Use of selected tropical feeds and additives as modulators of rumen fermentation and methanogenesis in ruminants

By

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UNIVERSITEIT VAN PRETORIA
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YUNIBESITHI YA PRETORIA

Dedicated to:

My beloved wife, Yenenesh Gizachew

and

My children, Hermoneal and Abenezer Belete

Declaration

I, **Belete Shenkute Gemeda**, declare that the thesis which I hereby submit for the degree of Doctor of Philosophy in Animal Science at the University of Pretoria, is my own work and has not previously been submitted by me for the degree at this or any other tertiary institution.

Date ----------

Signed----------

Preface

The dissertation is based on the following chapters, which have been published or are to be submitted for publication in peer review journals.

1. **Gemeda BS, Hassen A** (2014) *In vitro* fermentation, digestibility and methane production of tropical perennial grass species. *Crop and Pasture Science* **65**, 479-488.
2. **Gemeda BS, Hassen A** (2014) Effect of tannin and species variation on *in vitro* digestibility, gas, and methane production of tropical browse plants. *Asian-Australasian Journal of Animal Science* **28**,188-199.
3. **Gemeda BS, Hassen A, Odongo NE** (2014) Effect of application of fibrolytic enzyme products at different levels on *in vitro* ruminal fermentation of low-quality feeds and total mixed ration. *Journal of Animal and Plant Sciences* **24**, 1293-1302.
4. **Gemeda BS, Hassen A** (2015) Methane production of two roughage and total mixed ration as influenced by cellulase and xylanase enzyme addition. *Scientia Agricola* **72**, 11-19.
5. **Gemeda BS, Hassen A, Morgavi DP** (2015) Effect of *Acacia nilotica, Grewia flava and Monechma genistifolium* foliage supplementation on digestibility and methane emission of sheep (to be submitted for publication in *Animal Feed Science and Technology*).
6. **Gemeda BS, Hassen A, Morgavi DP** (2015) Ruminal fermentation and methane production of Merino rams supplemented with enzyme and nitrate (to be submitted for publication in *Animal Feed Science and Technology*).

The main objective of this work was to identify potential rumen modulators or feed additives that improve fibre digestion while reducing enteric methane production from tropical feeds. The research was conducted in the Department of Animal and Wildlife Sciences, University of Pretoria, South Africa. To accomplish this, feed additives and feeding technologies that were tested elsewhere to reduce methane in various ruminant production systems were reviewed in Chapter one. In Chapter two, the *in vitro* fermentability and methane production of commonly used tropical perennial grasses were studied. In Chapter three, 19 tropical browses collected from Pretoria were studied for their potential fermentation and methane production under *in vitro* conditions. In Chapter four, three types

of tannin-containing browse foliage that were collected from the Kalahari Desert in South Africa were used to replace *Medicago sativa* hay in a total mixed ration fed to Merino rams in order to investigate their effect on rumen fermentation and enteric methane production. In Chapter five, cellulase and xylanase fibrolytic enzymes were studied for their feed fermentation potential and methane production at seven dose rates. In Chapter six, the effects of fibrolytic enzymes, nitrate and enzyme-nitrate mixture were evaluated by measuring rumen fermentation and enteric methane production in Merino rams. Finally, general conclusions, recommendations and critical evaluation based on this experimental work were presented in Chapter seven.

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- Mr Paul Sonnoder programmed the software for the methane recovery and methane recording methods in an open circuit methane chamber unit and deserves my special acknowledgement.
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We know that all things work together for good to them that love God, to them who are the called according to [His] *purpose* (Rom 8:29 KJV).

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List of acronyms and initialisms

