 4.4 – 7.0 % when fed at maintenance levels. Different measurement techniques have been developed to quantify enteric methane emissions

across different ruminant species (Hammond *et al*., 2016). Generally, methane predictive models are an alternative to measurement techniques to quantify methane emissions across different geographical regions. This review focuses on factors affecting enteric methane production, mitigation strategies using vegetable oils and forage legumes.

2.2 RUMEN FERMENTATION

A diverse group of bacteria, ciliate protozoa and fungi are involved in nutrient degradation and interspecies substrate transfer in the rumen. The activity of microbial species in the rumen is influenced by dietary components of the diet consumed (Zhang *et al*., 2015). Rumen microbes degrade feed polymers to simple monomers and oligomers during digestion with ruminal pH ranging from 5.6 to 6.7, and an internal temperature around 39 ^{o}C (Kolver and de Veth, 2002; Janssen, 2010). In the rumen, methanogens constitute between 2 and 4 % of the bacterial population and they utilise different substrates in methane formation with hydrogen as a primary substrate. *Methanosarcina barkeri* is a gram-positive bacterium, it uses methyl groups from methanol or acetate during methane production (Nagaraja, 2016). Methanogens belong to the genus of *Archea*, and *Euryarchoeta* family (Hook, *et al*., 2010; Nagaraja, 2016). Some of the common methanogen species are *Methanobrevibacter* species, *Methanobacterium* species and *Methanosarcina* species; they differ from other ruminal bacteria due to their metabolism and morphology (Table 2.2).

Ruminal microbes adhere to feed particles using specific and non-specific mechanism through receptors (Yang, 2017). Microbes penetrate feed particles for their desired nutrient components; whilst the degree of degradation of cell wall contents of feed particles reflects lag time of fermentation associated with different dietary components (Janssen, 2010). In addition, the composition of SCFAs produced is affected by microbial species available in the rumen, dietary components consumed and different production pathways for energy (den Besten *et al*., 2013). Therefore, to reduce methane yields of fibrous roughages their solubility can be improved through treatment with non-protein nitrogen (NPN) sources to increase digestibility and movement of digesta through the gastrointestinal tract.

Table2. 2 Different methanogens in different hosts and their characteristics in the rumen

Methanogens	**Host**	**Substrate**	**Optimum pH**	**Gram nature**
Methanobacterium formicicum	Cattle, sheep	H_2, CO_2 formate	–	Variable
Methanobacterium bryantii	Cattle	H_2, CO_2	6.9 – 7.2	Positive/variable
Methanobrevibacter ruminantium	Cattle	H_2, CO_2, formate	6.3 – 6.8	Positive
Methanobrevibacter smithii	Cattle	H_2 or formate	6.9 – 7.4	Positive
Methanomicrobium mobile	Cattle	H_2, CO_2, formate	5.9 – 7.7	Negative
Methanosarcina barkeri	Goat, cattle	H_2, CO_2, methanol	7.0	Positive

Source: Sirohi *et al*. (2010); Bodas *et al*. (2012).

2.3 FACTORS THAT AFFECT RUMEN METHANE PRODUCTION INCLUDE DRY MATTER INTAKE AND RESIDUAL FEED INTAKE, PASSAGE RATE AND RETENTION TIME, RUMINAL PH AND HYDROGEN CONCENTRATION.

Methane production is produced in the process of feed energy utilisation in the rumen. The utilisation of feed nutrient by ruminal microbes influences methane emissions of the animals. Feed efficiency depends on type of diet, nutrient quality, animals and environmental conditions. Mitigation strategies have to consider these factors to improve feed utilisation and growth performance (Shibata and Terada, 2010).

2.3.1 *Dry matter intake (DMI) and residual feed intake (RFI)*

The increase in dry matter intake (DMI) has led to a decrease in mean retention time with higher passage rate of digesta which are inversely proportional to methane yield (Janssen, 2010). Poor-quality roughages are supplemented with forage legumes to improve feed intake, digestibility and reduce methane yields. Therefore, dietary components (NDF and CP) influences feed intake, movement of particulate and liquid fraction of digesta through the gastrointestinal tract. Hammond *et al*. (2013) reported that both white clover and ryegrass fed to sheep reduced methane yield. In pastoral system, maturity of grasses affects intake and digestibility. Grass harvested early would have a high digestibility due to its low fibre content

compared to a mature grass. Most mature grass are less nutritious due to nutrients translocated to the root system, while fibre in the stalks increases to support growth. The quality of pastures declines with maturity (Gracía *et al*., 1995; Ribeiro *et al*., 2014).

Residual feed intake (RFI) is the difference between the actual and expected feed intake, as feed efficiency with variations due to maintenance requirements (Arthur and Herd, 2008). Animals with a low residual feed intake (RFI) have a good feed efficiency and low methane yields depending on quality of diets (Hegarty *et al*., 2007; Muro-Reyes *et al*., 2011). Fitzsimons *et al*. (2013) reported a correlation of residual feed intake (RFI) to methane production which is affected by the type of diet, ruminant species, and body weight. The feeding time, efficiency of rumination will vary between small and large ruminants. Large ruminants (cattle) are able to utilise large fibre particles (straws) as they are efficient in ruminating compared to small ruminants. This is possibly explained by high methane yields in large ruminants. The selective capacity of small ruminants (goats) for digestible diets with a higher crude protein, lower fibre content improves passage rate and reduces their methane yields (Dulphy *et al*., 1980; Alcaide *et al*., 2000).

2.3.2 Passage rate and retention time

The movement of particulate and liquid fraction of digesta is inversely proportional to enteric methane yield that is influenced by DMI and dietary components (Allen, 1996; Moyo and Nsahlai, 2018). The accumulation of cell wall contents in the rumen reduces digestibility and passage rates. The prolonged period of fibre degradation in the rumen increases methane yield as a result of cellulolytic (acetate-producing) bacteria. The consumption of straws increases rumen loads in large ruminants, due to a high content of lignin, low nitrogen content, this negatively affects digestibility and passage rate (Moyo and Nsahlai, 2018). Provision of diets with more digestible nutrients reduces mean retention time and lowers methane yields (Goopy *et al*., 2014; Hammond *et al*., 2014). The quality of roughages can be improved by treating them with NPN (urea), this increases their nitrogen content and solubility of cell wall contents. The physical processing of straws increases surface area for microbial degradation. The high nitrogen content and small particle size increases the proliferation of microbes, digestibility and passage rate eventually reducing methane yields.

2.3.3 Rumen pH

Stone (2004) reported that nutrient composition affect rumen pH and microbial species in the rumen. Ruminal acidosis (sub-acute) results when ruminants consume highly soluble feeds (starch or high energy) at high intake levels, resulting in a sudden drop in ruminal pH. The high concentration of acid in the rumen damages the ruminal lining (Garrett *et al*., 1999; Mentschel *et al*., 2001; AlZahal *et al*., 2008). It is important to provide diets which stimulate saliva production containing bicarbonate for buffering. Buffering (saliva production) helps neutralize high acid production to avoid low rumen pH (Dijkstra *et al*., 2012). In addition, competitive methanogens are able to lower their growth rates with changing ruminal pH and maintain relative growth rates to available H_2 concentrations (Janssen, 2010; Wenner *et al*., 2017). Therefore, feeding diets with a nutrient balance maintains a ruminal pH (~ 6.4) to allow proper microbial digestion and reduce methane production.

2.3.4 The concentration of hydrogen in the rumen

The interaction between rumen microbes is important for hydrogen transfer and methane production (Bodas *et al*., 2012). Ciliate protozoa and fungi interact with methanogens providing substrate (H_2) for methanogenesis. The accumulation of hydrogen in the rumen is detrimental for proper fermentation and NADH-linked hydrogenase that is sensitive to increasing hydrogen (H_2) concentrations. In addition, methanogens making up a small fraction of microbial biomass, play an important role in reducing excess hydrogen molecules in the rumen (Janssen and Kirs, 2008). Methanogenesis is affected by the rate and amount of hydrogen passing through dissolved pool of total hydrogen concentrations (Johnson *et al*., 1972; Wang *et al*., 2014). Therefore, removal of ciliate protozoa is beneficial to reducing methanogenesis and growth rate of methanogens through the addition of vegetable oils in ruminant diets (Grainger and Beauchemin, 2011). Stoichiometric carbon-hydrogen balance equations have been used to display substrate utilised in methanogenesis (Baldwin *et al*., 1970) (Table 2.3). Wolin (1960) reported that the amount of substrate and molar proportion of SFCA (acetate, propionate and butyrate) affect the proportion of fermentative gases produced (CO_2 and CH_4).

$$C_6H_{12}O_6 \rightarrow 2CH_3COOH + 2\ CO_2 + 8H$$

$$C_6H_{12}O_6 \rightarrow C_4H_8O_2 + 2\ CO_2 + 4H$$

$$C_6H_{12}O_6 + 4H \rightarrow 2CH_3CH_2CO$$

$$CO_2 + 8H \rightarrow CH_4 + 2H_2O$$

NET: 3Glucose → 2 Acetate + Butyrate + 2 Propionate + $3CO_2 + CH_4 + 2\ H_2O$

Table2. 3 Different substrates utilised by methanogens for methanogenesis

Substrates	**Reactions performed by methanogens**
Hydrogen	$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$
Acetate	$CH_3COOH \rightarrow CH_4 + CO_2$
Formate	$4HCOOH \rightarrow CH_4 + 3CO_2 + 2H_2O$
Methanol	$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$
Carbon monoxide	$4CO + 2H_2O \rightarrow CH_4 + 3H_2CO_3$

Source: Choubey *et al*. (2015)

2.4 THE TECHNIQUES USED TO MEASURE AND PREDICTIVE MODELS FOR METHANE PRODUCTION

Methanogenesis is an important part of energy metabolism in animals and measuring emissions is important in understanding livestock productivity along with energy utilisation. In order to quantify rumen methane emissions, data can be combined relating to the rumen microbiome, metabolic processes, and digestion to understand the efficiency of livestock systems. The reduction in greenhouse gas emissions through methane (CH_4) mitigation strategies and more efficient production systems through assessment techniques have influenced anthropogenic climate change. The measurement of methane emitted by animals forms the basis for research on ruminant energetics and impact of livestock on greenhouse gas emissions (Hill *et al*., 2016).

2.4.1 Respiration chamber and ventilated hood system

A closed-circuit chamber is a sealed system that measures changes in air composition, it uses an air conditioner to control humidity and temperature. Water, feed bins, faecal and urine collectors are placed inside the chamber (Hellwing *et al*., 2012). The system is completely sealed to avoid negative pressure from external air and leaks that can affect gas measurement. Each run ranges between 30 and 100 minutes depending on the size and physiological state of the animal (Storm *et al*., 2012). Methane concentration is measured by airflow, the difference between inhaled and expelled air (Pinares-Patiño *et al*., 2011). A desiccant is used to remove

all moisture from air samples before being passed through the infrared and paramagnetic analyser (Pinares-Patiño *et al*., 2011). The chamber is opened twice daily during feeding or milking and cleaning (Histrov *et al*., 2017). The chamber differs from the ventilated hood system which does not measure whole animal emissions.

The ventilated hood system measures emissions emitted through the mouth and nostrils without considering hindgut emissions which may affect its accuracy. The ventilated hood system has metabolic cage with an airtight head box (Bhatta *et al*., 2007). The head box has automatic water system, with water metre to measure amount of water provided. Dimensions of metabolic cage, head box are 280 × 150 cm and 80 × 105 × 173 cm, respectively. The ventilated hood system has a flow metre to measure outflow rate under normal conditions. The air filters and dryers are used to remove dust and moisture. This system has gas analysers that are used to measure concentrations of carbon dioxide and methane (Tomkins *et al*., 2011). The disadvantage is that both measuring techniques are expensive and not easily applicable in developing countries. These techniques are not easily applicable in developing countries, since they are expensive and labour intensive.

2.4.2 In-vitro gas production technique and sulphur hexafluoride tracer gas

In-vitro gas production technique is used to estimate fermentation characteristics of ruminant feedstuff using rumen inoculum (Theart, 2015). This technique provides useful data on digestion mechanism for both soluble and insoluble fractions of feedstuffs (Tilley and Terry, 1963; Theart, 2015). It measures total gas produced and nutrient degradation (McCrabb, and Hunter, 1999). Different types of gases produced and SCFAs are analysed using gas chromatography. The composition of gases analysed differ based on the rate of fermentation and dietary components. This technique allows for SCFAs sample analysis at different time intervals. In addition, the total gas produced can be collected (through a desiccant) and passed through sodium hydroxide to remove carbon dioxide, thus purifying the gas for methane emission to be estimated. The *in vitro* gas technique is disadvantageous due to the collection of rumen fluid that may negatively affect the diversity and interactions of ruminal microbes.

Sulphur-hexafluoride (SF_6) tracer gas technique involves measuring the concentration of rumen gases, this is possible with a known rate of gas released from the rumen. It uses a small tube filled with $SF_{6,}$ which is an inert, non-toxic gas (Bhatta *et al*., 2007). The gases produced

in the rumen and SF_6 are analysed (Bhatta, *et al*., 2007). Failure and poor sampling can result from blockage of the tube due to feed particles, saliva or mucus (Berndt *et al*., 2014). It is a mobile technique that allows the animal to roam freely on the farm. It also eliminates the process of directly sampling through the throat or rumen. It is disadvantageous due to SF_6 is also a greenhouse gas, with a high global warming potential than carbon dioxide (Machmüller and Hegarty, 2006). It requires training or adapting the animal to the collar and canister (Boadi *et al*., 2002). This measurement techniques are applied by research institutions in developing countries. Predictive models are recommended for resource limited institutions to determine ruminant methane emissions based on different feedstuffs.

2.4.3 Methane predictive models

Tables 2.3 and 2.4 show the variation in average methane production using different prediction equations from high-forage and high-grain data sets, respectively. The average methane production was higher in high-forage (153.04 g/day) than for high-grain (143.47 g/day) diets. High methane production (g/day) in high-forage diet is a result of high fibre content which favours methanogenesis through low digestibility and acetate-producing bacteria. The variations among ruminant species are possibly be due to difference in feeding behaviour and feed efficiency. Prediction models are important in developing countries with limited access to methane measuring equipment or facilities. They also allow for determination of methane emissions from different feedstuffs and wide range of geographical regions.

Table2. 4 Evaluation of methane prediction equations using high forage data set

Prediction equations	**Average CH_4 (g/day)**	**Means bias (g/day)**	***P*-value mean bias**	**Linear bias (g/day)**	***P*-value linear bias**
IPCC (2006) Tier 2	163.5	- 4.42	ns	0.01	ns
Moraes *et al*. (2014) S-AL	156.6	13.02	**	0.19	ns
Moraes *et al*. (2014) SIM S-AL	154.1	6.30	ns	0.20	ns
Moraes *et al*. (2014) S-GEL	153.0	16.60	**	0.25	*
Ellis *et al*. (2009) N	138.0	11.64	**	0.27	**

Escobar-bahamondes *et al*. (2016); N - ordinal number assigned to each equation (Supplementary Table S2); S-steers; AL- animal level; SIM S-AL- calculated gross energy for animal level equations; GEL-gross energy level, ns- not significant ($P > 0.05$), * significant ($P < 0.05$); ** high significant ($P < 0.01$).

Table2. 5 Evaluation of methane prediction equations using high-grain data set

Prediction equations	**Average CH_4 (g/day)**	**Means bias (g/day)**	***P*-value mean bias**	**Linear bias (g/day)**	***P*-value linear bias**
Ellis *et al*. (2009) I	140.4	9.59	ns	-0.06	ns
Ricci *et al*. (2013) GEI	158.8	-12.11	ns	0.65	ns
Moraes *et al*. (2014) S-GEI/S-DL	165.8	-13.82	ns	0.32	ns
Moraes *et al*. (2014) S-AL	136.0	16.01	ns	0.44	ns
Moraes *et al*. (2014) S-SIM GEL	161.9	-11.27	ns	1.02	**
IPCC (2006) Tier 2	97.9	50.38	***	-0.70	**

Escobar-bahamondes *et al*. (2016) N - ordinal number assigned to each equation (Supplementary Table S2); GEI - gross energy intake; S - steers; GEL - gross energy level; DL -dietary level, AL - animal level, ns- not significant ($P > 0.05$), * significant ($P < 0.05$); ** high significant ($P < 0.01$), *** extremely significant ($P < 0.001$).

2.5 THE EFFECT OF SUNFLOWER OIL ON RUMEN FERMENTATION AND METHANE PRODUCTION

Dietary lipids are hydrolysed by rumen microbial enzymes through lipolysis (Dehority, 2003). *Anaerovibrio zipolytica* and *Butyrivibrio fibrisolvens* are strains of bacteria that together with protozoa produce enzymes involved in lipolysis of esters. During lipolysis, non-esterified fatty acids undergo biohydrogenation that utilises available hydrogen molecules in the rumen (Buccioni *et al*., 2012). Hence, the inclusion of vegetable oils with a high composition of unsaturated fatty acids (linoleic acid) are hydrolysed to reduce methane production. In addition, long-chain fatty acids in vegetable oils negatively affect both gram-positive and gram-negative bacteria in the rumen involved in fibre degradation (Fievez *et al*., 2010). In the rumen, fatty acids impair transport of essential nutrients through bacterial cell membrane. The effect of unsaturated fatty acids on rumen microbes is inconsistent with different species of microbes in the rumen reacting differently depending on the diet fed to ruminant species.

A group of gram-negative bacteria (*Anaerovibrio* species, *Selenomonas* species, *Peptostreptococcus* species and *Bacteroides* species) were not affected by presence of fatty acids (Fievez *et al*., 2010). The influence of vegetable oils on rumen methane production is affected by the source, fatty acid length, inclusion level and type of diet (Beauchemin *et al*., 2008; Benchaar *et al*., 2008). Plant oils are effective against methane production through defaunation (removal of ciliate protozoa), which would reduce growth of methanogens and

concentration of hydrogen in the rumen. Oil-rich feedstuffs such as coconut oil and whole crushed oilseeds (sunflower seeds, rapeseed and linseed) have proven effective in reducing protozoa count and methane production (Girón *et al*., 2016). Vasta *et al*. (2007) reported that polyphenolic compounds lowered intramuscular fat when lambs consumed tannin-rich diets and lipid combinations. Polyphenolic compounds negatively affect microbes involved in biohydrogenation.

The interaction of PSM with fats affects microbial biohydrogenation of polyunsaturated fatty acids (PUFA), thereby improving fatty acid (FA) composition in ruminant products (Shingfield *et al*., 2008; Carreño *et al*., 2015). Polyphenolic compounds have rumen modulating properties that allows them to modify the last step of biohydrogenation (Toral *et al*., 2013). Therefore, combination of condensed tannin with fats would be effective against methane production due to their antimicrobial properties. Most research on combination of condensed tannin and vegetable oils is focused on improving fatty acid profile of animal products. Dey *et al*. (2018) reported that sesame and mustard oil linearly decreased methane production, NDF digestibility and microbial biomass production.

2.6 THE EFFECT OF *VACHELLIA TORTILIS* LEAF MEAL ON FERMENTATION AND METHANE PRODUCTION

Forage legumes are known for their astringency due to the presence of plant secondary metabolites. Dormans and Deans (2000) reported that plant secondary metabolites (PSM) in forage legumes are an essential survival mechanism against herbivory. The astringency of forage legume leaves deters herbivores due to their bitter taste which reduces the palatability of forage legume and tree leaves. The antimicrobial activity of plant secondary metabolites is that they are micro-static, they intrude into the microbial cell membranes resulting in ion leakage and poor microbial growth (Bodas *et al*., 2012). Common PSM identified are polyphenolic compounds (hydrolysable and condensed) (Bhatta *et al*., 2009). Polyphenolic compounds affect nutrient utilisation in the rumen through formation of tannin-protein or fibre complexes. This is beneficial since nutrients are bypassed to the small intestines with a pH that allows dissociation of complexes and assimilation of nutrients (Mezzomo *et al*., 2011). Patra and Saxena (2009) reported that effectiveness of PSM against methanogenesis depends on

rumen pH, diet, method of preparation and extraction. The rumen pH (5.0 – 7.0) allows formation of tannin complexes with different dietary components.

In high crude protein diets, polyphenolic compounds improve nutrient degradation; tannin rich feeds depressed the rate of gas production and prolonged the lag time preceding the onset of gas production from neutral detergent fibre (NDF) (Nsahlai *et al*., 1995). Based on tannin molecular weight, polyphenolic compounds negatively affected microbes in the rumen and methane production. McAllister *et al*. (2005) reported that low molecular weight polyphenolic compounds had inhibitory effects on microbes. Saminathan *et al*. (2015) reported that different molecular weights of polyphenolic compounds from *Leucaena leucocephala* were effective against *in vitro* gas production, with polyphenolic compounds fractions of high molecular weight decreasing methane production. The effect of condensed tannin on rumen microbes and gas production is also dependent on their molecular weight. It is noteworthy to identify and classify polyphenolic compounds according to their molecular weight across different forage species. Essential oils are also a group of PSMs that are effective against methane production. Saponins are common essential oils that reduce rumen CH_4 production by negatively affecting ciliate protozoa. They form irreversible complexes and damage cell membrane (cholesterol) leading to the destruction of ciliate protozoa (Francis *et al*., 2002).

Frutos *et al*. (2004) reported that inclusion of forage legumes reduced ammonia production, through tannin-protein complexes which helped to recover amino acids nitrogen (N) for assimilation in the small intestines. The reduction in ammonia concentration is due to lower degradation of feed proteins and inhibit ammonia-producing bacteria (Alexander *et al*., 2008). Tannin-protein complexes helps to partition N compounds from urine to faeces, with less urinary N excreted as faecal N in organic form to avoid eutrophication (Mlambo and Mnisi, 2019). The loss of N through urinary N is leached and responsible for nitrous oxide (N_2O) emissions (de Klein and Ledgard, 2005). The constant exposure of rumen microbes to PSM would eventually lead to rumen microbes adapting to these compounds, developing a tolerance-mechanisms, dissociation of tannin-substrates complexes and modifications to form new resistant microbial strains (Smith *et al*., 2005).

2.7 LIVESTOCK METHANE EMISSION ACROSS DIFFERENT GEOGRAPHICAL REGIONS

2.7.1 Global greenhouse gas (GHG) emissions

Global GHG emissions vary across regions based on different factors like livestock numbers, species, production systems and type of feed (Bouwman *et al*., 2013). Table 2.6 provide data on global methane emissions based on global livestock distribution across different geographical regions. Most arid regions (Oceania (OCE), sub-Saharan Africa (SSA) and Middle East-North Africa (MNA)) are dominated by grazing systems, with poor productivity, limited resources as most livestock held in small-holder production are for socio-economic needs. In the arid to temperate regions, feed biomass varies due to different environmental conditions. Herrero *et al*. (2013) stated that approximately 4.7 billion tons of feed was consumed by livestock in the year 2000, with roughage consumption contributing 48 % and grains 28 % of biomass used. In developed countries (Europe and Russia (EUR), North America (NAM), Latin America and the Caribbean (LAMC) and Eastern Asian (EAS)), humid and temperate regions have a smaller number of animals coupled with more resources and good quality forages which improve performance and low methane yields. Whereas in arid and semi-arid regions across developing countries, are dominated by poor-quality roughages (grasses and stovers) that results in high enteric emissions (Blümmel *et al*., 2003). Global non-CO_2 GHG emissions from livestock production were recorded at 2.45 Gt CO_2-eq in 2000, with enteric emissions being the major source of GHG emissions. Methane produced from livestock rumen was calculated at 1.60 Gt CO_2-eq (Herrero *et al*., 2013). Developing countries contribute higher emissions due to pastoral production systems (low CP, high NDF and lignin content) relative to developed countries feeding high quality diets in intensive systems (FAO, 2009).

Table2. 6 Comparison of global livestock (per million heads) methane emissions (1900-2000)

Geographical regions	**Cattle**	**Pigs**	**Sheep**	**Global methane production (Mt CO_2 -eq)**
EUR	100	180	180	350
NAM	105	80	10	200
OCE	30	5	190	110
SEA	450	540	550	80
EA	450	540	550	200
LAMC	350	90	110	410
SSA	220	20	490	330
MNA	220	20	490	95

Source: Herrero *et al*. (2013); Bouwman *et al*. (2013): EUR (Europe, Russia), NA (North America), OCA (Oceania), SEA (Southeast Asia), EA (Eastern Asia), LAMC (Latin America- Caribbean), SSA (South Saharan Africa) & MNA (Middle East-North Africa), Mt – metric tons, eq-equivalent.

2.7.2 Agricultural production of greenhouse gases (GHG)

2.7.2.1 Nitrous oxide and ammonia emissions from livestock production

Nitrogen gas (NO_2) and ammonia (NH_4) are common greenhouse gases produced from livestock production (Grainger *et al*., 2009; Snyder *et al*., 2014). Agricultural production contributes to GHG emissions due to livestock emissions (CH_4 & N_2O), high application of urea fertilizer and poor manure management (Dudley, 2012; Erisman *et al*., 2008). Agricultural practices account for 90 % of nitrous oxide emitted (Snyder *et al*., 2014). Fertilization in crop production is responsible for nitrous oxide emissions, which vary with type of crops and tillage systems. Crop rotation of legumes and grains is an effective cropping system to reduce fertilization also improve nitrogen fixation in the soil. Composting can also reduce the production of GHG from manure and mixing manure with other waste material can help reduce methane formation by altering nutrient composition (Hristov *et al*., 2013). Manure management is a mitigation strategy against greenhouse gaseous emissions. Management of

manure involves processing manure to slurry, composting with straws, application of manure to crops and solid composting (Arogo *et al*., 2006; Rigolot *et al*., 2010).

Manure management in South Africa contributes the most to GHG emissions, responsible for CH_4 and N_2O emissions (Moeletsi and Tongwane, 2015). In South Africa, methane and nitrous oxide emissions are 3104 Gg and 2272 Gg, respectively; based on CO_2 global warming equivalent (Moeletsi and Tongwane, 2015). IPCC (2006) highlights that methane emissions are higher in intensive production systems (confined), due to poor manure management systems. The manure management methods determine the type of GHG emitted based on temperature, moisture and pH (EPA, 1999; Dalal *et al*., 2003; IPCC, 2006). Table 2.7 shows different livestock and their GHG emissions. Moeletsi and Tongwane (2015) reported that dairy and beef cattle had high rumen methane emissions due to higher emission factor compared to other livestock. The comparison of methane emissions from 1990, 2000, and 2004 showed an increase in CH_4 emissions from poor manure management at 83.41, 90.65 and 134.97 (Gg), respectively. Management of manure in liquid form results in higher emissions of methane compared to solid-form with less N-compound volatilisation (Van der Merwe and Scholes, 1998). Compositing treatment is the most effective measure to reduce manure GHG emissions, with result discrepancies between two research groups (Rigolot *et al*., 2010) and (Van der Merwe and Scholes, 1998) that are attributed to animal species.

Table2. 7 Different livestock species and their GHG emissions (CH_4 and N_2O)

Livestock category	CH_4 Emissions (Gg)	N_2O Emissions (Gg)
Dairy cattle	32.01	0.53
Beef cattle	15.47	1.80
Communal cattle	19.50	1.69
Sheep	7.74	0.56
Goats	2.71	0.28
Pigs	54.50	0.53
Poultry	1.84	1.72

Source. Moeletsi and Tongwane, (2015); IPCC (2006), Gg- gigagram.

2.8 FUTURE PROSPECTS IN MITIGATING ENTERIC METHANE EMISSIONS

2.8.1 Breeding for lower enteric emissions

De Haas *et al*. (2011) reported that genetic improvement in reducing enteric emissions should focus on selecting feed efficient animals, establish phenotypic and genotypic variations of methane output. It is a cost-effective technology that results in permanent and progressive changes on animal performance. Herd *et al*. (2002) reported that selection for low residual feed intake in beef cattle will lead to low enteric emissions as a consequence of daily feed intake. The challenge with genetic selection currently is direct measurements of enteric emissions in practical conditions. The focus is achieving direct or indirect measurements from normal conditions favouring genetic selection (Wall *et al*., 2010). Most advances have been made in genetic selection for feed efficient animals (residual feed intake) and predicting methane emissions using Intergovernmental Panel on Climate Change (IPCC) Tier 2.

2.8.2 Propionate-forming bacteria

Propionibacterium species and *Lactobacillus* species are effective in reducing acid accumulation or rumen acidosis in concentrate-fed animals. They have been exploited in their use to increase animal productivity (Seo *et al*., 2010). Recently, *Propionibacterium jensenii* and *Lactobacillus species* are used as direct fed microbials that have the potential to reduce methane production (Jeyanathan *et al*., 2014). Direct fed microbials (DFM) are used as supplements to modify rumen activities, fermentative and digestive functions. They effectively reduce protozoa numbers, increasing butyrate or propionate production and stimulating growth of acetogenic bacteria that utilises hydrogen diverting it from methanogens (Elanthamil and Bandeswaran 2017).

2.8.3 Nitrate/nitrite-reducing bacteria

Nitrate has been identified as a hydrogen sink in the rumen. Nitrate reduction is a two-step process whereby nitrate is converted to nitrite and nitrite to ammonia formation. The negative aspect of nitrate as a hydrogen sink is the toxicity of nitrite. With the slow process of nitrite to

ammonia, nitrite accumulation poses a risk to the animal (Jeyanathan *et al*., 2014). Anderson and Rasmussen (1998) tested nitrate-reducing bacteria as DFM, nitrite-reducing bacteria are present in the rumen such as *Wolinella succinogenes* and *Selenomonas ruminantium*. The number of nitrite-reducing bacteria has to increase to be able to compete with methanogens; also reducing nitrite accumulation using nitrite-reducing bacteria as DFM has proven to be effective (Iwamoto *et al*., 2002).

2.8.4 Sulphate-reducing bacteria

Anaerobic environment favour sulphate-reducing bacteria, which compete with methanogens for similar substrate (H_2). Sulphate is not limiting in anaerobic environments and sulphate-reducing bacteria have a co-operative relationship with methanogens through interspecies H_2 transfer (Jeyanathan *et al*., 2014). Some sulphate-reducing bacteria in the rumen are *Desulfovibrio* and *Desulfotomaculum*. Cummings *et al*. (1995) reported that feeding diets with sulphate did not increase sulphate-reducing bacteria population as expected. Sulphate-reducing bacteria have been proposed for methane mitigation due to their versatility in different diets and ruminant species. Paul *et al*. (2011) reported reduced methane production when sulphate-reducing bacteria (SRB) (*Fusobacterium species*) as DFM, in high sulphate diet *in vitro* without hydrogen sulphate accumulation.

2.9 SUMMARY

With increasing global population and consumer needs, it is imperative to improve livestock productivity and reduce enteric emissions. A lot of pressure is put on the livestock sector to increase production this would require improving feed efficiency and quality of feedstuff. The supplementation of dietary treatments with forage legumes and vegetable oil may be effective against methane emissions. However, animal productivity is negatively affected with inconsistencies due to different inclusion levels, ruminant species and type of diets. Therefore, more research is required to identify optimum inclusion levels in all ruminant species and production systems without affecting feeding intake and growth performance.

CHAPTER 3

Inclusion of *Vachellia tortilis* leaf meal and sunflower oil on *in vitro* short-chain fatty acids and methane production

Abstract

This experiment assessed the effect of *Vachellia tortilis* leaf meal and sunflower oil on *in vitro* total short-chain fatty acid (SCFA) production, individual SCFAs, acetate to propionate ratio, methane and carbon dioxide emissions, and *in vitro* dry matter digestibility (IVDMD). Five dietary treatments used were the control diet (CT), *Vachellia tortilis* leaf meal diet (121.5 g/kg DM) (VT), sunflower oil diet (40.8 g/kg DM) (SFO), combination of *Vachellia tortilis* leaf meal and sunflower oil diet (63.4 g/kg + 19.5 g/kg DM) (VSFO) and maize grain - lucerne (300 g/kg + 180 g/kg DM) (ML) diet. A semi-automated, incubation system was used to incubate samples at 39 ^{o}C for 48 hours. Crude protein content was high ($P < 0.05$) in VT and VSFO diet compared to the control. Ether extract content was high ($P < 0.05$) in VSFO and SFO diet compared to the control. Maize-lucerne diet had the lowest ($P < 0.05$) dry matter and organic matter content compared to other diets including the control. The production of total SCFAs, individual SCFAs (acetate and propionate), acetate to propionate ratio and proportion of methane were similar ($P < 0.05$) across all dietary treatments. Butyrate production and proportion of carbon dioxide were highest ($P < 0.05$) at 16 hours in VSFO diet. *In vitro* dry matter digestibility (IVDMD) was high ($P < 0.05$) in dietary treatments (VT and VSFO) compared to the control. During incubation, production of propionate was the highest ($P < 0.05$) at 2 hours, while acetate production was the highest ($P < 0.05$) at 16 hours across all dietary treatments. Total SCFA production was similar ($P > 0.05$) across all dietary treatments. Inclusion of sunflower oil had a negative effect on *in vitro* digestibility. In conclusion, the inclusion of *Vachellia tortilis* leaf meal and sunflower oil did not affect total SCFA production, acetate to propionate ratio and methane yields.

Key words: Fatty acids, ether extract, methane, proanthocyanidins, sunflower oil.

3.1 Introduction

Rumen microbial fermentation allows for the utilisation of plant feedstuffs; it produces and emits some greenhouse gases (GHG) which contribute to global warming leading to climate change (Hartung and Monteny, 2000; Lassey, 2007). Moss *et al*. (2000) reported that hydrogen is a major end product of fermentation by protozoa, fungi and bacteria. Ciliate protozoa and methanogens have a symbiotic relationship through interspecies hydrogen transfer, and hydrogen is a primary substrate for methanogenesis. Therefore, a reduction of hydrogen concentration decreases the rate of methane production. Among feed additives that can reduce methane emissions are nitrates, sulphates, vegetable oils, and fish oils. Inclusion of vegetable oils in ruminant diets has been reported to reduce total SCFAs, acetate to propionate ratio and methane production (Wanapat *et al*., 2011). Vegetable oils have a high composition of unsaturated fatty acids that disrupt digestibility and microbial activity (Toral *et al*., 2009). The hydrolysis of unsaturated fatty acids in the rumen reduces hydrogen concentration and reduce ciliate protozoa numbers (Bodas *et al*., 2012). The inclusion of oils at > 5 % DM in ruminant diets negatively affect feed intake, digestibility and passage rate of digesta in the gastrointestinal tract (Szumacher-Strabel *et al*., 2004).

Acetate, propionate, and butyrate account for a large portion of SCFAs identified in rumen fluid (Lan and Yang, 2018). Diets with inherently high fibre content would increase acetate production, concentrations of hydrogen, carbon dioxide and methane yield relative to diets with a low fibre content. This is due to propionate production and methanogenesis competing for substrates (Moss *et al*., 2000). Growth rate of methanogens and availability of primary substrates affects the proportion of methane produced in the rumen. Therefore, it is important to consider alternative dietary sources that reduces hydrogen concentration, acetate to propionate ratio and eventually methane production in the rumen (Eckard *et al*., 2010). In sub-Saharan regions, ruminants consume poor-quality pastures during cold-dry seasons. These pastures in certain seasons (dry, winter seasons) have a high fibre and low crude protein content which negatively affects animal performance. The amount of poor-quality roughages fed to ruminants is responsible for a significant proportion of the estimated 18 % of global anthropogenic GHG (Thornton and Herrero, 2010).

Forage legumes have a high nutritive value due to their high crude protein contents. However, proanthocyanidins in forage legumes inhibit fibre digestion by forming complexes with structural carbohydrates and disrupt microbial activity (Patra and Saxena, 2009). The

depression in fibre digestion also reduces acetate production, total SCFA and methane yield (Blümmel *et al*., 1997; Silanikove *et al*., 2001). The supplementation of forage legumes in poor-quality roughages is an effective mitigation strategy against methane production and livestock health. The effect of sunflower oil against enteric methane is affected by type of diet (Toral *et al*., 2009). The study aimed to assess the inclusion of *Vachellia tortilis* leaf meal and sunflower oil on SCFA composition and methane yields, with the objectives of determining: (1) the effect of *Vachellia tortilis* leaf meal and sunflower oil on total SCFAs production, individual SCFAs, acetate to propionate ratio and (2) the effect of *Vachellia tortilis* leaf meal and sunflower oil on methane and carbon dioxide yields. It was hypothesised that inclusion of *Vachellia tortilis* leaf meal and sunflower oil diets would reduce SCFA production, acetate: propionate ratio and proportion of methane in sheep.

3.2 Materials and Methods

3.2.1 *Leaf meal collection and preparation*

Vachellia tortilis leaves were harvested at Makhathini Research Station, Jozini in KwaZulu-Natal province (27º 43'S and 32° 14' E), and South Africa. *Vachellia tortilis* leaves were selected based on their nutritive value (crude protein content) and presence of polyphenolic compounds (Khanyile *et al*., 2014). Harvesting of leaves occurred after the rainy season at an advanced stage of maturity. These leaves were dried separately under shade for three days and sieved to get rid of thorns, pods and twigs. Thereafter, the leaf meal was immediately sampled pending chemical analysis.

3.2.2 *Chemical composition*

The chemical composition of dietary treatments was determined at the Animal and Poultry Science laboratory of the University of KwaZulu-Natal, Pietermaritzburg campus in KwaZulu-Natal province, and South Africa. Samples were analysed for dry matter (method: 945.15), ash (method: 942.05), crude protein (method: 979.09), and crude fat (method: 920.39) according to the Association of Official Analytical Chemists (AOAC, 1990). The nitrogen content of feed samples was determined using Leco Truspec nitrogen (N) analyser (St Joseph MI, USA) and crude protein (CP) was determined by multiplying N content by 6.25 (CP = N × 6.25). Neutral

detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) were determined according to Van Soest *et al*. (1991) using Ankom fibre Analyser (ANKOM^{200}, Ankom Technology, New York, USA). Condensed tannins were analysed using the butanol-hydrochloric acid (HCl) method according to Reed *et al*. (1995).

Table 3. 1 Ingredient and chemical composition (g/kg DM) for dietary treatments with the inclusion of Vachellia tortilis leaf meal and sunflower oil

	Dietary treatments						
Ingredient composition	**CL**	**VT**	**ML**	**VSFO**	**SFO**	**RMSE**	***P*-value**
Vachellia tortilis	0.0	121.5	0.0	63.4	0.0	-	-
Sunflower oil	0.0	0.0	0.0	19.5	40.8	-	-
Maize grain	297.9	261.7	625.0	273.2	285.7	-	-
Soybean meal	212.8	186.9	0.0	195.1	204.1	-	-
Lucerne	276.6	243.0	375.0	253.7	265.3	-	-
Wheat bran	148.9	130.8	0.0	136.6	142.9	-	-
Sunflower cake	63.8	56.1	0.0	58.5	61.2	-	-
Chemical composition							
DM	894^{b}	894^{b}	885^{c}	899^{ba}	901^{a}	2.16	***
ASH	54.3^{b}	67.8^{b}	194.0^{a}	61.6^{b}	55.1^{b}	4.13	**
OM	946^{a}	932^{a}	806^{b}	938^{b}	945^{b}	4.13	**
CP	229^{c}	241^{b}	117^{d}	257^{a}	253^{ab}	6.85	***
EE	18.4^{c}	20.3^{c}	22.3^{c}	37.8^{b}	55.8^{a}	0.30	***
NDF	314^{b}	311^{b}	261^{d}	302^{c}	346^{a}	2.02	**
ADF	143^{b}	149^{b}	192^{a}	145^{b}	147^{b}	1.27	**
ADL	34^{ba}	36^{ba}	22^{b}	38^{a}	32^{ba}	0.60	*
CT (mg/kg DM)	2.6	7.1	0.0	5.6	1.4	1.69	ns

CL-control diet; VT-*Vachellia tortilis* leaf meal; ML-Maize-Lucerne; VSFO-*Vachellia tortilis* leaf meal with sunflower oil; SFO- sunflower oil; CT- condensed tannin; DM-dry matter; OM- organic matter; CP- crude protein; EE-ether extract; NDF-neutral detergent fibre; ADF-acid detergent fibre; ADL- acid detergent lignin; RMSE- root mean square error; ns - not significant ($P > 0.05$); * significant ($P < 0.05$), ** highly significant ($P < 0.01$), *** extremely significant ($P < 0.001$);

a, b, c means within each row with different superscripts differ significantly ($P < 0.05$).

3.2.3 In vitro incubation measurements

3.2.3.1 Collection of rumen fluid from donor sheep

Rumen fluid was collected before morning feeding from two cannulated Merino sheep (mean body weight: 49 ± 1.06 kg). Sheep were offered urea-treated hay (UTH) as basal diet and a concentrate mixture (480 g/kg DM) in the morning (08:50 am) to meet their nutrient requirements for maintenance. Sheep were fed the control dietary treatment without *Vachellia tortilis* leaf meal or sunflower oil for 14 days of adaptation. Rumen fluid was collected with a prewarmed flask flushed with CO_2 to maintain anaerobic condition. Rumen fluid was pooled together from both sheep, strained and filtered through four layers of cheesecloth into a prewarmed flask to maintain the temperature at 39 ºC (Theart, 2015).

3.2.3.2 Buffer preparation and incubation system

A gas production system was used for incubating samples. The system comprised of data recording system (Wave-view) connected to temperature controller (OMRON E5F2 Corporation 2007- 2018). A crison micro*pH* 2000 was used for pH readings. Approximately 300 mg of feed sample was weighed into 250 ml duran bottles. A buffer mineral solution was prepared by dissolving 19.60, 7.40, 1.14, 0.94 and 0.26 g of $NaHCO_3$, Na_2HPO4, KCI, NaCI and $MgCI_2$ $6H_2O$, respectively in 2 litres of distilled water as buffer A and 5.30 g of $CaCI_2$ $2H_2O$ dissolved in 100 ml of distilled water as buffer B. Two millilitres of buffer B was titrated to buffer A, and stirred with magnetic hotplate stirrer for 15 minutes while bubbled with carbon dioxide using a gas tube. The buffer solution was placed in an incubator to maintain a temperature of 39 ºC (Tilley and Terry, 1963). Rumen fluid was added (33 ml) into each duran bottle containing 67 ml salivary buffer continuous flushing with CO_2. A magnetic stirrer was placed into each duran bottle. Twenty Duran bottles were used; each dietary treatment was replicated four times. Two duran bottles containing rumen fluid and buffer solution were incubated as blanks. The lids of duran bottles were lubricated with vaseline and tightened. Samples and pH readings were collected at sequential time intervals of 2, 4, 16, and 48 hours. During each time interval, 10 ml of fluid sample was collected using syringes and its pH was read. The incubation was terminated after 48 hours, by refrigerating duran bottles to avoid

further fermentation. Then after cooling in the refrigerator, samples were emptied into centrifuge tubes and the residue rinsed from lids using distilled water.