a: soluble fraction of fibre

ADF: acid detergent fibre

ADL: acid detergent lignin

ADFN: acid detergent fibre nitrogen

AOAC: Association of Official Analytical Chemists

b: the insoluble, but slowly fermentable fraction of fibre

$BW^{0.75}$: metabolizable bodyweight

c: rate of fermentation of b

CH_4: methane

CO_2: carbon dioxide

CP: crude protein

CT: condensed tannins

DM: dry matter

DMI: dry matter intake

ED: effective degradability

EE: ether extract

EFE: exogenous fibrolytic enzymes

FCR: feed conversion ratio

g: gram

GE: gross energy

Gg: giga gram

GHG: greenhouse gas

GLM: general linear model

GP: gas production

IVDDM: *in vitro* digestible dry matter

kg: kilogram

m asl: metres above sea level

ME: metabolizable energy

mg: milligram

MJ:mega joules

ml: millilitres

MW: molecular weight

N: nitrogen

NDF: neutral detergent fibre

NDFN: neutral detergent fibre nitrogen

NFC: non-fibre carbohydrates

NH_3-N: ammonia nitrogen

NPN: non-protein nitrogen

NRC: National Research Council

NSC: non-structural carbohydrate

NTT: non-total tannins

OM: organic matter

OMI: organic matter intake

PD: potential degradability

PEG: polyethylene glycol

PVPP: polyvinyl-polypyrrolidone

SAS: Statistical Analyses Software

SCVFA: short-chain volatile fatty acid

TDN: total digestible carbohydrate

TMR: total mixed ration

TP: total phenols

TT: total tannins

VFA: volatile fatty acid

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Summary

This set of *in vitro* and *in vivo* experiments evaluated the rumen fermentation, digestibility and methane (CH_4) production of certain tropical feeds as incubated alone and with some additives. This was followed by supplementation of selected additives (enzymes, tannins and nitrate) to Merino rams to investigate their effects on nitrogen retention, organic matter digestibility, volatile fatty acid (VFA) production and enteric CH_4 production.

The fermentation potential and CH_4 production of sixteen perennial grasses were correlated with their chemical composition. It was noted that fermentation and CH_4 production were both influenced by nitrogen and fibre content of grasses. Perennial grasses with high nitrogen and low fibre content resulted in better fermentation and produced less CH_4. Thus, it is important to focus on screening and selecting perennial grasses with high N and low fibre content to mitigate CH_4 production under tropical conditions.

Under the communal production system in Africa, ruminants have free access to trees and shrubs throughout the year, especially in the dry season. These browses contain high nitrogen, in addition to tannins, which can suppress rumen methanogenesis. As tannin composition and concentration vary among browses, 19 tanniferous browses were studied for their effect on rumen fermentation and CH_4 production with and without the inclusion of the tannin-binding agent polyethylene glycol (PEG). The study indicated that tannin decreased

gas production, IVOMD, total VFA and CH_4 production. The low methanogenesis and substantial ammonia-N generation observed with some browses indicate their usefulness as rumen-manipulating agents. However, systematic evaluation is needed to determine the optimum levels of supplementation in a mixed diet in order to attain a maximal decreasing effect on enteric CH_4 production with a minimal negative effect on rumen fermentation of poor-quality roughage-based diet. Based on this *in vitro* finding, a study was conducted in a 4 x 4 Latin square design with the foliage of three tannin-rich browses *(Acacia nilotica, Grewia flava* and *Monechma genistifolium)* that were collected from the Kalahari Desert of South Africa, and used to replace lucerne hay in a total mixed ration diet. The study investigated the effect of the inclusion of these browses in the diets of sheep on CH_4 mitigation and rumen fermentation characteristics. Addition of the browses to the diets of sheep, however, led to net body N retention, and the observed values were lower than those on the control diet. The inclusion of the tannin-rich plants reduced CH_4 emission per day and per unit of feed intake up to 39% relative to the control diet. In conclusion, *Monechma genistifolium*, *Grewia flava* and *Acacia nilotica* could be regarded as alternative protein supplements, and may have the potential to reduce CH_4 emission in ruminants.

Crop residues such as stover and hay are common ruminant feeds in the tropics and subtropics of Africa. Improving their utilization and reducing associated CH_4 emission is a current research priority. The inclusion of exogenous fibrolytic enzymes such as cellulase and xylanase at different levels resulted in substantially improved fermentation and digestibility. The observed improvement in increasing levels of enzymes was significant for its linear and quadratic effect. However, there was an associated increase in CH_4 production. Considering the efficiency of DM and NDF fermentation and production of associated VFA with the various levels of enzymes, the use of 1 mg enzyme g^{-1} DM of feed could be a good option with these feeds. Although fermentation and digestibility were improved with supplementation of these enzymes, increased CH_4 production is a big concern, as it is wastage of energy. In this regard, a study was conducted to investigate the effects of the inclusion of enzyme, nitrate and enzyme-nitrate mixture in a total mixed ration (TMR) of Merino rams on rumen fermentation and CH_4 production. It was observed that nitrate could be used alone or mixed with enzymes to reduce enteric CH_4 emissions and improve N retention, but it is important to consider the amount of nitrate mixed with enzyme to utilize such combinations effectively for improving feed fermentation and reducing CH_4 production.

In general, the various trials carried out in this thesis revealed that supplementation of browse with low tannin concentration, nitrate and enzyme-nitrate mixture as an additive can be an alternative option to modulate rumen fermentation in sheep fed tropical and sub-tropical forage-based diets to improve the fermentability of feed and mitigate enteric methane production.