3.2.3.3 Estimation of short-chain fatty acids

Samples collected from duran bottles were deposited in 10 ml centrifuge tubes for centrifuging using Beckman Coulter Centrifuge (Avanti J-26 XPI) at 6804 × *g* for 15 minutes, minimum temperature 4 ^{o}C and maximum temperature of 8 ^{o}C. The supernatant (4 ml) was transferred to 5 ml blue caped tubes containing 1 ml of 25% (w/v) meta-phosphoric acid (H_2SO_4) as internal standards, and then refrigerated prior to SCFA analysis.

3.2.3.4 Gas chromatography

Short-chain fatty acids (SCFAs) were analysed using coupled Varian 3800 gas chromatography (Varian Palo Alto, California, USA) and Varian 1200 mass spectrometry (GC-MS). The GC was equipped with an Alltech EC-WAX column of 30 m x 0.25 mm internal diameter x 0.25 µm film thickness (Alltech Associates Inc., Deerfield, Illinois, USA). Helium was used as the carrier gas at a flow rate of 1 ml min^{-1}. From each pre-treated sample, 2 µl was injected into a chromatoprobe trap prepared by cutting glass tubes equalling the size of chromatoprobe quartz microvials (length: 15 mm; inner diameter: 2 mm) and filled with 2 mg of a 50:50 mixture of Tenax TA (Alltech Associates, USA) and graphitized carbon (Carbotrap™, Supelco, USA) and closed on both ends with glass wool. The chromatoprobe traps were placed in a Varian 1079 injector by means of a chromatoprobe fitting and thermally desorbed. The temperature of the injector was 40 °C, and was held for 2 minutes with a 20:1 split ratio and then increased to 200 °C, and then held at 200 °C min^{-1} in split-less mode for thermal desorption.

Compound detection was delayed for 6 minutes. After a 3 minutes hold at 40 °C, the GC oven was ramped up to 240 °C at 10 °C min^{-1} and held there for 12 minutes. Compound identification was carried out using the NIST05 mass spectral library and by comparisons with retention times of chemical standards, as well as comparisons between calculated Kovats retention indices and those published in the literature. Clean chromatoprobe traps were run in GC-MS as controls to identify background contamination. Compounds present at higher or similar percentages in the blanks were considered as contaminants and excluded from the analysis. For

quantification of compounds, known amounts of standards of dominant compounds (acetate, propionate and butyrate) were injected into cartridges and thermally desorbed under identical conditions. The peak area of compounds in these samples were compared with those of the standards and used to calculate the total amount of compound per gram of substrate (Suinyuy *et al*., 2013).

3.2.3.5 Determination of IVDMD

The sediment from duran bottles after 48 hours and centrifuge tubes were scooped into labelled vials and dried for 48 hours in the oven at 70 ^{o}C. Thereafter, vials were removed from the oven and placed in a desiccator to cool before weighing.

$$\text{IVDMD \%} = \frac{[(\text{sample mass} - \text{undigestible residue mass}) - \text{blank mass}] \times 100}{\text{sample mass}}$$

3.2.4 Calculations and statistical analysis

The proportion of carbon dioxide and SCFAs was determined using equations by Wolin (1960) based on stoichiometric laws of chemical balance. The short-chain fatty acids (SCFAs): acetate, propionate, butyrate, along with carbon dioxide (Y), and methane (Z) were all expressed in percentages. The equations for balancing the oxidative state of this system algebraically, commencing from a hexose gives a balance of net oxidations equivalents should be equal to zero.

$$Y + Z + Ma + Mp + Mb = 0$$

The net oxidation values are Y = +2, Z = -2, Ma = 0, Mp = -1, Mb = -2. where Y- carbon dioxide, Z- methane, Ma- acetate, Mp- propionate and Mb- butyrate.

When substituted for each product into

$$2Y - 2Z - Mp - 2Mb = 0$$

Solving for Z: $Z = Y - Mp/2 - Mb$

Since methane is produced at the expense of carbon dioxide, acetate and butyrate. Butyrate in turn is formed from two moles of acetate. To calculate the proportion of carbon dioxide substituting for Z.

$$Y + Z = Ma + 2Mb$$

$$Y = Ma/2 + Mp/4 + 3\ Mb/2$$

Secondly, methane was also determined using an equation reported by Moss *et al*. (2000), the proportion of methane was calculated as follows:

$$CH_4 = 0.45C_2 – 0.275C_3 + 0.40C_4$$

C_2 – proportion of acetate, C_3- proportion of propionate and C_4- proportion of butyrate.

Effects of dietary treatments on total SCFAs produced, individual SCFAs, acetate to propionate ratio, proportion of methane and carbon dioxide, incubation time also IVDMD were analysed using the general linear model (GLM) procedure of SAS (9.4). The Student- Newman-Keuls (SNK) test was used to separate sample means that significantly differed from each other at $P < 0.05$. Statistical contrast was used to test the effect of condensed tannin (0 1 0 -1 0), ether extract with inclusion of sunflower oil (0 0 0 -1 1) and sunflower cake (1 0 -1 0 0). Where -1, 0 and 1 represent the lowest level, absence and the highest level, respectively. A regression (PROC REG) analysis was used to determine the variation in the proportion of methane produced calculated using Wolin (1960) method (as observed) and the equation by Moss *et al*. (2000).

3.3 Results

3.3.1 *Chemical composition of dietary treatments*

The analysed dry matter and organic matter content of maize-lucerne (ML) diet was lower ($P < 0.05$) due to absence of sunflower cake compared to the control. *Vachellia tortilis* leaf meal (VT) and VSFO diets had a high ($P < 0.05$) crude protein content compared to the control. Ether extract content were higher ($P < 0.05$) in VSFO and SFO diets compared to the control. Condensed tannin content ranged between 1.4 and 7.1 mg/kg DM (Table 3.1).

3.3.2 *In vitro fermentation and incubation time*

The production of total SCFAs, individual SCFAs (acetate and propionate), and A: P ratio were similar ($P > 0.05$) at 2, 4, 16 and 48 hours of sampling time across all dietary treatments. The production of butyrate differed across dietary treatments at 16 hours of incubation. The combination of *Vachellia tortilis* leaf meal and sunflower oil (VSFO) diet had the highest ($P < 0.05$) butyrate production. Individual SCFAs, total SCFAs, A: P ratio were not influenced ($P > 0.05$) by condensed tannin, ether extract or sunflower cake content (Table 3.2). The proportions of methane according to Wolin (1960) and Moss *et al*. (2000) and proportion of carbon dioxide were similar ($P > 0.05$) at 2, 4, and 48 hours of incubation across all dietary treatments. However, proportion of carbon dioxide was the highest ($P < 0.05$) at 16 hours in VSFO diet. Proportions of methane and carbon dioxide were not influenced ($P > 0.05$) by condensed tannin, ether extract or sunflower cake content across all dietary treatments (Table 3.3). Proportion of acetate was highest ($P < 0.05$) at 16 hours of incubation time, and proportion of propionate was highest ($P < 0.05$) at 2 hours of incubation time. Butyrate and total SCFA were not affected ($P > 0.05$) by incubation time. Proportion of methane according to both Moss *et al*., (2000) and Wolin (1960) showed that highest ($P < 0.05$) proportion of methane was produced from 4 to 48 hours. Production of carbon dioxide was not affected ($P > 0.05$) by incubation time (Table 3.4). The inclusion of *Vachellia tortilis* leaf meal improved ($P < 0.05$) IVDMD compared to the control diet. The productions of furfural, isovaleric acid, valeric acid, dimethylsulfone and hydrocinnamic acid were similar ($P > 0.05$) across all dietary treatments. The production of isobutyric acid was higher ($P < 0.05$) in diets containing sunflower oil. The production of furfural, isovaleric acid, isobutyric acid, valeric acid, dimethylsulfone and hydrocinnamic acid was not influenced ($P > 0.05$) by condensed tannin, ether extract or sunflower cake content across all dietary treatments (Table 3.5).

Table 3. 2 Effect of *Vachellia tortilis* leaf meal and sunflower oil on individual SCFAs, acetate to propionate ratio and total SCFAs (molar proportions) during in vitro fermentation

	Dietary treatments						*P*-value[2]		
	CL	**VT**	**ML**	**VSFO**	**SFO**	**RMSE**	***P*-value**	**CT-effect**	**EE-effect**	**SF-effect**
2 (h)										
Acetate	63.19	45.37	55.03	56.67	55.33	6.5	ns	ns	ns	ns
Propionate	25.14	32.35	28.87	27.50	27.66	3.1	ns	ns	ns	ns
Butyrate	11.67	22.28	16.09	15.83	17.01	3.5	ns	ns	ns	ns
A:P ratio	2.51	1.47	1.97	2.13	2.05	0.5	ns	ns	ns	ns
TSCFAs	273.50	61.24	107.77	233.30	97.86	60.5	ns	ns	ns	ns
4 (h)										
Acetate	65.21	64.84	60.09	54.35	63.21	9.5	ns	ns	ns	ns
Propionate	23.08	22.96	26.98	27.57	23.74	4.3	ns	ns	ns	ns
Butyrate	11.71	12.20	12.93	18.07	13.05	5.2	ns	ns	ns	ns
A:P ratio	2.83	2.83	2.23	2.22	2.67	0.7	ns	ns	ns	ns
TSCFAs	315.60	149.40	179.00	201.50	154.00	110.5	ns	ns	ns	ns
16 (h)										
Acetate	68.76	64.71	69.35	59.04	68.76	3.9	ns	ns	ns	ns
Propionate	21.47	22.12	19.87	24.03	19.45	2.4	ns	ns	ns	ns
Butyrate	9.77^{b}	13.16^{ab}	10.79^{b}	16.94^{a}	12.34^{ab}	1.5	*	ns	ns	ns
A:P ratio	3.20	2.97	3.52	2.51	3.51	0.5	ns	ns	ns	ns
TSCFAs	188.73	178.65	180.66	100.61	161.38	39.2	ns	ns	ns	ns
48 (h)										
Acetate	57.87	66.28	61.33	60.47	67.62	8.2	ns	ns	ns	ns
Propionate	25.81	21.37	24.00	23.50	24.32	3.5	ns	ns	ns	ns
Butyrate	16.30	12.35	14.66	16.03	13.06	4.9	ns	ns	ns	ns
A:P ratio	2.40	3.10	2.56	2.63	2.59	0.7	ns	ns	ns	ns
TSCFAs	111.10	138.89	170.80	101.61	149.08	53.6	ns	ns	ns	ns

CL- control diet; VT- *Vachellia tortilis* leaf meal diet; ML- maize with lucerne diet; VSFO- *Vachellia tortilis* with sunflower oil diet; SFO- sunflower oil diet; A:P ratio - acetate to propionate ratio; TSCFAs – total short-chain fatty acids; h - hours; RMSE- root mean square error; ns- not significant ($P > 0.05$); * significantly different ($P < 0.05$).

[2] Contrast probability: Condensed tannin (CT) - effect of *Vachellia tortilis* supplementation (control vs VT + VSFO); Ether extract (EE) - effect of sunflower oil supplementation (VSFO + SFO); SF-Sunflower cake (SF) - addition of sunflower cake to diet (CT vs ML).

[a, b, c] means within each row with different superscripts differ significantly ($P < 0.05$).

Table 3. 3 Effect of *Vachellia tortilis* leaf meal and sunflower oil on proportion of methane, carbon dioxide, and *in-vitro* dry matter digestibility (IVDMD) during *in vitro* fermentation

	Dietary treatments							*P*-value[2]		
	CL	**VT**	**ML**	**VSFO**	**SFO**	**RMSE**	***P*-value**	**CT-effect**	**EE-effect**	**SF-effect**
2 (h)										
CH_4(W)	31.15	25.74	28.34	29.38	29.26	2.350	ns	ns	ns	ns
CH_4(M)	26.19	20.43	23.26	24.27	24.10	2.440	ns	ns	ns	ns
CO_2	55.39	64.19	58.87	58.96	60.09	2.760	ns	ns	ns	ns
4 (h)										
CH_4(W)	32.69	32.78	29.76	29.32	32.19	3.210	ns	ns	ns	ns
CH_4(M)	27.68	27.74	24.79	24.11	27.13	3.360	ns	ns	ns	ns
CO_2	55.94	56.46	56.18	61.18	57.11	4.190	ns	ns	ns	ns
16 (h)										
CH_4(W)	33.90	33.41	35.10	31.99	35.41	1.800	ns	ns	ns	ns
CH_4(M)	28.95	28.30	30.06	26.74	30.28	1.810	ns	ns	ns	ns
CO_2	54.40^{b}	57.63^{b}	55.82^{b}	60.93^{a}	57.51^{b}	0.980	**	ns	ns	ns
48 (h)										
CH_4(W)	30.64	33.97	32.00	32.37	31.76	2.63	ns	ns	ns	ns
CH_4(M)	25.47	28.89	26.86	27.16	26.71	2.77	ns	ns	ns	ns
CO_2	59.85	57.01	58.66	60.15	56.98	4.05	ns	ns	ns	ns
IVDMD	44.17^{ab}	64.50^{a}	34.33^{b}	53.57^{ab}	37.51^{b}	12.46	**	*	ns	ns

CL-control diet; VT-*Vachellia tortilis* leaf meal diet; ML- maize with lucerne diet; VSFO- *Vachellia tortilis* leaf meal with sunflower oil diet; SFO-sunflower oil diet; W-Wolin, (1960) using stoichiometric laws; M - Moss *et al.* (2000) equation; h – hours; IVDMD – in vitro dry matter digestibility; RMSE: root mean square error; ns- not significant ($P > 0.05$); * significantly ($P < 0.05$); ** highly significant ($P < 0.01$).

[2] Contrast probability: Condensed tannin (CT) - effect of *Vachellia tortilis* supplementation (control vs VT + VSFO); Ether extract (EE) - effect of sunflower oil supplementation (VSFO + SFO); SF-Sunflower cake (SF) - addition of sunflower cake to diet (CT vs ML).

[a, b, c] means within each row with different superscripts differ significantly ($P < 0.05$).

Table 3. 4 Effect of incubation time on production of individual SCFA (acetate, propionate and butyrate), total SCFA and AP ratio, proportions of methane and carbon dioxide during in vitro fermentation.

	Incubation time (h)					
	2	**4**	**16**	**48**	**RMSE**	**P-value**
Acetate %	55.12^{b}	61.54^{ba}	66.01^{a}	61.72^{ba}	7.342	*
Propionate %	28.30^{a}	24.89^{b}	21.39^{b}	23.80^{b}	3.399	**
Butyrate %	16.58^{a}	13.59^{a}	12.61^{a}	14.48^{a}	4.051	ns
Total SCFAs	154.72^{a}	199.91^{a}	162.01^{a}	134.30^{a}	71.174	ns
AP ratio	2.00^{b}	2.56^{ba}	3.14^{a}	2.66^{ba}	0.589	**
CH_4 (M)	23.65^{b}	26.29^{a}	28.86^{a}	27.02^{a}	2.656	**
CH_4 (W)	28.77^{b}	31.35^{a}	33.96^{a}	32.15^{a}	2.549	**
CO_2	59.50^{a}	57.38^{a}	57.26^{a}	58.53^{a}	3.262	ns

W- Wolin, (1960) using stoichiometric laws; M - Moss *et al*. (2000) equation; CH4- methane; CO2- carbon dioxide; AP-acetate to propionate; h – hours; RMSE: root mean square error; ns- not significant ($P > 0.05$); * significantly ($P < 0.05$); ** highly significant ($P < 0.01$).

a, b, c means within each row with different superscripts differ significantly ($P < 0.05$).

Table 3. 5 Effect of *Vachellia tortilis* leaf meal and sunflower oil on production of furfural, iso-branched acids (isobutyric and isovaleric acid), valeric acid, dimethylsulfone and hydrocinnamic acid (mM/l) during *in vitro* fermentation

	Dietary treatments							***P*-value[2]**		
	CL	**VT**	**ML**	**VSFO**	**SFO**	**RMSE**	***P*-value**	**CT-effect**	**EE-effect**	**SF-effect**
Furfural	1.5	0.9	1.0	1.1	1.0	0.76	ns	ns	ns	ns
Isovaleric acid	12.5	7.7	9.4	9.7	16.4	10.59	ns	ns	ns	ns
Isobutyric acid	0.2^{b}	1.4^{ab}	1.1^{ab}	1.7^{a}	1.5^{ab}	0.97	*	ns	ns	ns
Valeric acid	8.0	4.4	5.2	6.0	4.9	2.99	ns	ns	ns	ns
Dimethylsulfone	0.1	0.0	0.1	0.1	0.0	0.05	ns	ns	ns	ns
Hydrocinnamic acid	4.4	2.1	2.9	4.3	2.1	3.35	ns	ns	ns	ns

CL-control diet; VT-*Vachellia tortilis* leaf meal diet; ML- maize with lucerne diet; VSFO - *Vachellia tortilis* leaf meal with sunflower oil diet; SFO - sunflower oil diet; RMSE-root mean square error; ns - not significant ($P > 0.05$); * significant ($P < 0.05$).

[2] Contrast probability: Condensed tannin (CT) - effect of *Vachellia tortilis* supplementation (control vs VT + VSFO); Ether extract (EE) - effect of sunflower oil supplementation (VSFO + SFO); SF - Sunflower cake (SF) - addition of sunflower cake to diet (CT vs ML).

[a, b, c] means within each row with different superscripts differ significantly ($P < 0.05$).

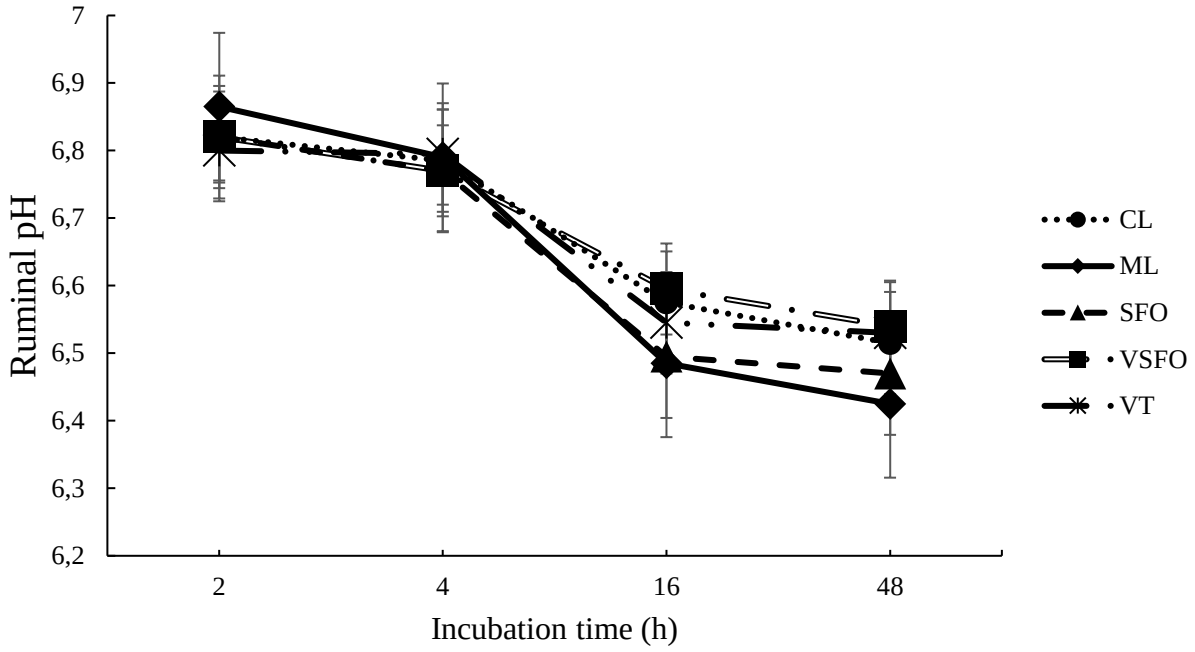

Figure 3. 1 Effect of *Vachellia tortilis* leaf meal and sunflower oil on ruminal pH during incubation

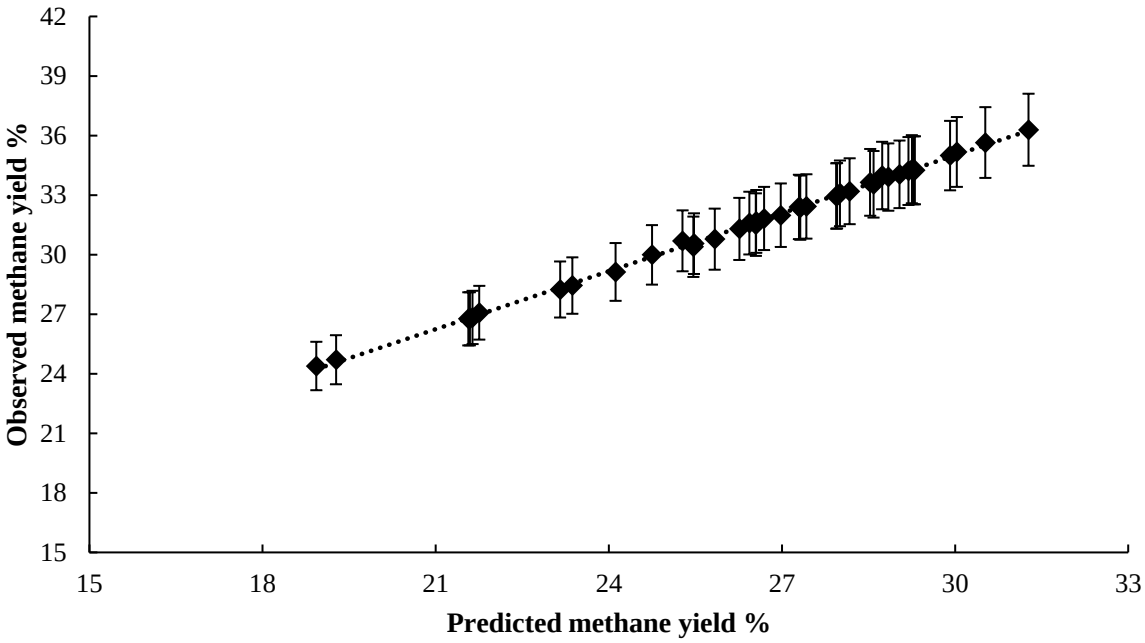

Figure 3. 2 The regression relationship between proportion of methane calculated using equations by Wolin, (1960) (Y) and Moss *et al*. (2000) (X) (molar proportions) was Y = 1.026 (± 0.006) – 5.916 (± 0.183) X (n = 40, R^2 = 0.998, RMSE = 0.11, P-value = 0.0001)

3.4 Discussion

3.4.1 Effect of sunflower oil and Vachellia tortilis leaf meal, incubation time, relationship of predicted and observed methane

The effects of vegetable oils are depended on the source, inclusion level, fatty acids composition, and animal species (Dey *et al*., 2018; Koenig *et al*., 2018). Inclusion of sunflower oil did not affect total SCFAs production. The rate of digestion is directly affected by the degradability of the substrates (Kozloski, 2011). Therefore, easily degradable nutrients were rapidly utilised, and insoluble nutrients had a prolonged period of digestion producing high proportion of acetate due to their fibre content. The inclusion of sunflower oil and high sunflower cake content increased ether extract levels negatively affecting IVDMD. The high ether extract content lead to coating of feed particles creating a physical barrier that reduces microbial degradation of digesta. Also, sunflower oil is a rich source of polyunsaturated fatty acids (PUFAs) for which its negative effects on ruminal bacteria population are known (Silva *et al*., 2019). Unsaturated fatty acids are antimicrobial because of their cytotoxic effects that could destroy the cell membrane of bacteria (Zhang *et al*., 2006). The effects of vegetable oils are predominately observed in roughage diets compared to concentrate diets; this is due to a high cellulolytic bacterium count in roughage diets that are susceptible to unsaturated fatty acids in vegetable oils (Toral *et al*., 2009).

The high amount of NDF in SFO diet allowed the proliferation of acetate-producing bacteria. It is possible that some microbial species were not affected by sunflower oil, which was evident due to the high butyrate production and proportion of carbon dioxide at 16 hours of incubation. Palmquist and Griinari (2006) reported an increase in butyrate production with a combination of sunflower oil with fish oil. *Butyrivibrio* bacteria were reported to be tolerant to high concentrations of unsaturated fatty acids (Toral *et al*., 2010). Kim *et al*. (2008) reported that fish oil supplementation had no effect on similar group of *Butyrivibrio* bacteria. The effect of lipid supplementation and response of bacteria is also dependent on the type of diets and differences in the bacteria composition that vary among ruminant species. Paillard *et al*. (2007) reported that some species (*Butyrivibrio hungatei*) were not sensitive to linoleic acid in sunflower oil compared to *Butyrivibrio* and *Pseudobutyrivibrio* species.

A combination of *Vachellia tortilis* leaf meal and sunflower oil improved IVDMD compared to the control. The inclusion of *Vachellia tortilis* leaf meal and sunflower cake increased crude protein content in VSFO diet. Hristov *et al*. (2004) reported similar results where diets with high crude protein content increased concentrations of iso-butyrate and iso-valerate. In this study, iso-butyric acid production was the highest in VSFO diet compared to other diets including the control. Our results agree with Wolin *et al*. (1997) where the increased dietary crude protein resulted to high concentration of branched-chain SCFAs in diets containing soybean meal, silage, or alfalfa. The concept of 'free tannin' states that 90 % of proanthocyanidins interact with dietary constituents (i.e. fibre and protein) allowing bypass of ruminal protein to the small intestines (McSweeney *et al*., 2001), while 10 % of free tannins in solution inactive microbial enzymes. The fact that proanthocyanidins have a high affinity for dietary components (fibre and proteins) reducing their digestibility, this may be the possible explanation proanthocyanidins did not affect the production of SCFAs. Additionally, proanthocyanidins reportedly increase butyrate production and biohydrogenation when supplemented with oils (Costa *et al*., 2018).

Pal *et al*. (2015) reported that *Vachellia tortilis* leaf meal had a high microbial biomass production that was positively correlated with polyphenolic fractions but negatively affected by NDF content. Polyphenolic compounds have antioxidant (oxygen radical scavenger) properties that may stimulate microbial growth (Alberto *et al*., 2012; Pal *et al*., 2015). The high production of butyrate in *Vachellia tortilis* leaf meal diets may be due to the bacterial population that is positively correlate to proanthocyanidins (Jayanegara *et al*., 2011). Ondiek *et al*. (2010) reported that *Vachellia tortilis* had a high degradability (70.6 – 90.7 %) relative to indigenous Kenyan browses. Furfural, valeric acid, dimethyl sulphide, and hydrocinnamic acid are produced during fermentation. Furfural is produced from hemicellulose present in lignocellulose biomass in diets. This composition affects the effective utilization of biomass due to rigid structures (Luo *et al*., 2019). The hemicellulose content of dietary treatments did not affect the production of furfural. Valeric acid is a straight chain, saturated fatty acid with a short length. It is synthesized from acetic and propionic acid; otherwise it would arise as a consequence of deamination. The production of valeric acid was similar in all dietary treatments. Dimethyl sulphide is a metabolic product of methionine. It is produced during rumen microbial protein synthesis and oxidation of methionine that occurs during conversion and storage of nutrients (Ellis *et al*., 2008). The production of hydrocinnamic acid was similar with dietary treatments. The presence of hydrocinnamic acid is directly related to *Clostridium*

sporogenes that is involved in the synthesis of amino acids (isoleucine) from propionate or α-methylbutyrate (Monticello *et al*., 1984). The high degradability of *Vachellia tortilis* leaf meal and its nutritive value (high crude protein) is beneficial for supplementation of poor-quality roughages fed to ruminants during the cold-dry season. They biohydrogenation of sunflower oil may have favoured butyrate production, butyrate is converted to D-3-hydroxybutyrate, which may be used as a source of energy by skeletal and heart muscles of the animal. Therefore, addition of sunflower oil would increase the energy density of the diets, through the production of fatty acids which are assimilated in the small intestines as energy sources. The ruminal pH tended to decrease after 4 to 16 hours in all dietary treatments, due to utilisation of readily fermentable carbohydrates producing less methane.

Incubation time influenced the production of acetate and propionate. The production of propionate was high between 2 and 4 hours of incubation, while acetate production increased between 16 and 48 hours in all dietary treatments. This was due to different nutrient components utilised during incubation; dietary treatments favoured propionate production between 2 and 4 hours producing low proportions of methane. Butyrate and total SCFAs were similar during incubation. Butyrate production is influenced by both acetate and propionate concentrations in the rumen fluid. The production of acetate and propionate displayed an inversely proportional relationship at 16 hours. Both equations Moss *et al*. (2000) and Wolin (1960) display a similar trend with proportion of methane increasing up to 16 hours, while proportion of carbon dioxide was not affected during incubation. The regression analysis of methane from both methods by Wolin (1960) as X-axis and Moss *et al*. (2000) as Y-axis displayed a linear relationship with an R^2 value of (0.998). This implies that both of these equations were precise in determining the proportion of methane based on the composition of SCFAs. The low (0.11) root mean square error (RMSE) value indicates that the predicted and observed proportions of methane produced were accurate and similar. Therefore, proportion of methane could be determined using either of the methods based on molar proportions of SCFAs.

3.5 Conclusion

The addition of *Vachellia tortilis* leaf meal and sunflower oil did not affect proportions of total SCFAs and methane yields. It is possible that some butyrate-producing bacteria were not affected by unsaturated fatty acids in sunflower oil. Based on results, the addition of *Vachellia*

tortilis leaf meal has the potential to reduce SCFAs and proportion of methane at higher inclusion levels without negatively affecting overall fermentation. Also, the effect of sunflower oil on SCFAs and proportion of methane may vary with microbial species. The high degradability of *Vachellia tortilis* leaf meal is beneficial for growth of ruminants during cold, dry seasons when feed resources are scarce. This will improve the utilisation of fibrous diets and the high digestibility improves nutrient availability for growing animals.

CHAPTER 4

The effect of *Vachellia tortilis* leaf meal and sunflower oil on the growth performance of lambs

Abstract

This experiment assessed the effect of *Vachellia tortilis* leaf meal and sunflower oil on dry matter intake (DMI), average daily gain (ADG), feed conversion ratio (FCR), apparent total tract digestibility (AD), neutral detergent fibre digestibility (NDFD), outflow rate, total mean retention time (TMRT) of particulate and liquid fraction of digesta. Five dietary treatments were formulated for growth and maintenance requirements of Merino lambs comprising of the control diet (CT), *Vachellia tortilis* leaf meal diet (121.5 g/kg DM) (VT), sunflower oil diet (40.8 g/kg DM) (SFO), combination of *Vachellia tortilis* leaf meal and sunflower oil diet (63.4 g/kg + 19.5 g/kg DM) (VSFO) and maize grain - lucerne (300 g/kg + 180 g/kg DM) (ML) diet. Dietary treatments were fed to 10 Merino lambs (mean body weight: 27.15 ± 3.0 kg). These lambs (n= 6) were allocated using an incomplete Latin square design of three periods. Sheep were offered 480 g/kg DM of concentrate daily with *ad libitum* access to urea-treated hay (UTH). The experiment lasted for 126 days with three periods each lasting 42 days. Each period had an adaption period of 14 days and 28 days of experimental measurements. For passage rate, five lambs were selected one from each dietary treatment to determine the fractional passage rate of particulate and liquid fraction of digesta through reticulorumen (RR) and hindgut (HG). Crude protein content was higher ($P < 0.05$) in VT and VSFO diets compared to the control. Dry matter intake was lower ($P < 0.05$) in maize-lucerne diet relative to the control. Urea-treated hay intake (UTHI), total dry matter intake (TDMI), average daily gain (ADG), feed conversion ratio (FCR) and NDFD were similar ($P > 0.05$) across all dietary treatments. The inclusion of sunflower oil in dietary treatments (VSFO and SFO) reduced ($P < 0.05$) apparent digestibility compared to the control. Total dry matter intake (TDMI), ADG, UTHI, FCR and NDFD were not influenced ($P > 0.05$) by condensed tannin, ether extract or sunflower cake content. Fractional passage rate of particulate and liquid fractions was similar ($P > 0.05$) across all dietary treatments. The high crude protein of dietary treatments and improvement of roughage with urea improved growth performance of lambs across all dietary treatments. In conclusion, *Vachellia tortilis* leaf meal and sunflower oil did not affect the growth performance of lambs. **Keywords**: digestibility, feed intake, ether extract, passage rate, sunflower cake, tannins, weight gain

4.1 Introduction

Generally, scarcity of nutritious feed during cold-dry seasons negatively affects productivity of free-ranging ruminants in the tropical regions (Khanyile *et al*., 2014; Theart, 2015). Therefore, improving ruminant performance has led to the utilisation of forage legumes in tropical regions to supplement poor-quality roughage diets mostly during adverse climatic conditions. Crop residues are mainly fed to ruminants during the cold-dry season due to feed shortage and high availability; however, their high lignin content reduces voluntary feed intake and consequently animal performance (Baloyi *et al*., 2008). Both voluntary dry matter intake and digestibility have been improved in small ruminants due to supplementing poor-quality roughages with leguminous forage plants, which has led to intensive research on utilisation of forage legumes (Salem *et al*., 2005). Presence of secondary plant metabolites (SPM) in forage legumes have negatively affected feed intake, due to astringency that reduces nutrient digestibility and availability to the animal. However, the inclusion of secondary plant metabolites in rations is beneficial for livestock health and reduced protein degradation (Asante *et al*., 2017; Naumann *et al*., 2018).

Crop residues and mature pastures in tropical regions during winter seasons are deficient in essential nutrients (crude protein and minerals), which causes loss in body weight in Pedi goats (Jalajakshi *et al*., 2016). Access to nutritious roughages in tropical regions is limited, leading resource-limited farmers to rely on supplementing forage legumes to improve livestock productivity (Tolera, 2007). Forage legumes would be widely used in livestock production if they were simple and inexpensive to grow, allowing for easy processing (pelleting) and availability for incorporation into ruminant rations (Kronberg *et al*., 2018). Lipids are a source of essential fatty acids that have wide variations in proportion of fatty acids (unsaturated, monounsaturated and polyunsaturated). They are modified in the rumen to produce bioactive lipids beneficial to human health (Mlambo and Mnisi, 2019). Sunflower oil contains both linoleic acid and oleic acid at 68 % and 19 %, respectively (Wanapat *et al*., 2012).

Therefore, addition of sunflower oil in ruminant diets has the potential to reduce methanogenesis through biohydrogenation of unsaturated fatty acids as hydrogen sinks (Patra, 2013; Szczechowiak *et al*., 2018). The combination of proanthocyanidins and vegetable oils modulates biohydrogenation intermediates, increases fatty acid composition in the rumen and improves the quality of metabolizable protein ultimately increasing weight gain of ruminants (Shingfield *et al*., 2008; Kamel *et al*., 2019). The objectives of this study were to determine:

(1) the effect of *Vachellia tortilis* leaf meal and sunflower oil on growth performance of Merino lambs and (2) the effect of *Vachellia tortilis* leaf meal and sunflower oil on fractional passage rate kinetics. It was hypothesised that the inclusion of *Vachellia tortilis* leaf meal and sunflower oil improves growth performance and fractional passage rate kinetics in lamb.

4.2 Materials and Methods

4.2.1 Study Site

The experiment was conducted at Ukulinga research farm, University of KwaZulu-Natal, Pietermaritzburg, South Africa. The area is a subtropical hinterland at 29º24'E and 30º24'S with elevation of 775 m above sea level. The characteristic vegetation involves various tree and grass species that include *Vachellia karroo* (Sweet thorn), *Vachellia nilotica* (thorn mimosa), *Vachellia sieberiana (*Paperbark thorn), *Themedia trianda* (Kangaroo grass) and *Heteropogon contortus* (Black-spear grass). The climate is characterised by mean annual maximum and minimum temperature of 25.7 ºC and 8.9 ºC, respectively. During summer season there is a mean annual rainfall of 735 mm, most of which occur between October and April, whilst light to moderate frost occurs in winter.

4.2.2 Experimental design and diets

The study was conducted in an incomplete Latin square design using 10 Merino lambs (mean initial weight: 27.15 ± 3.0 kg), which were randomly grouped into five pairs and allocated to five treatment rations based on their initial body weights. The experiment lasted for 126 days with three periods each lasting for 42 days. Each period had an adaptation of 14 days and 28 days of experimental measurements. Five concentrate rations were the control diet (CT), *Vachellia tortilis* leaf meal diet (VT) (121.5 g/kg DM), sunflower oil (SFO) (40.8 g/kg DM) diet, combination of *Vachellia tortilis* leaf meal and sunflower oil (VSFO) (63.4 g/kg + 19.5 g/kg DM) diet and maize grain - lucerne (300 g/kg + 180 g/kg DM) (ML) diet, with *ad libitum* access to urea-treated hay (*Themeda trianda*) and water. The concentrate portion of the diet (480 g/kg DM) was offered once daily between 08:20 and 08:40 am. Lambs were housed in individual pens (dimensions: 70 cm wide, 150cm long, and 90cm high) with slatted wooden floors, installed in a well-ventilated shed and roofed to protect animals against sun and rain.

Prior to the experiment, animals were treated with Prodose® Orange Oral suspension (Virbac Endoparasiticides) against liverfluke, roundworm and Nasalworm. The experimental protocol was specifically approved with the compliance of UKZN Animal Ethics Committee (REF: AREC/057/017M) on research in animals following international principles for animal use and care.

4.2.3 Chemical composition

Mineral contents were determined using Inductively Coupled Plasma emission spectroscopy (ICP-OES, Varian 720, Frankfurt, Germany). Analysis of all other chemical compositions followed procedures described in chapter 3. Ingredient and chemical compositions are similar to those described in chapter 3. Urea-treated hay (UTH) had a dry matter content of (815 ±108.09), ASH content (82 ±4.16), organic matter content (76 ±6.56), crude protein content (85 ±8.37), neutral detergent fibre content (734 ±13), acid detergent fibre content (488 ±124.72) and hemicellulose content (335 ±5.57).

Table 4. 1 Mineral composition of dietary treatments

	Dietary treatments						
	CL	**VT**	**ML**	**VSFO**	**SFO**	**RMSE**	***P*-value**
Macro-minerals (g/kg DM)							
Calcium	48.3	51.3	25.7	64.7	42.2	10.09	ns
Phosphorus	41.3^{a}	43.3^{a}	5.8^{b}	43.0^{a}	42.0^{a}	3.26	***
Sodium	16.1^{a}	15.3^{a}	0.2^{b}	17.1^{a}	16.1^{a}	1.37	***
Potassium	99.4^{a}	103.7^{a}	33.9^{b}	104.5^{a}	103.3^{a}	6.98	***
Magnesium	26.2^{a}	27.4^{a}	3.3^{b}	27.8^{a}	26.2^{a}	0.99	***
Micro-minerals (mg/kg DM)							
Copper	0.50	0.49	0.28	0.44	0.47	0.08	ns
Iron	2.34^{b}	2.31^{b}	8.0^{a}	2.67^{b}	2.16^{b}	0.42	***
Manganese	0.43^{b}	0.44^{b}	0.71^{a}	0.45^{b}	0.41^{b}	0.03	***
Zinc	0.50	0.52	0.64	0.48	0.47	0.05	ns

CL – control diet; VT- *Vachellia tortilis* leaf meal; ML- maize-lucerne; VSFO – *Vachellia tortilis* leaf meal with sunflower oil; SFO – sunflower oil; RMSE - root mean square error; ns – not significant ($P > 0.05$); *** extremely significant ($P < 0.001$)

a, b, c means within each row with different superscripts differ significantly ($P < 0.05$)

4.2.4 Feed intake, body weight gain and feed conversion ratio

Each lamb was first allowed to adapt to dietary treatments for 14 days and experimental trial 28 days in three consecutive periods. Daily feed offers, refusals and spillage were recorded throughout each experimental period. Dry matter intake (DMI) was determined as the difference between amounts of feed offered and refusal on DM basis. While average daily feed intake of hay was determined as difference of hay offered and refused divided by seven. Body weight data was measured weekly before morning feeding. Average daily gain was determined as difference between final body weight end of each week and initial body weight beginning of each week divided by seven.

$$\text{ADG} = \frac{\text{Final body weight (g)} - \text{initial body weight(g)}}{7 \text{ days}}$$

Feed conversion ratio (FCR) was calculated by dividing average daily gain (ADG) by total dry matter intake (DMI) for each animal.

4.2.5 Apparent total tract digestibility and neutral detergent fibre digestibility (NDFD)

Digestibility trial was conducted at the end of each period for 7 consecutive days after 3 days of adaptation of lambs to harness and faecal bags. The amount of daily feed offers, refused and spillage along with total wet faeces for experimental lambs were recorded. Daily faecal defecations for each lamb was mixed thoroughly, weighed and 10 % of the sample was kept in airtight plastic bags which was eventually oven dried on foil trays at 60 ^{o}C for 72 hours to determine total dry matter content of total faecal output. Apparent total tract digestibility (AD) was recorded for each animal as the difference between total dry matter intake and total dry matter of faecal output divided by the total dry matter intake

Apparent total tract digestibility

$$= \frac{\text{total dry matter intake} - \text{total faecel output}}{\text{total dry matter intake}}$$

Faecal samples were used to determine neutral detergent fibre digestibility (NDFD). Neutral detergent fibre (NDF) content of diet was multiplied by intake of diet to determine the NDF intake in each diet. Faecal NDF was a product of NDF content in faeces and total faecal output.

Neutral detergent fibre digestibility (NDFD) was calculated as difference between the NDF intake and faecal NDF divided by NDF intake of diet.
Neutral detergent fibre digestibility

$$\text{NDFD} = \frac{\text{NDF intake} - \text{Faecal NDF}}{\text{NDF intake}}$$

4.2.6 Liquid and solid passage rate measurements

Passage rate parameters were measured in lambs allocated to five concentrate diets. Five lambs were randomly selected from each of the five dietary treatments, dosed with ytterbium and cobalt-ethylenediaminetetraacetic acid (Co-EDTA) markers for solid and liquid passage parameters, respectively. Solid passage rate marker (Ytterbium mordant roughage) was prepared according to Hatfield *et al*. (1990). Ytterbium solution was prepared by dissolving 7.5 g $YbCl_3.6H_2O$ in 3 litres of distilled water. Roughage was ground to pass 12 mm screen, soaked in distilled water overnight to remove soluble material and dried overnight in the oven at 60 ^oC. Ytterbium marked roughages was prepared by soaking roughage in 2.5 g l^{-1} of $YbCl_3.6H_2O$ solution at a rate of 50 g of roughage per litre of solution for 120 hours. Residues were washed using distilled water until the colour of water turned clear to remove any unbound ytterbium. The residue was dried in an oven at a temperature of 50 ^oC for 48 hours. Ytterbium-labelled hay was stored in zip plastic bags pending administration to sheep.