General introduction

Ruminants play a significant role in the livelihoods of farmers and national economies in tropical and sub-tropical regions of Africa. In these regions, ruminants are fed mainly fibrous feeds that are low in protein, energy, minerals and vitamin content. As these feeds have a high content of ligno-cellulosic materials, their degradation in the rumen is very low, resulting in low nutrient release and high enteric CH_4 emission per animal (McDonald *et al.,* 2011). In the rumen, CH_4 is produced under anaerobic conditions by methanogenic *archaea* that gain energy by reducing CO_2 with H_2 to form CH_4 (Leng, 2008). Its production depends primarily on the quantity and quality of the diet (Van Soest, 1994; Beauchemin *et al.,* 2008) consumed by the animals. In particular, the nature of fermented carbohydrates and their fibre concentration play a vital role in the amount of CH_4 produced from diets (Santoso *et al.,* 2003; Hindrichsen *et al.,* 2003). Methane represents about 8% of energy intake, with a range from 3% to 15% GE, depending on diet characteristics (Robertson & Waghorn, 2002; Hristov *et al.,* 2013).

Altering the diet and improving feed utilization efficiency are effective ways of mitigating CH_4 production from ruminants (Smith *et al.,* 2007; Singh *et al.,* 2012). In the tropics, fibre constitutes a major portion of the diet consumed by ruminant livestock species. Thus, it is crucial to consider improving its fermentation to increase nutrient flow, decrease retention time by the animal, and reduce CH_4 emission per unit of animal product. In this regard, the use of exogenous fibre-fermenting enzymes has resulted in potentially positive outcomes in terms of feed utilization (Beauchemin *et al.,* 2003; Adesogan *et al.,* 2007; Krueger *et al.,* 2008). In particular, the inclusion of cellulase and xylanase in diets of ruminants was reported to improve DM intake (Beauchemin *et al.,* 2000; Pinos-Rodriguez *et al.,* 2002), increase digestibility (Krueger *et al.,* 2008), and increase the average daily gain of beef cattle (Beauchemin *et al.,* 1999) and milk production of dairy cows (Yang *et al.,* 2000; Adesogan *et al.,* 2007). However, there is limited information about associated CH_4 production and, where available, the reports were contrasting. In addition, the effects of exogenous enzymes on feed substrate vary with the proportion of concentrate in the diet (Giraldo *et al.,* 2008), with enzyme doses (Jalilvand *et al.,* 2008), pH (Yang *et al.,* 2002), the moisture content of feed (Wang *et al.,* 2002) and with methods of supplementation (Beauchemin *et al.,* 1999; Yang *et al.,* 2000; Krueger *et al.,* 2008). Their interaction with different sources and levels of nitrogen

is not clear either. Additionally, the optimal level of enzymes depends on the diet under consideration, indicating the need to determine the optimum application rate of enzyme preparation for individual feeds (Yang *et al.,* 1999).

One important strategy for improving fibre fermentation in ruminants is to supplement poor-quality diets with browses rich in nitrogen (Tolera *et al.,* 1997; FAO, 2007). Tropical browses contain reasonably high nitrogen and low fibre content, and can be good candidates for supplementing poor-quality feeds. However, they contain tannins in variable amounts as a defence mechanism against herbivores. Several studies have indicated that tannins have anti-methanogenic activity, either by direct inhibition of methanogens or indirectly through inhibition of protozoa (Animut *et al.,* 2008; Bhatta *et al.,* 2009; Jayanegara *et al.,* 2009). Interestingly, tannins can be beneficial or detrimental to ruminants, depending on their type, the amount consumed, structure and molecular weight, and the physiology of the animal (Hagerman & Butler, 1989). It has been reported that consumption of low or moderate concentrations of tannin does not affect voluntary feed intake, but can suppress CH_4 production (Waghorn *et al.,* 1994). Tannins vary in structure, depending on source, type and level, which further variably influence methanogenesis, fermentation and rumen function (Mueller-Harvey, 2006; Patra *et al.,* 2006). This was evident for tannins from different plantsources that exhibited different effects in terms of the magnitude of gas production and digestibility at the same concentration level (Makkar, 2003). This indicates that tannins from different plants might also show different effects in rumen fermentation and methanogenesis. Thus, tropical tanniferous browses in different ecologies have to be characterized for the effects of tannins on fermentation and methanogenesis. With such knowledge, optimal utilization of tannin-containing browses for different ruminant production systems could be recommended. Moreover, supplementation of tannin-containing browses could be regarded as a way forward to increase poor-quality feed utilization and reduce enteric CH_4 emission (Monteny & Chadwick, 2006; Beauchemin *et al.,* 2008).