Cobalt-ethylenediaminetetraacetic acid (Co-EDTA) was prepared according to Udén *et al*. (1980), which was used as a liquid marker. Sodium-ethylenediaminetetraacetic acid (Na-EDTA, 297.2 g), acyl chloride ($CoCl_2.6H_2O$) (190.4 g) and sodium hydroxide (NaOH) (32 g) were dissolved in 1600 ml distilled water in 5-litre beaker. Sodium hydroxide (NaOH, 7 g) was added to ensure that all reagents dissolved. The solution was allowed to cool at room temperature and 160 ml of hydrogen peroxide (H_2O_2) was added. The mixture was allowed to stand at room temperature for 4 hours, and 2400 ml of 95 % (v/v) ethanol was added. For crystallization, the mixture was placed in the refrigerator for approximately 120 hours. The pH of solution was maintained at 9.95. The crystals from the solution were filtered and washed 3 times using 330 ml of 80 % (v/v) ethanol for each cycle. The resulting crystals were dried in an oven at 90 ^oC for 24 hours and stored in plastic bottles pending administration.

Prior to administration, sheep were starved overnight and 20 g of ytterbium marked roughage was weighed and offered to each sheep mixed uniformly with a portion of dietary treatments for sheep to consume whole ytterbium roughage. Approximately, 120 g of cobalt-EDTA crystals were dissolved in 720 ml of water and each sheep was drenched with 60 ml of the solution containing cobalt-EDTA. Faecal sample collection was done over 7 days after administration, faecal samples were collected by rectal palpation at the following sequential time intervals 0, 3, 6, 9, 12, 24, 27, 30, 48, 54, 72, 77, 96, 102, 120, 144, and 168 hours post marker administration. Faecal samples from each sheep were dried in an oven at a temperature of 60 ^{o}C for 96 hours. Dried faecal samples were ground to pass through a 1 mm sieve using a hammer mill and stored pending analysis. Five grams (5 g) of faecal samples were weighed, placed in porcelain crucible and ashed in a furnace overnight at 550 ^{o}C. Ashed samples were allowed to cool in a desiccator, then boiled in 5 cm^3 HCI and evaporated to dryness using water bathe. The residue was cooled and 5 cm^3 of HNO_3 was added. The solution was heated to boiling using water bathe. The resulting solution was passed through filter paper into 100 cm^3 volumetric flask. The solution was diluted to the mark with deionised water and mixed well. Ytterbium and cobalt concentrations were determined using an Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) (Perkin Elmer, Precisely, Optima 5300 DV Spectrometer, Shelton, CT 06484, USA).

4.2.6.1 Digesta kinetics mathematical procedure

The ytterbium and cobalt concentrations data were used to plot a curve of concentration of each marker against time. A natural log-transformed curve of the descending slope of the marker concentration against time was plotted. Grovum and Williams (1973) and Blaxter *et al*. (1956) mathematical procedure was used as follows:

$$Y = 0 \text{ when } t < TT$$

$$Y = Ae^{-k1\,(t-TT)} - Ae^{-k2\,(t-TT)} \text{ when } t > TT$$

Where Y and A = adjusted marker concentrations in the faecal DM;

k1 and k2 = rate constants;

TT = the calculated time for first appearance of marker in the faeces; and

t = the sampling time (h).

A linear regression line of best fit was plotted between the log-transformed curve of marker concentrations plotted against time on the descending slope of the curve; the equation of the line was determined (descending slope). The regression coefficients (k_1) gave the slowest rate constant that corresponded to rate of passage in the rumen and y-intercept as A_1. Thereafter, residual marker concentrations were calculated for the ascending slope, natural log transformed and regressed on time during this phase giving the Y- intercept (A2) and the second slowest rate constant, k2. The two lines intersect at the point (TT, A) where TT is the transit time and can be calculated as:

$$TT = (A2-A1)/ (k1-k2)$$

The mean retention time in the reticulorumen (MRT_R) and hindgut (MRT_H) within each compartment was calculated as follows:

$$MRT = 1/k1 \text{ or } 1/k2$$

The total mean retention time (TMRT) was calculated as follows:

$$TMRT = (MRT_R) + (MRT_H) + TT.$$

This procedure was outlined by Osuji *et al*. (1993). The selectivity factor (SF) was calculated as $SF= MRT_{particles} / MRT_{liquid}$ (Clauss and Lechner-Doll, 2001).

4.2.7 Statistical analysis

Effects of dietary treatments on average daily gain, dry matter intake, feed conversion ratio, apparent digestibility, NDF digestibility, fractional passage rate and total mean retention time were analysed using the General Linear Model (GLM) procedure of SAS (9.4). The initial body weight was used as a covariate in the analysis. The statistical model was:

$Y_{ijk} = \mu + D_i + W_j + P_k + (D \times W)_{ij} + \varepsilon_{ijk}$

Where Y_{ijk} is the response variables: DMI, ADG, FCR, DMD, NDFD and passage rate (k-values, MRT and TMRT), μ is overall mean; D_i - effect of diet ($i=5$), W_j - effect of weeks ($j=4$); P_k - effect of period ($k=3$); $(D \times W)_{ij}$ – interaction of diet and weeks; ε_{ijk} – error due to random effects. Statistical contrasts were used to test the effect of condensed tannin (0 1 0 -1 0), ether extract with sunflower oil supplementation (0 0 0 -1 1) and sunflower cake (1 0 -1 0 0), where -1, 0 and 1 represent the lowest level, absence and the highest level, respectively.

The Student- Newman-Keuls (SNK) test was used to separate sample means that significantly differed from each other at $P < 0.05$.

4.3 Results

4.3.1 Chemical composition

Macro-mineral composition was lower ($P < 0.05$) in maize-lucerne diet; although, iron (Fe) and manganese (Mn) were highest in maize-lucerne diet across dietary treatments (Table 4.1).

4.3.2 Growth performance

Maize-lucerne (ML) diet had the lowest ($P < 0.05$) dry matter intake across all dietary treatments. Urea-treated hay (UTH) intake, TDMI, ADG, FCR and NDFD were similar ($P > 0.05$) across all dietary treatments. Sunflower oil (SFO) had the lowest ($P < 0.05$) apparent DM digestibility compared to the control. This was due to the influence ($P < 0.05$) of ether extract content. Urea-treated hay (UTH) intake, TDMI, ADG and FCR were not influenced ($P > 0.05$) by condensed tannin, ether extract or sunflower cake effect across all dietary treatments (Table 4.3). Urea-treated hay (UTH) intake and TDMI improved ($P < 0.05$) from week 2 to 4 in all dietary treatments (Figures 4.1 and 4.2). Average daily gain (AGD) in lambs improved ($P < 0.05$) from week 1 to 4 in all dietary treatments (Figure 4.3). Feed conversion ratio (FCR) improved ($P < 0.05$) from week 1 to 4 in all dietary treatments (Figure 4.4).

4.3.3 Fractional passage rate kinetics

The rate of particulate (k_p) and liquid (k_l) passage rate in the reticulorumen (RR) and hindgut (HG) were similar ($P > 0.05$) in all dietary treatments. The disappearance of particulate in the RR and HG ranged between 4 and 6 % h^{-1} and 5 and 7 % h^{-1} across diets, respectively. Liquid outflow rate in RR and HG ranged between 8 and 10 % h^{-1} and 9 and 10 % h^{-1} in all dietary treatments, respectively. Mean retention time (MRT) and total mean retention time (TMRT) of particulate and liquid fractions of digesta were similar ($P > 0.05$) in both RR and HG in all dietary treatments. Total mean retention time (TMRT) of particulate and liquid fractions of

digesta ranged between 40.6 and 57.5 hours and 23.8 and 26.9 hours with dietary treatments, respectively.

Digesta washing (selectivity factor index) in both RR and HG were similar ($P > 0.05$) in all dietary treatments. Selectivity factor for RR and HG ranged between 1.8 and 3.1 and 1.4 and 2.3 across dietary treatments, respectively (Table 4.4**)**. Based on the selectivity factor, the particulate fraction of digesta was retained for prolonged time compared to liquid fractions of digesta in all dietary treatments. In general, there were no significant differences in retention times of both markers in dietary treatments.

Table 4. 2 Effect of *Vachellia tortilis* leaf meal and sunflower oil on growth performance of Merino lambs

	Dietary treatments							*P* – value[2]		
	CL	**VT**	**ML**	**VSFO**	**SFO**	**RMSE**	***P*-value**	**CT-effect**	**EE-effect**	**SF-effect**
CON DMI (g DM)	433[a]	433[a]	425[b]	431[a]	433[a]	0.006	***	ns	ns	***
UTH INTAKE (g DM)	548	583	581	568	534	0.081	ns	ns	ns	ns
TDMI (g DM)	981	1016	1005	998	968	0.083	ns	ns	ns	ns
ADG (g)	181	170	170	173	161	0.071	ns	ns	ns	ns
FCR (g feed/ g gain)	4.1	4.8	5.0	4.9	4.7	2.594	ns	ns	ns	ns
AD	0.57[a]	0.48[ab]	0.50[ab]	0.44[b]	0.41[b]	0.082	*	ns	**	ns
NDFD	0.66	0.54	0.48	0.50	0.53	0.176	ns	ns	ns	ns

Con-concentrate diets; CL- control diet; VT- *Vachellia tortilis* leaf meal diet; ML- maize + lucerne diet; VSFO- *Vachellia tortilis* leaf meal with sunflower oil diet; SFO- sunflower oil diet; DMI- dry matter intake; UTH- urea-treated hay; TDMI- total dry matter intake; ADG-average daily gain; FCR-feed conversion ratio; AD-Apparent total tract digestibility; NDFD-neutral detergent fibre digestibility; RMSE-root mean square error; ns- not significant ($P > 0.05$), * significant ($P < 0.05$), ** highly significant ($P < 0.01$), *** extremely significant ($P < 0.001$).

[2] Contrast probability: Condensed tannin (CT) - effect of *Vachellia tortilis* supplementation (control vs VT + VSFO); Ether extract (EE) - effect of sunflower oil supplementation (VSFO + SFO); Sunflower cake (SF) - addition of sunflower cake to diet (CT vs ML).

[a, b, c] means within each row with different superscripts differ significantly ($P < 0.05$).

Table 4. 3 Effect of *Vachellia tortilis* leaf meal and sunflower oil on fractional passage rate, mean retention time and selectivity factor in the whole gastrointestinal tract of sheep

	Dietary treatments							*P*-value[2]		
	CL	**VL**	**ML**	**VSFO**	**SFO**	**RMSE**	***P*-value**	**CT-effect**	**EE-effect**	**SF-effect**
Fractional passage rate (per h)										
RR (k_P)	0.06	0.04	0.04	0.05	0.05	0.021	ns	ns	ns	ns
HG (k_P)	0.07	0.05	0.05	0.06	0.05	0.016	ns	ns	ns	ns
RR (k_l)	0.08	0.10	0.09	0.08	0.08	0.023	ns	ns	ns	ns
HG (k_l)	0.09	0.10	0.09	0.09	0.09	0.023	ns	ns	ns	ns
Mean Retention Time (h)										
RR_P	22.1	29.1	31.4	25.5	24.1	10.706	ns	ns	ns	ns
HG_P	16.9	21.4	21.3	18.8	19.6	5.238	ns	ns	ns	ns
$TMRT_P$	40.6	55.9	57.5	52.0	48.5	15.770	ns	ns	ns	ns
RR_l	12.4	11.3	11.9	12.5	13.2	2.871	ns	ns	ns	ns
HG_l	12.1	11.0	11.8	11.9	12.4	2.653	ns	ns	ns	ns
$TMRT_l$	26.9	24.2	23.8	24.4	24.2	7.068	ns	ns	ns	ns
Selectivity Factor										
RR	1.9	3.1	2.7	2.2	1.8	1.461	ns	ns	ns	ns
HG	1.4	2.3	1.9	1.6	1.6	0.859	ns	ns	ns	ns

CL- control diet; VT- *Vachellia tortilis* leaf meal diet; ML- maize + lucerne diet; VSFO- *Vachellia tortilis* with sunflower oil diet; SFO- sunflower oil diet; RR- reticulorumen; HG- hindgut; TMRT-total mean retention time; k_P- fractional passage rate of solid fraction; k_l-fractional passage rate of liquid fraction of digesta; RR_p -rumen solid fraction, HG_p -hindgut solid fraction; RR_l – rumen liquid fraction of digesta; HG_l -hindgut liquid fraction of digesta; $TMRT_p$ -total mean retention time of solid fraction; $TMRT_l$ – total mean retention time of liquid fraction of digesta; RMSE- root mean square error; ns - not significant ($P > 0.05$).

[2] Contrast probability: Condensed tannin (CT) - effect of *Vachellia tortilis* supplementation (control vs VT + VSFO); Ether extract (EE) - effect of sunflower oil supplementation (VSFO + SFO); Sunflower cake (SF) - addition of sunflower cake to diet (CT vs ML).

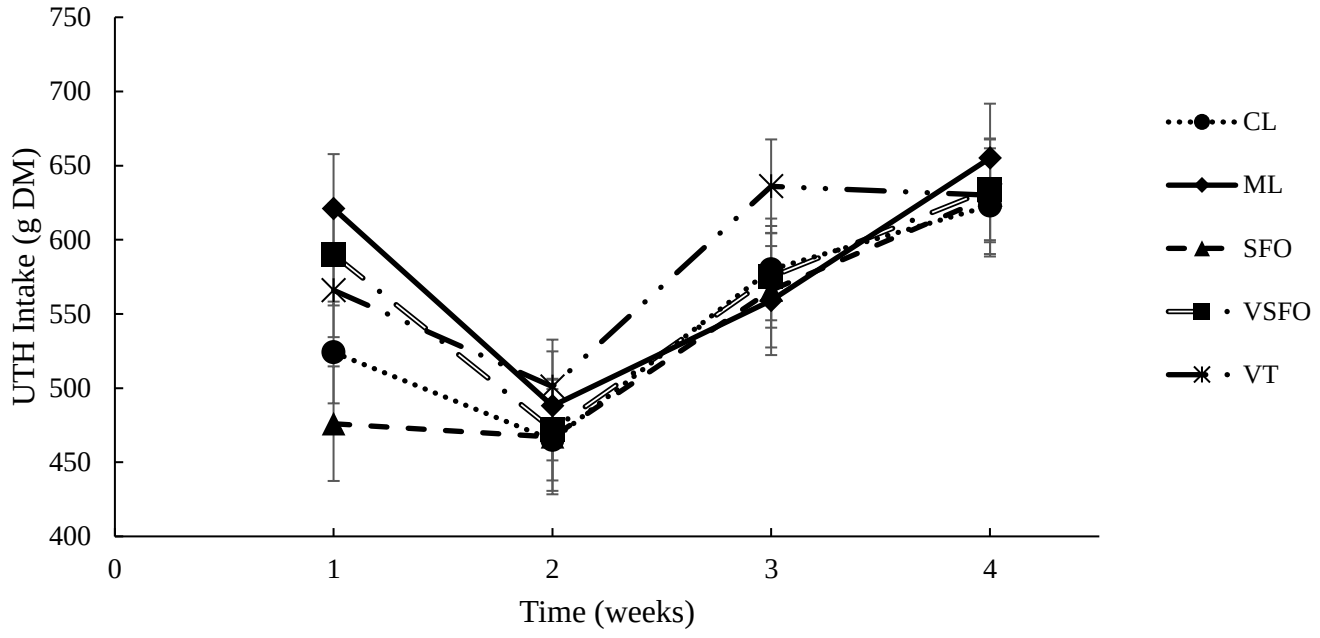

Figure 4. 1 Effect of *Vachellia tortilis* leaf meal and sunflower oil on UTH intake per week

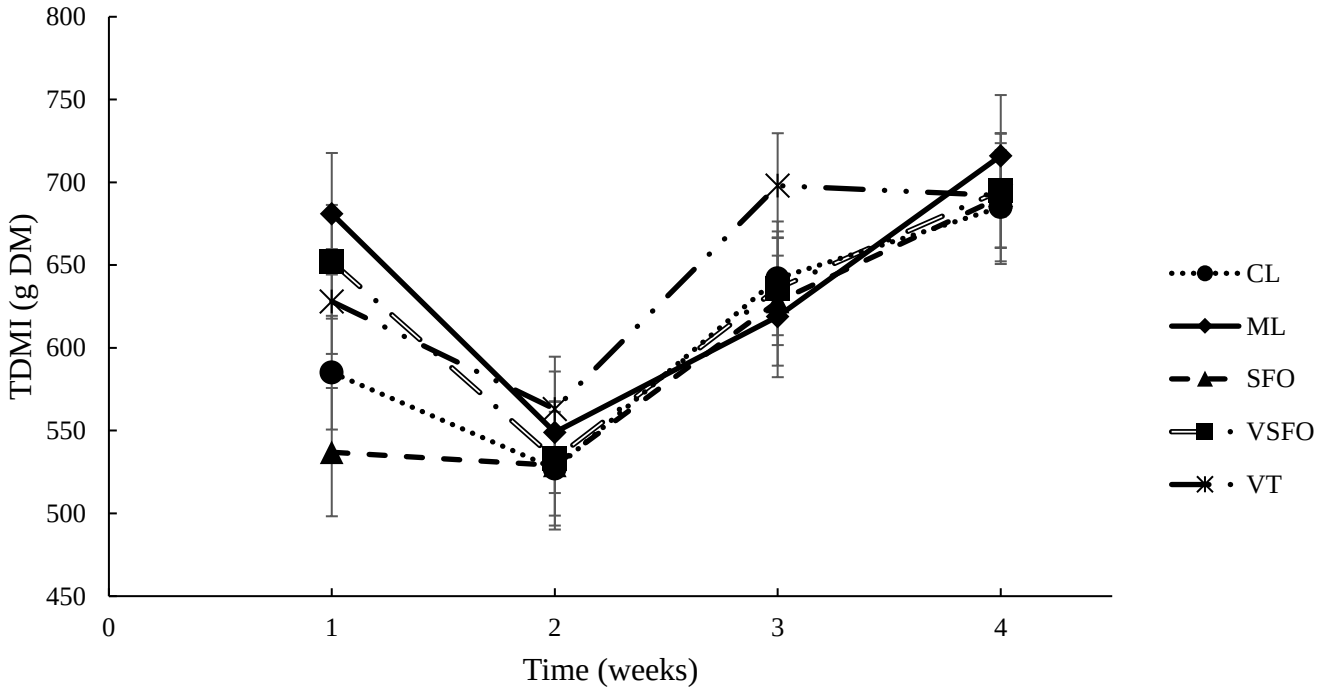

Figure 4. 2 Effect of *Vachellia tortilis* leaf meal and sunflower oil on TDMI per week

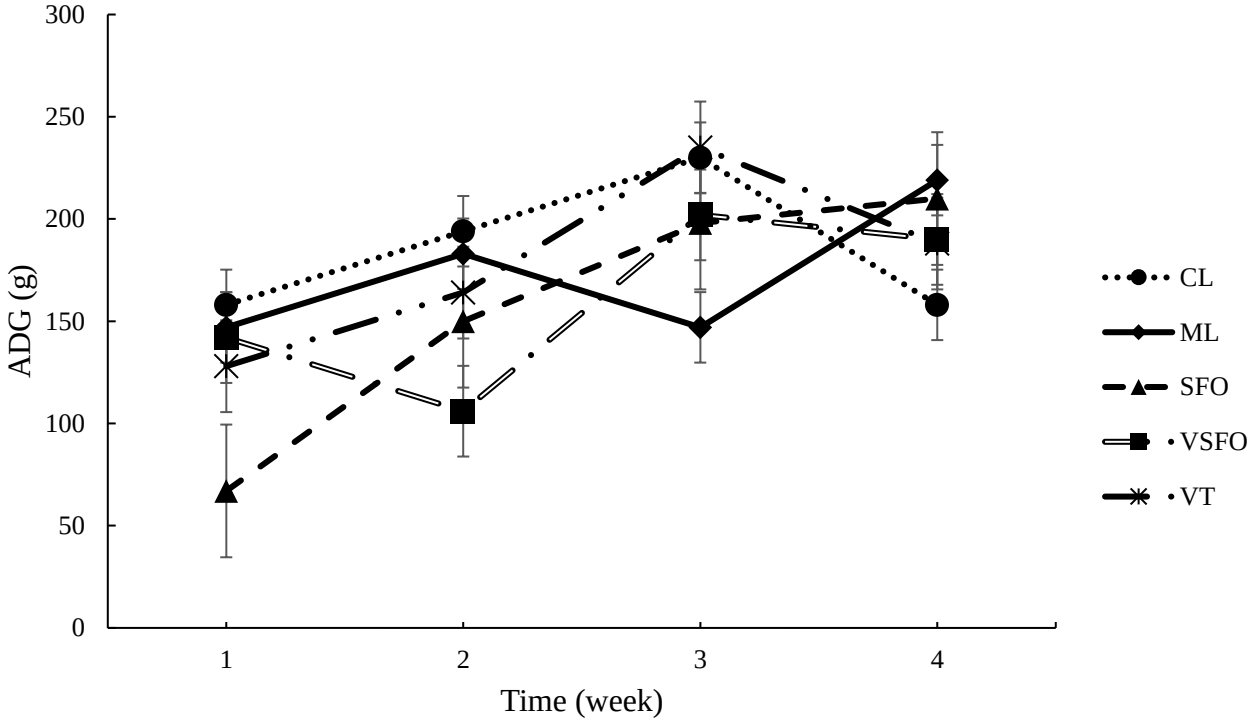

Figure 4. 3 Effect of *Vachellia tortilis* leaf meal and sunflower oil on ADG per week

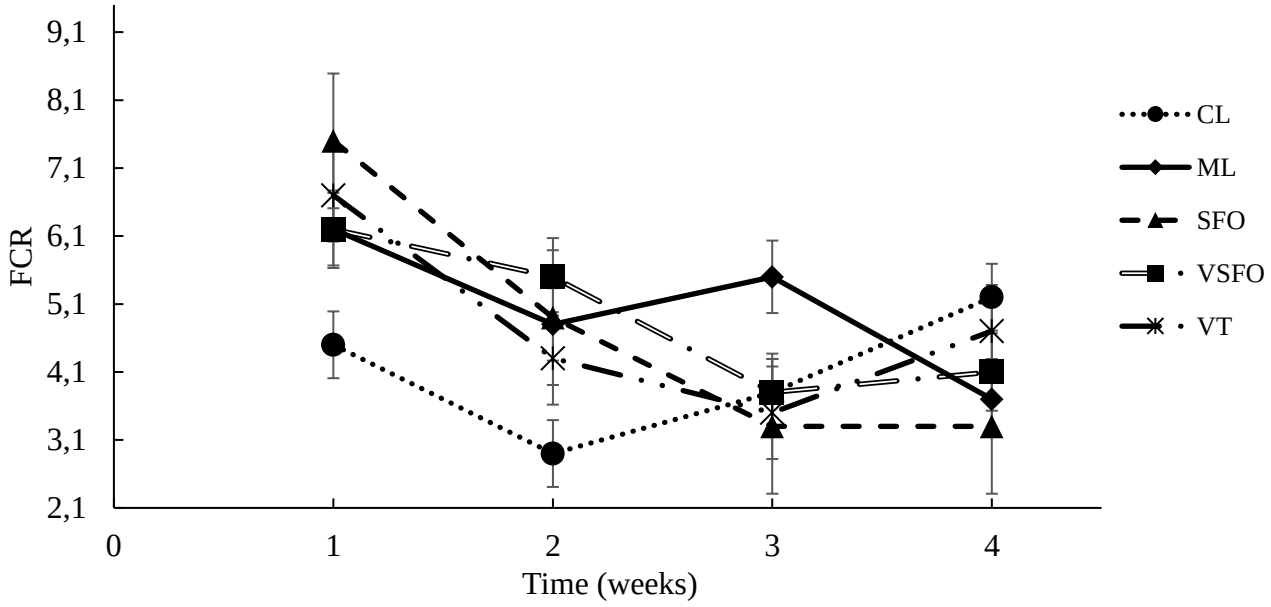

Figure 4. 4 Effect of *Vachellia tortilis* leaf meal and sunflower oil on FCR per week

4.4 Discussion

4.4.1 Effect of sunflower oil and Vachellia tortilis leaf meal, and mineral composition

Sunflower oil is rich in polyunsaturated fatty acids that adhere to feed particles, creating hydrophobic barrier that prevents the action of microbes and microbial enzymes; this can reduce dry matter digestibility (Wanapat *et al*., 2011). The inclusion of sunflower oil did not affect dry matter intake, yet the high ether extract levels impaired digestibility. In contrast, Patra *et al*. (2013) reported a quadratic response in dry matter intake of cattle fed a diet with 4.2 % DM fat concentration. The SFO diet had high ether extract levels (18.4 – 55.8 g/kg DM) above the recommended mean level (11.2 – 40.1 g/kg DM) for ruminant diets (Silva *et al*., 2019). This was due to the addition of sunflower oil and a high sunflower cake content in SFO diet. The high NDF and ADF content in SFO diet negatively affected digestibility. In addition, oils would reduce fibre digestibility by interfering on microbial activity; oils also tend to reduce the rate of passage of digesta. The poor digestibility of SFO diet may also be attributed to the removal of ciliate protozoa impaired by vegetable oils (Vargas-Bello-Pérez *et al*., 2016). The inclusion of sunflower oil did not affect growth parameters in lambs. These results are in agreement with Bhatt *et al*. (2011) who reported that coconut oil supplementation (25, 50, and 75 g/kg DM of concentrate) had no effect on ADG, DMI, and FCR.

The productivity of ruminants is affected by the quantity of protein reaching the small intestines. An increase in flow of dietary protein to the small intestines is affected by protein degradation by ruminal proteolytic micro-organisms. To improve the outflow of metabolizable protein, proteolysis has to be reduced while allowing for microbial nitrogen synthesis in the rumen. This was reported to be possible with the addition of proanthocyanidins or sunflower oil. Kamel *et al*. (2019) reported that the combination of 20 g/kg DM of sunflower oil and 40 g/kg DM of quebracho tannin had similar metabolizable protein compared to control diet. However, a combination of sunflower oil (20 g/kg DM) and quebracho tannin (40 g/kg DM) resulted in a higher average daily gain which was linked to the quality of metabolizable protein produced compared to the control. In the present study, a combination of *Vachellia tortilis* leaf meal with sunflower oil did not affect ADG. Liu *et al*. (2011) reported similar results in Rideau Arcott sheep, where a combination of chestnut tannins and coconut oil had no effect on growth performance. A high crude protein content of these diets and improvement of roughage with

non-protein nitrogen (NPN) source maintained a high dry matter intake and nutrient balance in lambs across dietary treatments.

Supplementing poor-quality roughage with forage legumes would reduce protein degradation and improves nitrogen retention, and could yet increase microbial protein synthesis (Getachew *et al*., 2000). *Vachellia tortilis* leaf meal has a high fibre content, which increased the ADF (cellulose and lignin) content and led to a reduction in digestibility compared to the control diet. Kronberg *et al*. (2018) reported a negative linear relationship between apparent DM digestibility and dietary fibre (NDF, ADF and ADL). The inclusion of *Vachellia tortilis* leaf meal did not affect TDMI, ADG and FCR. The effects of proanthocyanidins on performance of sheep may vary with different sources of proanthocyanidins, structure of the condensed tannin consumed and ruminant species (Kronberg *et al*., 2018). Dietary treatments had adequate crude protein content to support beyond the maintenance requirements of sheep which is above 100 g/kg DM (Mokoboki *et al*., 2005).

Supplementation of *Vachellia tortilis* leaf meal is recommended for ruminants to supplement poor-quality roughages as a source of crude protein. The crude protein content of dietary treatments may have inactivated proanthocyanidins by forming insoluble complexes; this would have reduced the effect of proanthocyanidins on performance of lambs (Francisco *et al*., 2015). The fact that a combination of maize-lucerne did not affect performance of lambs may be due to adequate dietary crude protein content and quality of UTH. In this study, maize-lucerne diet was chosen as a negative control to evaluate the feed efficiency of lambs, which was similar across all diets. Fractional passage rate and TMRT were similar in dietary treatments. The urea-treated hay (UTH) stimulated microbial activity, increasing N and digestible cell wall content in the rumen (Alcaide *et al*., 2000; Allen, 1996). Selectivity factor index is an expression of how much longer particles of a defined size (< 2mm) are retained in the ruminant digestive tract than fluids (Clauss and Lechner-Doll, 2001). The lambs had a selectivity ranging between 1.8 – 3.1 meaning they retained particulate longer in reticulorumen and hindgut for proper degradation than liquid fractions of digesta. The movement of particulate and liquid fraction of digesta was not affected by inclusion of *Vachellia tortilis* leaf meal and sunflower oil.

Phosphorus levels decrease with maturity of plants (Schillhorn van Veen and Loeffler, 1990). The calcium (Ca) and phosphorus (P) content in plants vary based on region and seasons. During dry seasons, slow growth rate and late maturity of ruminants results from dietary

deficiency of protein and P in pastures (Brinckman and de Leeuw, 1979). Concentrations of potassium are low in mature pastures as a result of uptake and availability of trace-minerals influenced by environmental factors (Schillhorn van Veen and Loeffler, 1990). The mineral composition of grasses and legumes vary with seasons. Pirhofer-Walzl *et al*. (2011) reported that pastures had high concentration of trace-minerals (Mn and Mo) that forage legumes; although, forage legumes had a high composition of macro-minerals. The variation in minerals composition of pastures and forage legumes allows for both plant feedstuffs to complement each other during winter and autumn when pastures have a poor nutrient composition due to translocating nutrients to the root system. The inclusion of *Vachellia tortilis* leaf meal tended to increase the macro-mineral composition, whereas ML diet had a low macro-mineral composition across all dietary treatments. Maize-lucerne diet had a high iron (Fe) and manganese (Mn) concentration due to high content of lucerne compared to other dietary treatments.

4.5 Conclusion

The inclusion level of *Vachellia tortilis* leaf meal and sunflower oil did not affect growth performance and fractional passage rate in lambs. There is a potential for *Vachellia tortilis* leaf meal to improve growth performance due to its high crude protein content without negatively affecting digestibility. The inclusion of sunflower oil did not affect growth performance of lambs. However, addition of both sunflower oil and sunflower cake results in high ether extract levels above 11.2 – 40.1 g/kg DM negatively affecting dry matter digestibility. Based on these results, the inclusion levels of *Vachellia tortilis* leaf meal and sunflower oil were below threshold levels since they did not affect growth performance of lambs. The combination of *Vachellia tortilis* leaf meal with sunflower oil has potential to improve average daily gain in lambs.

CHAPTER 5

General discussion, knowledge gaps and recommendations for future research

5.1 General discussion

Nutrient composition of diets can affect rumen microbial digestion, composition of short-chain fatty acids (SCFAs) and methane yield in the rumen. Supplementing forage legumes and vegetable oils is an effective strategy used to reduce rumen methane emissions; and also, to improve livestock performance and animal products (Mlambo and Mnisi, 2019). The main objective of this study was to reduce the proportion of methane based on the composition of SCFAs and improve growth performance of lambs. The effect of *Vachellia tortilis* leaf meal and sunflower oil on production of total SCFAs, individual SCFA, proportion of methane and carbon dioxide were assessed. Also, the effect of *Vachellia tortilis* leaf meal and sunflower oil on weight gain, feed intake and feed efficiency of lambs were evaluated. The specific objectives of this study were to determine (1) the effect of *Vachellia tortilis* leaf meal and sunflower oil on *in vitro* SCFA composition, total SCFA production, proportion of methane, carbon dioxide, and IVDMD; (2) the effect of *Vachellia tortilis* leaf meal and sunflower oil on growth performance of merino lambs and (3) the effect of *Vachellia tortilis* leaf meal and sunflower oil on fractional outflow rate of particulate and liquid fractions of digesta in sheep.

In chapter 3, results showed that the inclusion of *Vachellia tortilis* leaf meal improved the crude protein of dietary treatments compared to the control. While, the inclusion of sunflower oil in dietary treatments (VSFO and SFO) was responsible for a higher ether extract content compared to the other dietary treatments including the control. The study tested the hypothesis that the inclusion of *Vachellia tortilis* leaf meal will reduce total SCFAs, individual SCFA, acetate to propionate ratio and methane yield. However, the inclusion of *Vachellia tortilis* leaf meal and sunflower oil did not affect the proportion of acetate, propionate, acetate to propionate ratio, total SCFAs produced, proportion of methane at 2, 4, 16, and 48 hours during incubation. Although, production of butyrate and proportion of carbon dioxide was highest at 16 hours in the combination of *Vachellia tortilis* leaf meal and sunflower oil diet compared to the control. The inclusion of *Vachellia tortilis* leaf meal improved IVDMD. The hypothesis is partially rejected based on the fact that the inclusion of *Vachellia tortilis* leaf meal improved IVDMD yet total SCFAs, proportion of methane were not affected. Based on the objectives, inclusion

of *Vachellia tortilis* leaf meal and sunflower oil may be below threshold levels to reduced total SCFAs and proportion of methane. It was concluded that inclusion of *Vachellia tortilis* leaf meal and sunflower oil did not affect total SCFAs, acetate to propionate ratio and proportion of methane. Yet the proportion of butyrate and carbon dioxide were high in sunflower oil diets. The high *in vitro* digestibility of *Vachellia tortilis* leaf meal diets highlights the degradative potential of *Vachellia tortilis* not only as an alternative protein source but for improving nutrient utilisation.

In chapter 4, *Vachellia tortilis* leaf meal and sunflower oil diets had a lower apparent digestibility compared to the control. It was hypothesised that the inclusion of *Vachellia tortilis* leaf meal and sunflower oil will improve growth performance and fractional passage rate kinetics in lambs. The incorporation of *Vachellia tortilis* leaf meal in dietary treatments with other ingredients reduced the bitter taste of forage legumes, which may possibly be explained by similar dry matter intake. The lambs were provided good quality roughage due to its improvement with a non-protein nitrogen (NPN) source. This was crucial for nutrient requirements of lambs fed maize-lucerne diet which had a low crude protein and organic matter content compared to the control. Dietary treatments had adequate crude protein content for maintenance and growth requirements which may possibly be explained by similar performance of lambs. The average daily gain was similar in all dietary treatments; yet the combination of *Vachellia tortilis* leaf meal and sunflower oil has potential to improve weight gain. This may be due to a high degradative potential of *Vachellia tortilis* (Ondiek *et al*., 2010), its combination with sunflower oil allowed for outflow of metabolizable protein to the small intestine.

Fractional passage rate of particulate and liquid fractions of digesta were not affected by inclusion of *Vachellia tortilis* leaf meal and sunflower oil, which may be due to similar dry matter intake and quality of urea-treated hay. The hypothesis is rejected based on the view that inclusion of *Vachellia tortilis* leaf meal and sunflower oil had a negligible influence on the growth performance of lambs and fractional passage rate kinetics. The concentration of sunflower oil was not detrimental to growth performance of lambs, yet the addition of both sunflower oil and sunflower cake was responsible for higher ether extract content above recommended levels for ruminants (Silva *et al*., 2019). It was concluded that despite the inclusion of *Vachellia tortilis* leaf meal and sunflower oil, growth performance and fractional passage rate of lambs were not affected. *Vachellia tortilis* leaf meal and sunflower oil did not affect the growth performance of lambs due to the quality of diets and roughage. Therefore,

type of diets may have an influence on the effects of forage legumes and vegetable oils; which have been reported to be predominant in roughage diets compared to concentrate diets (Kamel *et al*., 2019).

5.2 Knowledge gaps

Identifying the optimum inclusion level of polyphenolic compounds against rumen methane emissions is important. A meta-analysis on different forage legumes from different regions, climatic conditions, soil types and their effect on ruminants is worth considering; together with the aim of comparatively analysing the impact of polyphenolic compounds on rumen SCFAs and methane emissions, while improve animal performance. The effect of sunflower oil on butyrate-producing bacteria, focusing on *Butyrivibrio* species or similar microbial species which are not sensitive to sunflower oil this warrants further research.

5.3 Recommendations for future research

It is evident that in both studies the effects of *Vachellia tortilis* leaf meal and sunflower oil were negligible. Further studies may analyse the influence of different type of diets on activity of polyphenolic compounds and nutrient availability. It is reported that the effect of phenolic compounds in forage legumes do not affect growth performance in isoenergetic and isoproteic rations (Franscisco *et al*., 2015). The main focus is identifying optimum inclusion levels of polyphenolic compounds (proanthocyanidins) and vegetable oils without negatively affecting nutrient degradation yet achieving the objective of reducing proportion of SCFAs and methane yields.

Aspects that require further research include the following:

1. Determine the effect of the mixture of *Vachellia tortilis* leaf meal with sunflower oil on metabolizable protein production and average daily gain.
2. Determining the effect of the mixture of *Vachellia tortilis* leaf meal with sunflower oil on butyrate-producing bacteria, proportion of methane at different inclusion levels.
3. Determine the effect of type of diets on the activity of polyphenolic compounds and unsaturated fatty acids.

REFERENCES

Abdulrazak, S.A., Orden, E.A., Ichinohe, T. and Fujihara, T., 2000. Chemical composition, phenolic concentration and in vitro gas production characteristics of selected Acacia fruits and leaves. *Asian-Australasian Journal of Animal Sciences*, *13*, pp.935-940.

Alberto, M.R., Manca de Nadra, M.C. and Arena, M.E., 2012. Influence of phenolic compounds on the growth and arginine deiminase system in a wine lactic acid bacterium. *Brazilian Journal of Microbiology*, *43*, pp.167-176.

Alcaide, E.M., García, A.M. and Aguilera, J.F., 2000. A comparative study of nutrient digestibility, kinetics of degradation and passage and rumen fermentation pattern in goats and sheep offered good quality diets. *Livestock Production Science*, *64*, pp.215-223.

Alexander, G., Singh, B., Sahoo, A. and Bhat, T.K., 2008. In vitro screening of plant extracts to enhance the efficiency of utilization of energy and nitrogen in ruminant diets. *Animal Feed Science and Technology*, *145*, pp.229-244.

Allen, M.S., 1996. Physical constraints on voluntary intake of forages by ruminants. *Journal of Animal Science*, *74*, pp.3063-3075.

Al-Masri, M.R., 2003. An in vitro evaluation of some unconventional ruminant feeds in terms of the organic matter digestibility, energy and microbial biomass. *Tropical Animal Health and Production*, *35*, pp.155-167.

AlZahal, O., Kebreab, E., France, J., Froetschel, M. and McBride, B.W., 2008. Ruminal temperature may aid in the detection of subacute ruminal acidosis. *Journal of Dairy Science*, *91*, pp.202-207.

Anderson, R.C. and Rasmussen, M.A., 1998. Use of a novel nitrotoxin-metabolizing bacterium to reduce ruminal methane production. *Bioresource Technology*, *64*, pp.89-95.

AOAC (Association of Official Analytical Chemists), 1990. Official methods of analysis. *Association of Official Analytical Chemists, Arlington*.

Arogo, J., Westerman, P.W., Heber, A.J., Robarge, W.P. and Classen, J.J., 2006. Ammonia emissions from animal feeding operations. *Animal agriculture and the environment: National Center for Manure and Animal Waste Management white papers*, pp.41-88.

Arthur, J.P. and Herd, R.M., 2008. Residual feed intake in beef cattle. *Revista Brasileira de Zootecnia*, *37*, pp.269-279.

Asante, B.O., Villano, R.A. and Battese, G.E., 2017. Integrated crop-livestock management practices, technical efficiency and technology ratios in extensive small-ruminant systems in Ghana. *Livestock science*, *201*, pp.58-69.

Attwood, G. and McSweeney, C., 2008. Methanogen genomics to discover targets for methane mitigation technologies and options for alternative H2 utilisation in the rumen. *Australian Journal of Experimental Agriculture*, *48*, pp.28-37.

Baldwin, R.R., Hopkins, D.E., Norris, A.C. and Walker, R.W., 1970. The addition of methane to slowly reacting hydrogen-oxygen mixtures: Reactions of methyl radicals. *Combustion and Flame*, *15*, pp.33-46.

Baloyi, J.J., Ngongoni, N.T. and Hamudikuwanda, H., 2008. The effect feeding forage legumes as nitrogen supplement on growth performance of sheep. *Tropical animal health and production*, *40*, pp.457-462.

Baümler, E.R., Carrín, M.E. and Carelli, A.A., 2016. Extraction of sunflower oil using ethanol as solvent. *Journal of Food Engineering*, *178*, pp.190-197.

Beauchemin, K.A., Kreuzer, M., O'mara, F. and McAllister, T.A., 2008. Nutritional management for enteric methane abatement: a review. *Australian Journal of Experimental Agriculture*, *48*, pp.21-27.

Bell, M.J., Eckard, R.J. and Cullen, B.R., 2012. The effect of future climate scenarios on the balance between productivity and greenhouse gas emissions from sheep grazing systems. *Livestock Science*, *147*, pp.126-138.

Benchaar, C., Calsamiglia, S., Chaves, A.V., Fraser, G.R., Colombatto, D., McAllister, T.A. and Beauchemin, K.A., 2008. A review of plant-derived essential oils in ruminant nutrition and production. *Animal Feed Science and Technology*, *145*, pp.209-228.

Berndt, A., Lee, S.Y., Ramakrishnan, C. and Deisseroth, K., 2014. Structure-guided transformation of channelrhodopsin into a light-activated chloride channel. *Science*, *344*, pp.420-424.

Bhatt, R.S., Soren, N.M., Tripathi, M.K. and Karim, S.A., 2011. Effects of different levels of coconut oil supplementation on performance, digestibility, rumen fermentation and carcass traits of Malpura lambs. *Animal Feed Science and Technology*, *164*, pp.29-37.

Bhatta, R., Enishi, O. and Kurihara, M., 2007. Measurement of methane production from ruminants. *ASIAN AUSTRALASIAN JOURNAL OF ANIMAL SCIENCES*, *20*, p.1305.

Bhatta, R., Uyeno, Y., Tajima, K., Takenaka, A., Yabumoto, Y., Nonaka, I., Enishi, O. and 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*, pp.5512-5522.

Birteeb, P.T., Addah, W., Jakper, N. and Addo-Kwafo, A., 2011. Effects of intercropping cereal-legume on biomass and grain yield in the savannah zone. *Livestock research for rural development*, *23*(9).

Blaxter, K.L., Graham, N.M. and Wainman, F.W., 1956. Some observations on the digestibility of food by sheep, and on related problems. *British Journal of Nutrition*, *10*, pp.69-91.

Blümmel, M., Steingaβ, H. and Becker, K., 1997. The relationship between in vitro gas production, in vitro microbial biomass yield and 15 N incorporation and its implications for the prediction of voluntary feed intake of roughages. *British Journal of Nutrition*, *77*, pp.911-921.

Blümmel, M., Zerbini, E., Reddy, B.V.S., Hash, C.T., Bidinger, F. and Ravi, D., 2003. Improving the production and utilization of sorghum and pearl millet as livestock feed: methodological problems and possible solutions. *Field Crops Research*, *84*, pp.123-142.

Boadi, D.A., Wittenberg, K.M. and Kennedy, A.D., 2002. Validation of the sulphur hexafluoride (SF6) tracer gas technique for measurement of methane and carbon dioxide production by cattle. *Canadian Journal of Animal Science*, *82*, pp.125-131.

Bodas, R., Prieto, N., García-González, R., Andrés, S., Giráldez, F.J. and López, S., 2012. Manipulation of rumen fermentation and methane production with plant secondary metabolites. *Animal Feed Science and Technology*, *176*, pp.78-93.

Bouwman, L., Goldewijk, K.K., Van Der Hoek, K.W., Beusen, A.H., Van Vuuren, D.P., Willems, J., Rufino, M.C. and Stehfest, E., 2013. Exploring global changes in nitrogen

and phosphorus cycles in agriculture induced by livestock production over the 1900–2050 period. *Proceedings of the National Academy of Sciences*, *110*, pp.20882-20887.

Brinkman, W.L. and De Leeuw, P. N., (1979). Wildlife Management in Savannah Woodland, (Eds S.S. Ajayi and L.B. Halstead), Taylor & Francis Ltd, London, pp 101 – 109.

Buccioni, A., Decandia, M., Minieri, S., Molle, G. and Cabiddu, A., 2012. Lipid metabolism in the rumen: New insights on lipolysis and biohydrogenation with an emphasis on the role of endogenous plant factors. *Animal Feed Science and Technology*, *174*, pp.1-25.

Bunglavan, S.J., Valli, C., Ramachandran, M. and Balakrishnan, V., 2010. Effect of supplementation of herbal extracts on methanogenesis in ruminants. *Livestock Research for Rural Development*, *22*, p.216.

Carreño, D., Hervás, G., Toral, P.G., Belenguer, A. and Frutos, P., 2015. Ability of different types and doses of tannin extracts to modulate in vitro ruminal biohydrogenation in sheep. *Animal Feed Science and Technology*, *202*, pp.42-51.

Choubey, M., Patel, V.R., Sorathiya, A.B. and Tabhani, P.M., 2015. Nutritional Interventions for Methane Mitigation in Ruminants. *Journal of Animal Nutrition and Physiology*| *Year*, *1*, pp.23-27.

Clauss, M. and Lechner-Doll, M., 2001. Differences in selective reticulo-ruminal particle retention as a key factor in ruminant diversification. *Oecologia*, *129*, pp.321-327.

Costa, M., Alves, S.P., Cappucci, A., Cook, S.R., Duarte, A., Caldeira, R.M., McAllister, T.A. and Bessa, R.J., 2018. Effects of condensed and hydrolyzable tannins on rumen metabolism with emphasis on the biohydrogenation of unsaturated fatty acids. *Journal of agricultural and food chemistry*, *66*, pp.3367-3377.

Cummings, B.A., Gould, D.H., Caldwell, D.R. and Hamar, D.W., 1995. Ruminal microbial alterations associated with sulfide generation in steers with dietary sulfate-induced polioencephalomalacia. *American journal of veterinary research*, *56*, pp.1390-1395.

Dalal, R.C., Wang, W., Robertson, G.P. and Parton, W.J., 2003. Nitrous oxide emission from Australian agricultural lands and mitigation options: a review. *Soil Research*, *41*, pp.165-195.

De Haas, Y., Windig, J.J., Calus, M.P.L., Dijkstra, J., De Haan, M., Bannink, A. and Veerkamp, R.F., 2011. Genetic parameters for predicted methane production and potential for

reducing enteric emissions through genomic selection. *Journal of dairy science*, *94*, pp.6122-6134.

De Klein, C.A. and Ledgard, S.F., 2005. Nitrous oxide emissions from New Zealand agriculture–key sources and mitigation strategies. *Nutrient cycling in Agroecosystems*, *72*, pp.77-85.

Degen, A.A., El-Meccawi, S. and Kam, M., 2010. Cafeteria trials to determine relative preference of six desert trees and shrubs by sheep and goats. *Livestock Science*, *132*, pp.19-25.

Dehority, B.A., 2003. *Rumen microbiology* (Vol. 372). Nottingham: Nottingham University Press.

Den Besten, G., van Eunen, K., Groen, A.K., Venema, K., Reijngoud, D.J. and Bakker, B.M., 2013. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *Journal of lipid research*, *54*, pp.2325-2340.

Dey, A., Paul, S.S., Dahiya, S.S. and Punia, B.S., 2018. Effects of vegetable oils supplementation on in vitro rumen fermentation, volatile fatty acid composition and methane production in buffaloes. *Buffalo Bulletin*, *37*, pp.37-44.

Dijkstra, J., Ellis, J.L., Kebreab, E., Strathe, A.B., López, S., France, J. and Bannink, A., 2012. Ruminal pH regulation and nutritional consequences of low pH. *Animal Feed Science and Technology*, *172*, pp.22-33.

Dorman, H.J.D. and Deans, S.G., 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *Journal of applied microbiology*, *88*, pp.308-316.

Dube, J.S., Reed, J.D. and Ndlovu, L.R., 2001. Proanthocyanidins and other phenolics in Acacia leaves of Southern Africa. *Animal Feed Science and Technology*, *91*, pp.59-67.

Dudley, Q.M., 2012. Life Cycle Boundaries and Greenhouse Gas Emissions from Beef Cattle.

Dulphy, J.P., Remond, B. and Theriez, M., 1980. Ingestive behaviour and related activities in ruminants. In *Digestive physiology and metabolism in ruminants*, Springer, Dordrecht, pp. 103-122.

Eckard, R.J., Grainger, C. and De Klein, C.A.M., 2010. Options for the abatement of methane and nitrous oxide from ruminant production: a review. *Livestock Science*, *130*, pp.47-56.

Elanthamil, R. and Bandeswaran, C. 2017. Methane Emission from Ruminants and Its Mitigating Measures Using Probiotic– A Review. *Int. J. Sci. Environ. Technol., 6,* pp. 319-325.

Ellis, J.L., Dijkstra, J., Kebreab, E., Bannink, A., Odongo, N.E., McBride, B.W. and France, J., 2008. Aspects of rumen microbiology central to mechanistic modelling of methane production in cattle. *The Journal of Agricultural Science*, *146*, pp.213-233.

Ellis, J.L., Kebreab, E., Odongo, N.E., Beauchemin, K., McGinn, S., Nkrumah, J.D., Moore, S.S., Christopherson, R., Murdoch, G.K., McBride, B.W. and Okine, E.K., 2009. Modeling methane production from beef cattle using linear and nonlinear approaches. *Journal of Animal Science*, *87*, pp.1334-1345.

Environmental Protection Agency (EPA). U.S. Methane Emissions 1990–2020: Inventories, Projections, and Opportunities for Reductions; U.S. Environmental Protection Agency: Washington, DC, USA, 1999.

Erisman, J.W., Sutton, M.A., Galloway, J., Klimont, Z. and Winiwarter, W., 2008. How a century of ammonia synthesis changed the world. *Nature Geoscience*, *1*, p.636.

Escobar-Bahamondes, P., Oba, M. and Beauchemin, K.A., 2016. An evaluation of the accuracy and precision of methane prediction equations for beef cattle fed high-forage and high-grain diets. *Animal*, *11*, pp.68-77.

FAO (2009). The State of Food and Agriculture (Food and Agriculture Organization of the United Nations, Rome, Italy).

Fitzsimons, C., Kenny, D.A., Deighton, M.H., Fahey, A.G. and McGee, M., 2013. Methane emissions, body composition, and rumen fermentation traits of beef heifers differing in residual feed intake. *Journal of Animal Science*, *91*, pp.5789-5800.

Francis, G., Kerem, Z., Makkar, H.P. and Becker, K., 2002. The biological action of saponins in animal systems: a review. *British journal of Nutrition*, *88*, pp.587-605.

Francisco, A., Dentinho, M.T., Alves, S.P., Portugal, P.V., Fernandes, F., Sengo, S., Jerónimo, E., Oliveira, M.A., Costa, P., Sequeira, A. and Bessa, R.J.B., 2015. Growth

performance, carcass and meat quality of lambs supplemented with increasing levels of a tanniferous bush (*Cistus ladanifer L.*) and vegetable oils. *Meat Science*, *100*, pp.275-282.

Franz, R.M., 2010. *Nutritional limits of gigantism-Allometry of digestive anatomy and physiology in herbivores with special reference to methane losses* (Doctoral dissertation, University of Zurich).

Frutos, P., Hervas, G., Giráldez, F.J. and Mantecón, A.R., 2004. Tannins and ruminant nutrition. *Spanish Journal of Agricultural Research*, *2*, pp.191-202.

Galyean, M.L. and Owens, F.N., 1991. Effects of diet composition and level of feed intake on site and extent of digestion in ruminants. In *Physiological aspects of digestion and metabolism in ruminants* pp. 483-514.

García, M.A., Aguilera, J.F. and Alcaide, E.M., 1995. Voluntary intake and kinetics of degradation and passage of unsupplemented and supplemented pastures from semiarid lands in grazing goats and sheep. *Livestock Production Science*, *44*, pp.245-255.

Garrett, E.F., Pereira, M.N., Nordlund, K.V., Armentano, L.E., Goodger, W.J. and Oetzel, G.R., 1999. Diagnostic methods for the detection of subacute ruminal acidosis in dairy cows. *Journal of dairy science*, *82*, pp.1170-1178.

Getachew, G., Makkar, H.P.S. and Becker, K., 2000. Effect of polyethylene glycol on in vitro degradability of nitrogen and microbial protein synthesis from tannin-rich browse and herbaceous legumes. *British Journal of Nutrition*, *84*, pp.73-83.

Girón, J.E.P., Restrepo, M.L.P. and Fornaguera, J.E.C., 2016. Supplementation with corn oil and palm kernel oil to grazing cows: ruminal fermentation, milk yield, and fatty acid profile. *Revista Brasileira de Zootecnia*, *45*(11), pp.693-703.

Goopy, J.P., Donaldson, A., Hegarty, R., Vercoe, P.E., Haynes, F., Barnett, M. and Oddy, V.H., 2014. Low-methane yield sheep have smaller rumens and shorter rumen retention time. *British Journal of Nutrition*, *111*, pp.578-585.

Grainger, C., Clarke, T., Auldist, M.J., Beauchemin, K.A., McGinn, S.M., Waghorn, G.C. and Eckard, R.J., 2009. Potential use of Acacia mearnsii condensed tannins to reduce methane emissions and nitrogen excretion from grazing dairy cows. *Canadian Journal of Animal Science*, *89*, pp.241-251.

Grainger, C. and Beauchemin, K.A., 2011. Can enteric methane emissions from ruminants be lowered without lowering their production? *Animal feed science and technology*, *166*, pp.308-320.

Grovum, W.L. and Williams, V.J., 1973. Rate of passage of digesta in sheep: 4. Passage of marker through the alimentary tract and the biological relevance of rate-constants derived from the changes in concentration of marker in faeces. *British journal of Nutrition*, *30*, pp.313-329.

Gxasheka, M., L.T. Thobela, Q. Ning, and L. Zhi-Chao (2015). An overview of tannins rich plants as alternative supplementation on ruminant animals: A Review. *Int. J. Agric. Res. Rev. 3*, pp. 343-349.

Hammond, K.J., Burke, J.L., Koolaard, J.P., Muetzel, S., Pinares-Patiño, C.S. and Waghorn, G.C., 2013. Effects of feed intake on enteric methane emissions from sheep fed fresh white clover (Trifolium repens) and perennial ryegrass (Lolium perenne) forages. *Animal Feed Science and Technology*, *179*, pp.121-132.

Hammond, K.J., Pacheco, D., Burke, J.L., Koolaard, J.P., Muetzel, S. and Waghorn, G.C., 2014. The effects of fresh forages and feed intake level on digesta kinetics and enteric methane emissions from sheep. *Animal Feed Science and Technology*, *193*, pp.32-43.

Hammond, K.J., Crompton, L.A., Bannink, A., Dijkstra, J., Yáñez-Ruiz, D.R., O'Kiely, P., Kebreab, E., Eugène, M.A., Yu, Z., Shingfield, K.J. and Schwarm, A., 2016. Review of current in vivo measurement techniques for quantifying enteric methane emission from ruminants. *Animal Feed Science and Technology*, *219*, pp.13-30.

Hartung, E. and Monteny, G.J., 2000. Methane (CH4) and nitrous oxide (N2O) emissions from animal husbandry. *Agrartechnische Forschung*, *6*, pp.62-115.

Hatfield, P.G., Clanton, D.C., Sanson, D.W. and Eskridge, K.M., 1990. Methods of administering ytterbium for estimation of fecal output. *Journal of Range Management*, pp.316-320.

Hegarty, R.S., Goopy, J.P., Herd, R.M. and McCorkell, B., 2007. Cattle selected for lower residual feed intake have reduced daily methane production. *Journal of animal science*, *85*, pp.1479-1486.

Hellwing, A.L.F., Lund, P., Weisbjerg, M.R., Brask, M. and Hvelplund, T., 2012. Test of a low-cost and animal-friendly system for measuring methane emissions from dairy cows. *Journal of dairy science*, *95*(10), pp.6077-6085.

Henry, B.K., Eckard, R.J. and Beauchemin, K.A., 2018. Adaptation of ruminant livestock production systems to climate changes. *Animal*, *12*, pp. 445-456.

Herd, R.M., Arthur, P.F., Hegarty, R.S. and Archer, J.A., 2002, August. Potential to reduce greenhouse gas emissions from beef production by selection for reduced residual feed intake. In *Proceedings of the 7th World Congress on Genetics Applied to Livestock Production'. Communication*, pp. 10-22.

Herrero, M., Havlík, P., Valin, H., Notenbaert, A., Rufino, M.C., Thornton, P.K., Blümmel, M., Weiss, F., Grace, D. and Obersteiner, M., 2013. Biomass use, production, feed efficiencies, and greenhouse gas emissions from global livestock systems. *Proceedings of the National Academy of Sciences*, *110*, pp.20888-20893.

Heuzé, V., Tran, G., 2011. Umbrella thorn (*Acacia tortilis*). Feedipedia.org. A project by INRA, CIRAD, AFZ and FAO. *http:// www.trc.zootechnie:fr/node./339*.

Hill, J., McSweeney, C., Wright, A.D.G., Bishop-Hurley, G. and Kalantar-zadeh, K., 2016. Measuring methane production from ruminants. *Trends in biotechnology*, *34*(1), pp.26-35.

Hook, S.E., Wright, A.D.G. and McBride, B.W., 2010. Methanogens: methane producers of the rumen and mitigation strategies. *Archaea*, *2010*.

Hristov, A.N., Etter, R.P., Ropp, J.K. and Grandeen, K.L., 2004. Effect of dietary crude protein level and degradability on ruminal fermentation and nitrogen utilization in lactating dairy cows. *Journal of Animal Science*, *82*, pp.3219-3229.

Hristov, A.N., Oh, J., Firkins, J.L., Dijkstra, J., Kebreab, E., Waghorn, G., Makkar, H.P.S., Adesogan, A.T., Yang, W., Lee, C. and Gerber, P.J., 2013. SPECIAL TOPICS—Mitigation of methane and nitrous oxide emissions from animal operations: I. A review of enteric methane mitigation options 1. *Journal of animal science*, *91*, pp.5045-5069.

Hristov, A.N., Harper, M., Meinen, R., Day, R., Lopes, J., Ott, T., Venkatesh, A. and Randles, C.A., 2017. Discrepancies and uncertainties in bottom-up gridded inventories of

livestock methane emissions for the contiguous United States. *Environmental science & technology*, *51*, pp.13668-13677.

Intergovernmental Panel on Climate Change (IPCC) 2006. Guidelines for National Greenhouse Gas Inventories. In National Greenhouse Gas Inventories Programme; Eggleston, H.S., Buendia, L., Miwa, K., Ngara, T., Tanabe, K., Eds.; IGES: Hayama, Kanagawa, Japan, 2006.

Iwamoto, M., Asanuma, N. and Hino, T., 2002. Ability of Selenomonas ruminantium, Veillonella parvula, and Wolinella succinogenes to reduce nitrate and nitrite with special reference to the suppression of ruminal methanogenesis. *Anaerobe*, *8*, pp.209-215.

Jalajakshi, K., Reddy, Y.R. and Prasad, A.R.V., 2016. Growth performance and carcass traits of Nellore Brown ram lambs kept on grazing with or without concentrate supplementation. *Inter. J Sci. Environ*, *5*, pp.2788-2797.

Janssen, P.H. and Kirs, M., 2008. Structure of the archaeal community of the rumen. *Applied and environmental microbiology*, *74*, pp.3619-3625.

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*, pp.1-22.

Jayanegara, A., Wina, E., Soliva, C.R., Marquardt, S., Kreuzer, M. and Leiber, F., 2011. Dependence of forage quality and methanogenic potential of tropical plants on their phenolic fractions as determined by principal component analysis. *Animal Feed Science and Technology*, *163*, pp.231-243.

Jeyanathan, J., Martin, C. and Morgavi, D.P., 2014. The use of direct-fed microbials for mitigation of ruminant methane emissions: a review. *Animal*, *8*(2), pp.250-261.

Johnson, G.E., US Department of the Interior, 1972. *Production of methane by bacterial action*. U.S. Patent 3,640,846.

Kamel, H.E., Al-Dobaib, S.N. and Salem, A.Z., 2019. Dietary supplementation of sunflower oil and quebracho tannins in sheep feeding: In vivo nutrient digestibility, nitrogen utilization and in vitro ruminal degradation kinetics. *Journal of the Science of Food and Agriculture*.

Khanyile, M., Ndou, S.P. and Chimonyo, M., 2014. Influence of *Acacia tortilis* leaf meal-based diets on growth performance of pigs. *Livestock Science*, *167*, pp.211-218.

Kim, E.J., Huws, S.A., Lee, M.R., Wood, J.D., Muetzel, S.M., Wallace, R.J. and Scollan, N.D., 2008. Fish oil increases the duodenal flow of long chain polyunsaturated fatty acids and trans-11 18: 1 and decreases 18: 0 in steers via changes in the rumen bacterial community. *The Journal of nutrition*, *138*, pp.889-896.

Knapp, J.R., Laur, G.L., Vadas, P.A., Weiss, W.P. and Tricarico, J.M., 2014. Invited review: Enteric methane in dairy cattle production: Quantifying the opportunities and impact of reducing emissions. *Journal of dairy science*, *97*, pp.3231-3261.

Koenig, K.M. and Beauchemin, K.A., 2018. Effect of feeding condensed tannins in high protein finishing diets containing corn distiller grains on ruminal fermentation, nutrient digestibility, and route of nitrogen excretion in beef cattle. *Journal of animal science*, *96*, pp.4398-4413.

Kolver, E.S. and De Veth, M.J., 2002. Prediction of ruminal pH from pasture-based diets. *Journal of dairy science*, *85*, pp.1255-1266.

Kozloski, G.V., 2011. Biochemistry of ruminants, edn. Publisher Federal University of Santa Maria, Santa Maria.

Kronberg, S.L., Zeller, W.E., Waghorn, G.C., Grabber, J.H., Terrill, T.H. and Liebig, M.A., 2018. Effects of feeding Lespedeza cuneata pellets with Medicago sativa hay to sheep: Nutritional impact, characterization and degradation of condensed tannin during digestion. *Animal Feed Science and Technology*, *245*, pp.41-47.

Lan, W. and Yang, C., 2018. Ruminal methane production: Associated microorganisms and the potential of applying hydrogen-utilizing bacteria for mitigation. *Science of the Total Environment*.

Lassey, K.R., 2007. Livestock methane emission: from the individual grazing animal through national inventories to the global methane cycle. *Agricultural and forest meteorology*, *142*, pp.120-132.

Le Houérou, H.N., 1980. Planting and management methods for browse trees and shrubs. *Browse in Africa*, pp.351-359.

Lima, P.R., Apdini, T., Freire, A.S., Santana, A.S., Moura, L.M.L., Nascimento, J.C.S., Rodrigues, R.T.S., Dijkstra, J., Neto, A.G., Queiroz, M.Á. and Menezes, D.R., 2019. Dietary supplementation with tannin and soybean oil on intake, digestibility, feeding behavior, ruminal protozoa and methane emission in sheep. *Animal Feed Science and Technology*, *249*, pp.10-17.

Liu, H., Vaddella, V. and Zhou, D., 2011. Effects of chestnut tannins and coconut oil on growth performance, methane emission, ruminal fermentation, and microbial populations in sheep. *Journal of dairy science*, *94*, pp.6069-6077.

Luo, Y., Li, Z., Li, X., Liu, X., Fan, J., Clark, J.H. and Hu, C., 2019. The production of furfural directly from hemicellulose in lignocellulosic biomass: A review. *Catalysis Today*, *319*, pp.14-24.

Machmüller, A. and Hegarty, R.S., 2006. Alternative tracer gases for the ERUCT technique to estimate methane emission from grazing animals. In *International Congress Series*, 1293, pp. 50-53.

Makkar, H.P.S., 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*, pp.241-256.

McAllister, T.A., Martinez, T., Bae, H.D., Muir, A.D., Yanke, L.J. and Jones, G.A., 2005. Characterization of condensed tannins purified from legume forages: chromophore production, protein precipitation, and inhibitory effects on cellulose digestion. *Journal of Chemical Ecology*, *31*, pp.2049-2068.

McCrabb, G.J. and Hunter, R.A., 1999. Prediction of methane emissions from beef cattle in tropical production systems. *Crop and Pasture Science*, *50*, pp.1335-1340.

McSweeney, C.S., Palmer, B., McNeill, D.M. and Krause, D.O., 2001. Microbial interactions with tannins: nutritional consequences for ruminants. *Animal Feed Science and Technology*, *91*, pp.83-93.

Mentschel, J., Leiser, R., Mülling, C., Pfarrer, C. and Claus, R., 2001. Butyric acid stimulates rumen mucosa development in the calf mainly by a reduction of apoptosis. *Archives of Animal Nutrition*, *55*, pp.85-102.

Mezzomo, R., Paulino, P.V.R., Detmann, E., Valadares Filho, S.C., Paulino, M.F., Monnerat, J.P.I.S., Duarte, M.S., Silva, L.H.P. and Moura, L.S., 2011. Influence of condensed tannin on intake, digestibility, and efficiency of protein utilization in beef steers fed high concentrate diet. *Livestock Science*, *141*, pp.1-11.

Mlambo, V. and Mnisi, C.M., 2019. Optimizing ruminant production systems for sustainable intensification, human health, food security and environmental stewardship. *Outlook on Agriculture*, p.0030727019840758.

Moate, P.J., Clarke, T., Davis, L.H. and Laby, R.H., 1997. Rumen gases and bloat in grazing dairy cows. *The Journal of Agricultural Science*, *129*, pp.459-469.

Moeletsi, M.E. and Tongwane, M.I., 2015. 2004 Methane and Nitrous Oxide Emissions from Manure Management in South Africa. *Animals*, *5*, pp.193-205.

Mokoboki, H.K., Ndlovu, L.R., Ng'ambi, J.W., Malatje, M.M. and Nikolova, R.V., 2005. Nutritive value of Acacia tree foliages growing in the Limpopo Province of South Africa. *South African Journal of Animal Science*, *35*, pp.221-228.

Monticello, D.J., Hadioetomo, R.S. and Costilow, R.N., 1984. Isoleucine synthesis by Clostridium sporogenes from propionate or α-methylbutyrate. *Microbiology*, *130*, pp.309-318.

Moraes, L.E., Strathe, A.B., Fadel, J.G., Casper, D.P. and Kebreab, E., 2014. Prediction of enteric methane emissions from cattle. *Global change biology*, *20*, pp.2140-2148.

Morgavi, D.P., Forano, E., Martin, C. and Newbold, C.J., 2010. Microbial ecosystem and methanogenesis in ruminants. *Animal*, *4*, pp.1024-1036.

Moss, A.R., Jouany, J.P. and Newbold, J., 2000, May. Methane production by ruminants: its contribution to global warming. In *Annales de zootechnie*, EDP Sciences, *49*, pp. 231-253.

Moyo, M. and Nsahlai, I.V., 2018. Rate of Passage of Digesta in Ruminants; Are Goats Different? *Goat Science*, p.39.

Muro-Reyes, A., Gutierrez-Banuelos, H., Diaz-Garcia, L.H., Gutierrez-Pina, F.J., Escareno-Sanchez, L.M., Banuelos-Valenzuela, R., Medina-Flores, C.A. and Corral Luna, A., 2011. Potential environmental benefits of residual feed intake as strategy to mitigate methane emissions in sheep. *J Anim Vet Adv*, *10*, pp.1551-1556.

Nagaraja, T.G., 2016. Microbiology of the Rumen. In *Rumenology*, Springer, Cham, pp. 39-61.

Naumann, H.D., Stewart, W.C. and Whitney, T.R., 2018. The effect of maturity on concentration and biological activity of protein precipitating polyphenolics in ground juniper is dependent upon species. *Animal Feed Science and Technology*, *246*, pp.11-17.

Nsahlai, I.V., Umunna, N.N. and Negassa, D., 1995. The effect of multi-purpose tree digesta on in vitro gas production from napier grass or neutral-detergent fibre. *Journal of the Science of Food and Agriculture*, *69*, pp.519-528.

Nyamukanza, C.C. and Scogings, P.F., 2008. Sprout selection and performance of goats fed Acacia karroo coppices in the False Thornveld of the Eastern Cape, South Africa. *South African Journal of Animal Science*, *38*, pp.83-90.

Ondiek, J.O., Abdulrazak, S.A. and Njoka, E.N., 2010. Chemical and mineral composition, in-vitro gas production, in-sacco degradation of selected indigenous Kenyan browses. *Livestock Research for Rural Development*, *22*, p.2010.

Orwa C., Mutua, A., Kindt, R., Jamnadass, R. and Anthony, S., 2009. Agroforestree database: a tree reference and selection guide version 4.0 *Pharmacognosy and Phytochemistry*, *1*, pp. 45-50.

Osuji, P.O., Nsahlai, I.V. and Khalili, H., 1993. *Feed evaluation* (Vol. 5). ILRI (aka ILCA and ILRAD).

Paillard, D., McKain, N., Chaudhary, L.C., Walker, N.D., Pizette, F., Koppova, I., McEwan, N.R., Kopečný, J., Vercoe, P.E., Louis, P. and Wallace, R.J., 2007. Relation between phylogenetic position, lipid metabolism and butyrate production by different Butyrivibrio-like bacteria from the rumen. *Antonie Van Leeuwenhoek*, *91*, pp.417-422.

Pal, K., Patra, A.K., Sahoo, A. and Kumawat, P.K., 2015. Evaluation of several tropical tree leaves for methane production potential, degradability and rumen fermentation in vitro. *Livestock Science*, *180*, pp.98-105.

Palmquist, D.L. and Griinari, J.M., 2006. Milk fatty acid composition in response to reciprocal combinations of sunflower and fish oils in the diet. *Animal Feed Science and Technology*, *131*, pp.358-369.

Patra, A.K., Kamra, D.N. and 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*, pp.276-291.

Patra, A.K. and Saxena, J., 2009. The effect and mode of action of saponins on the microbial populations and fermentation in the rumen and ruminant production. *Nutrition Research Reviews*, *22*, pp.204-219.

Patra, A.K., 2013. The effect of dietary fats on methane emissions, and its other effects on digestibility, rumen fermentation and lactation performance in cattle: a meta-analysis. *Livestock science*, *155*, pp.244-254.

Paul, S.S., Deb, S.M. and Singh, D., 2011. Isolation and characterization of novel sulphate-reducing *Fusobacterium* sp. and their effects on in vitro methane emission and digestion of wheat straw by rumen fluid from Indian riverine buffaloes. *Animal feed science and technology*, *166*, pp.132-140.

Pérez-Barbería, F.J., 2017. Scaling methane emissions in ruminants and global estimates in wild populations. *Science of the Total Environment*, *579*, pp.1572-1580.

Pinares-Patiño, C.S., Lassey, K.R., Martin, R.J., Molano, G., Fernandez, M., MacLean, S., Sandoval, E., Luo, D. and Clark, H., 2011. Assessment of the sulphur hexafluoride (SF6) tracer technique using respiration chambers for estimation of methane emissions from sheep. *Animal Feed Science and Technology*, *166*, pp.201-209.

Pirhofer-Walzl, K., Søegaard, K., Høgh-Jensen, H., Eriksen, J., Sanderson, M.A., Rasmussen, J. and Rasmussen, J., 2011. Forage herbs improve mineral composition of grassland herbage. *Grass and Forage Science*, *66*, pp.415-423.

Reed, J.D., 1995. Nutritional toxicology of tannins and related polyphenols in forage legumes. *Journal of animal science*, *73*, pp.1516-1528.

Ribeiro Jr, G.O., Teixeira, A.M., Velasco, F.O., Júnior, W.F., Pereira, L.G.R., Chaves, A.V., Gonçalves, L.C. and McAllister, T.A., 2014. Production, nutritional quality and in vitro methane production from Andropogon gayanus grass harvested at different maturities and preserved as hay or silage. *Asian-Australasian journal of animal sciences*, *27*(3), p.330.

Ricci, P., Rooke, J.A., Nevison, I. and Waterhouse, A., 2013. Methane emissions from beef and dairy cattle: quantifying the effect of physiological stage and diet characteristics. *Journal of Animal Science*, *91*, pp.5379-5389.

Rigolot, C., Espagnol, S., Robin, P., Hassouna, M., Béline, F., Paillat, J.M. and Dourmad, J.Y., 2010. Modelling of manure production by pigs and NH_3, N_2O and CH_4 emissions. Part II: effect of animal housing, manure storage and treatment practices. *Animal*, *4*, pp.1413-1424.

Roy, A.S., Chingkheihunba, A. and Pakshirajan, K., 2016. An Overview of Production, Properties, and Uses of Biodiesel from Vegetable Oil. In *Green Fuels Technology* (pp. 83-105). Springer, Cham.

Rubanza, C.D.K., Shem, M.N., Otsyina, R., Bakengesa, S.S., Ichinohe, T. and Fujihara, T., 2005. Polyphenolics and tannins effect on in vitro digestibility of selected Acacia species leaves. *Animal Feed Science and Technology*, *119*, pp.129-142.

Salem, H.B., Nefzaoui, A., Salem, L.B. and Tisserand, J.L., 1999. Intake, digestibility, urinary excretion of purine derivatives and growth by sheep given fresh, air-dried or polyethylene glycol-treated foliage of Acacia cyanophylla Lindl. *Animal Feed Science and Technology*, *78*, pp.297-311.

Salem, H.B., Makkar, H.P.S., Nefzaoui, A., Hassayoun, L. and Abidi, S., 2005. Benefit from the association of small amounts of tannin-rich shrub foliage (Acacia cyanophylla Lindl.) with soya bean meal given as supplements to Barbarine sheep fed on oaten hay. *Animal Feed Science and Technology*, *122*, pp.173-186.

Saminathan, M., Sieo, C.C., Abdullah, N., Wong, C.M.V.L. and Ho, Y.W., 2015. Effects of condensed tannin fractions of different molecular weights from a Leucaena leucocephala hybrid on in vitro methane production and rumen fermentation. *Journal of the Science of Food and Agriculture*, *95*, pp.2742-2749.

Schillhorn van Veen, T.W. and Loeffler, I.K., 1990. Mineral deficiency in ruminants in subsaharan Africa: A review. *Tropical Animal Health and Production*, *22*, pp.197-205.

Seo, J.K., S.W. Kim, M.H. Kim, S.D. Upadhaya, D.K. Kam and J.K. Ha, 2010. Direct fed microbials for ruminant animals. *Asian-Australasian Journal of Animal Sciences*, *23*, pp. 1657-1667.

Shibata, M. and Terada, F., 2010. Factors affecting methane production and mitigation in ruminants. *Animal Science Journal*, *81*(1), pp.2-10.

Shingfield, K.J., Chilliard, Y., Toivonen, V., Kairenius, P. and Givens, D.I., 2008. Trans fatty acids and bioactive lipids in ruminant milk. In *Bioactive components of milk*, Springer, New York, NY. pp. 3-65.

Silanikove, N., Perevolotsky, A. and Provenza, F.D., 2001. Use of tannin-binding chemicals to assay for tannins and their negative postingestive effects in ruminants. *Animal Feed Science and Technology*, *91*, pp.69-81.

Silva, L.H.X.D., Diaz, T.G., Branco, A.F., Teodoro, A.L. and Ferreira, G.R., 2019. Sunflower cake in diets for beef cattle: digestibility, kinetics and in vitro ruminal fermentation parameters. *Acta Scientiarum. Animal Sciences*, *41*.

Sirohi, S.K., Pandey, N., Singh, B. and Puniya, A.K., 2010. Rumen methanogens: a review. *Indian journal of microbiology*, *50*, pp.253-262.

Śliwiński, B.J., Kreuzer, M., Wettstein, H.R. and 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*, pp.379-392.

Smith, A.H., Zoetendal, E. and Mackie, R.I., 2005. Bacterial mechanisms to overcome inhibitory effects of dietary tannins. *Microbial ecology*, *50*, pp.197-205.

Smith, P., Martino, D., Cai, Z., Gwary, D., Janzen, H., Kumar, P., McCarl, B. and Ogle, S., O, Mara F, Rice C, Scholes B, Sirotenko O (2007) Agriculture. In 'Climate change 2007: mitigation. Contribution of Working Group III to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change, (Eds B Metz, OR Davidson, PR Bosch, R Dave, LA Meyer) pp. 497-540.

Snyder, C.S., Davidson, E.A., Smith, P. and Venterea, R.T., 2014. Agriculture: sustainable crop and animal production to help mitigate nitrous oxide emissions. *Current Opinion in Environmental Sustainability*, *9*, pp.46-54.

Stone, W.C., 2004. Nutritional approaches to minimize subacute ruminal acidosis and laminitis in dairy cattle. *Journal of Dairy Science*, *87*, pp. E13-E26.

Storm, I.M., Hellwing, A.L.F., Nielsen, N.I. and Madsen, J., 2012. Methods for measuring and estimating methane emission from ruminants. *Animals*, *2*, pp.160-183.

Suinyuy, T.N., Donaldson, J.S. and Johnson, S.D., 2013. Patterns of odour emission, thermogenesis and pollinator activity in cones of an African cycad: what mechanisms apply? *Annals of botany*, *112*, pp.891-902.

Szczechowiak, J., Szkudelska, K., Szumacher-Strabel, M., Sadkowski, S., Gwozdz, K., El-Sherbiny, M., Kozłowska, M., Rodriguez, V. and Cieslak, A., 2018. Blood hormones, metabolic parameters and fatty acid proportion in dairy cows fed condensed tannins and oils blend. *Annals of Animal Science*, *18*, pp.155-166.

Szumacher-Strabel, M., Martin, S.A., Potkanski, A., Cieslak, A. and Kowalczyk, J., 2004. Changes in fermentation processes as the effect of vegetable oil supplementation in in vitro studies. *Journal of Animal and Feed Sciences*, *13*, pp.215-218.

Tamir, B. and Asefa, G., 2009. Effects of different forms of Acacia saligna leaves inclusion on feed intake, digestibility and body weight gain in lambs fed grass hay basal diet. *Animal feed science and technology*, *153*, pp.39-47.

Thabethe, F., Khanyile, M., Ncobela, C.N. and Chimonyo, M., 2019. Relationship between inclusion level of Vachellia tortilis leaf meal and behavioral activities of finishing pigs. *Asian-Australasian journal of animal sciences*.

Theart, J.J.F., 2015. *Forage quality of some Kalahari browse species and its ability to reduce methane emission* (Doctoral dissertation, University of Pretoria).

Thornton, P.K. and Herrero, M., 2010. Potential for reduced methane and carbon dioxide emissions from livestock and pasture management in the tropics. *Proceedings of the National Academy of Sciences*, *107*, pp.19667-19672.

Tilley, J.M.A. and Terry, R.A., 1963. A two-stage technique for the in vitro digestion of forage crops. *Grass and forage science*, *18*, pp.104-111.

Tolera, A. and Abebe, A., 2007. Livestock production in pastoral and agro-pastoral production systems of southern Ethiopia. *Livestock Research for Rural Development*, *19*, pp.4-7.

Tomkins, N.W., McGinn, S.M., Turner, D.A. and Charmley, E., 2011. Comparison of open-circuit respiration chambers with a micrometeorological method for determining

methane emissions from beef cattle grazing a tropical pasture. *Animal Feed Science and Technology, 166*, pp.240-247.

Toral, P.G., Belenguer, A., Frutos, P. and Hervás, G., 2009. Effect of the supplementation of a high-concentrate diet with sunflower and fish oils on ruminal fermentation in sheep. *Small Ruminant Research, 81*, pp.119-125.

Toral, P.G., Frutos, P., Hervás, G., Gómez-Cortés, P., Juárez, M. and De la Fuente, M.A., 2010. Changes in milk fatty acid profile and animal performance in response to fish oil supplementation, alone or in combination with sunflower oil, in dairy ewes. *Journal of Dairy Science, 93*, pp.1604-1615.

Toral, P.G., Hervás, G., Belenguer, A., Bichi, E. and Frutos, P., 2013. Effect of the inclusion of quebracho tannins in a diet rich in linoleic acid on milk fatty acid composition in dairy ewes. *Journal of Dairy Science, 96*, pp.431-439.

Udén, P., Colucci, P.E. and Van Soest, P.J., 1980. Investigation of chromium, cerium and cobalt as markers in digesta. Rate of passage studies. *Journal of the Science of Food and Agriculture, 31*, pp.625-632.

Van der Merwe, M.; Scholes, R.J. Greenhouse Gas Emissions Inventory of 1990 for South Africa; National Research Foundation: Pretoria, South Africa, 1998.

Van Soest, P.V., Robertson, J.B. and Lewis, B.A., 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *Journal of dairy science, 74*, pp.3583-3597.

Vargas-Bello-Pérez, E., Cancino-Padilla, N., Romero, J. and Garnsworthy, P.C., 2016. Quantitative analysis of ruminal bacterial populations involved in lipid metabolism in dairy cows fed different vegetable oils. *Animal, 10*, pp.1821-1828.

Vasta, V., Pennisi, P., Lanza, M., Barbagallo, D., Bella, M. and Priolo, A., 2007. Intramuscular fatty acid composition of lambs given a tanniniferous diet with or without polyethylene glycol supplementation. *Meat Science, 76*, pp.739-745.

Waghorn, G., 2008. Beneficial and detrimental effects of dietary condensed tannins for sustainable sheep and goat production—Progress and challenges. *Animal Feed Science and Technology, 147*, pp.116-139.

Waghorn, G.C. and Hegarty, R.S., 2011. Lowering ruminant methane emissions through improved feed conversion efficiency. *Animal Feed Science and Technology*, *166*, pp.291-301.

Wallace, M. and Charles, M., 2013. What goes in does not always come out: the impact of the ruminant digestive system of sheep on plant material, and its importance for the interpretation of dung-derived archaeobotanical assemblages. *Environmental Archaeology*, *18*(1), pp.18-30.

Wall, E., Simm, G. and Moran, D., 2010. Developing breeding schemes to assist mitigation of greenhouse gas emissions. *Animal*, *4*, pp.366-376.

Wanapat, M., Mapato, C., Pilajun, R. and Toburan, W., 2011. Effects of vegetable oil supplementation on feed intake, rumen fermentation, growth performance, and carcass characteristic of growing swamp buffaloes. *Livestock Science*, *135*, pp.32-37.

Wanapat, M., Kongmun, P., Poungchompu, O., Cherdthong, A., Khejornsart, P., Pilajun, R. and Kaenpakdee, S., 2012. Effects of plants containing secondary compounds and plant oils on rumen fermentation and ecology. *Tropical Animal Health and Production*, *44*, pp.399-405.

Wang, M., Sun, X.Z., Janssen, P.H., Tang, S.X. and Tan, Z.L., 2014. Responses of methane production and fermentation pathways to the increased dissolved hydrogen concentration generated by eight substrates in in vitro ruminal cultures. *Animal Feed Science and Technology*, *194*, pp.1-11.

Wang, Y., McAllister, T.A., Yanke, L.J. and Cheeke, P.R., 2000. Effect of steroidal saponin from Yucca schidigera extract on ruminal microbes. *Journal of Applied Microbiology*, *88*, pp.887-896.

Wenner, B.A., de Souza, J., Batistel, F., Hackmann, T.J., Yu, Z. and Firkins, J.L., 2017. Association of aqueous hydrogen concentration with methane production in continuous cultures modulated to vary pH and solids passage rate. *Journal of dairy science*, *100*, pp.5378 - 5389.

Wolin, M.J., 1960. A theoretical rumen fermentation balance. *Journal of Dairy Science*, *43*, pp.1452-1459.

Wolin, M.J., Miller, T.L. and Stewart, C.S., 1997. Microbe-microbe interactions. In *The rumen microbial ecosystem* Springer, Dordrecht, pp. 467- 491.

Yang, H.E., 2017. *Comparsion of digestion and particle-associated bacteria after in situ incubation of different barley varieties in the rumen of cattle* (Doctoral dissertation, University of Saskatchewan).

Yáñez-Ruiz, D.R., Bannink, A., Dijkstra, J., Kebreab, E., Morgavi, D.P., O'Kiely, P., Reynolds, C.K., Schwarm, A., Shingfield, K.J., Yu, Z. and Hristov, A.N., 2016. Design, implementation and interpretation of in vitro batch culture experiments to assess enteric methane mitigation in ruminants—a review. *Animal feed science and technology*, *216*, pp.1-18.

Zhang, R.H., Mustafa, A.F. and Zhao, X., 2006. Effects of feeding oilseeds rich in linoleic and linolenic fatty acids to lactating ewes on cheese yield and on fatty acid composition of milk and cheese. *Animal Feed Science and Technology*, *127*, pp.220-233.

Zhang, X., Zhang, H., Wang, Z., Zhang, X., Zou, H., Tan, C. and Peng, Q., 2015. Effects of dietary carbohydrate composition on rumen fermentation characteristics and microbial population in vitro. *Italian Journal of Animal Science*, *14*(3), p.3366.

APPENDIX

Ethical Clearance

UNIVERSITY OF
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02 March 2018

Mr Mahlogonolo Daniel Serumula (217078104)
School of Agricultural, Earth & Environmental Sciences
Pietermaritzburg Campus

Dear Mr Serumula,

Protocol reference number: AREC/057/017M
Project title: Effects of indigenous plants and chemical inhibitors on methanogenesis

Full Approval — Research Application

With regards to your revised application received on 12 February 2018. The documents submitted have been accepted by the Anima,[i] Research Ethics Committee and FULL APPROVAL for the protocol has been granted with the following conditions:

CONDITIONS:

1. A prior gas test must be conducted to determine the gas collection time of the gas pump.
2. Animals must be closely monitored while they will be on the metabolic crate for discomfort and distress (if any).

Please note: Any Veterinary and Para-Veterinary procedures must be conducted by a SAVC registered VET or SAVC authorized person.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 02 March 2019.

Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health / wellbeing.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

Prof S Islam, PhD
Chair: Animal Research Ethics Committee

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Founding Campuses: Edgewood Pietermaritzburg