During microbial fermentation of feeds in the rumen, the H gas produced is used as a substrate for methanogens to produce CH_4. Especially when VFA production is shifted towards acetate, there will be more H^+ and CH_4. In many research reports, where enzymes were supplemented, the increase in fibre digestion was associated mainly with an acetate-shifted volatile fatty acid production (Arriola *et al.,* 2011). Under such conditions, stimulation of hydrogen utilization towards other pathways or supplementation of an electron acceptor

(hydrogen sink) might be beneficial for the animal. In this regard, nitrate has been reported to be a promising methane mitigation agent and has received wide research efforts as an alternate electron acceptor in the rumen (Van Zijderveld *et al.,* 2010; Hulshof *et al.,* 2012). According to Gerber *et al.* (2013), the addition of nitrate could be attractive in developing countries, where forages contain negligible levels of nitrate and low crude protein. It could serve as a source of rumen-degradable nitrogen for microbial protein synthesis. This might be because of the conversion of nitrate to ammonia-N, during which more (8) electrons are consumed or trapped by out-competing methanogens for electrons (Leng, 2008; Leng & Preston, 2010). Thus in assimilatory nitrate reduction, energy is conserved for microbial use instead of loss in CH_4 generation (Leng, 2008). It has been indicated that nitrate reduced enteric CH_4 production by up to 50% from sheep (Van Zijderveld *et al.,* 2010) and cattle (Hulshof *et al.,* 2012). Nitrate supplementation increased N retention per unit of OM digested, with subsequent reduction of CH_4 (Sophal *et al.,* 2013). However, gradual adaptation of animals to dietary nitrate is crucial for successful utilization of nitrates as a CH_4-mitigating strategy (Leng, 2008).

Theoretically, it is possible to combine exogenous fibrolytic enzymes with soluble nitrate sources to supplement ruminant feeds in the tropics in order to improve rumen fibre fermentation and nitrogen utilization, and at the same time to reduce CH_4. Here, the additional amount of hydrogen produced because of improved organic matter fermentation due to exogenous fibrolytic enzyme inclusion might be trapped by nitrate. However, it is difficult to know whether the net effect is additive or synergetic. In addition, the types of nitrate source and the optimum amount of nitrate to be mixed with enzyme and vice versa vary with the nitrogen level of the feed, and other factors. There is limited information about such techniques, their effect on degradability, rumen fermentation and CH_4 production from different feeds, ruminant production system, and species of animals. To quantify the exact benefits, more data need to be generated using *in vitro* and *in vivo* experiments.

Any nutritional management interventions that can contribute to the reduction of wastage of nutrients, loss of energy and reduced enteric CH_4 production can generally be regarded as win-win solutions (Boadi *et al.,* 2004; Monteny & Chadwick, 2006; Beauchemin *et al.,* 2008). More importantly, it is crucial to identify low-cost and low-risk mitigation strategies, as these strategies can be easily adapted by the majority of resource-poor farmers, pastoralists and emerging commercial farmers residing in tropical areas. Furthermore, the

competitiveness and sustainability of the recommended strategy or intervention is important. This research study was undertaken with the overall objective of identifying potential rumen modulators or feed additives that improve fibre fermentation, while reducing enteric CH_4 production.

CHAPTER 1

Review of literature

1.1. The role of livestock in food security and livelihoods

The global human population is estimated to be 9.15 billion in 2050 and most of the increase is expected to be in developing countries (United Nations, 2010). This might result in a huge discrepancy in terms of food security, especially foods of animal origin. As a result, the livestock sector will face the challenge of increasing production to meet the growing demand for animal protein (Thornton, 2010). According to the Food and Agriculture Organization (FAO) of the United Nations (2010), cereal, fish, meat and egg production in developing countries increased by 78, 113, 127 and 331%, respectively, in the past two decades. This growth was driven by the rapidly increasing demand for livestock products, coupled with population growth, urbanization and increasing incomes. Despite this, the animal product consumption level is still lower in developing countries than in developed countries (Thornton & Gerber, 2010), indicating substantial need for expansion of livestock production.

The global livestock industry represents US$ 1.4 trillion worth of assets, employs at least 1.3 billion people and directly supports the livelihoods of 800 million smallholder farmers in developing countries (FAO, 2010). Livestock production can also diversify risks by decreasing the vulnerability of people to climate, disease and market shocks. In smallholder crop-livestock, agro-pastoral, and pastoral livestock systems of tropical Africa, livestock production is one the options that could increase income and sustain the livelihoods of farmers. Above all, ruminants are capable of converting plant fibres (structural carbohydrates), which constitute the planet's most renewable abundant resource, into useful end products (high-quality protein, fibre, etc).

1.2. Current challenges associated with livestock production

Livestock production is a critical component of agriculture by generating income and improving the nutrition of people in developing countries. Despite such contributions, currently livestock production faces climate change challenges globally, in addition to feed shortages in the developing and least developing countries. The primary problem of livestock production in Africa is lowquality and quantity of feed throughout most of the year. In this region, livestock production is dominated by smallholder farmers with communal and small-sized private grazing areas (Ajayi *et al.,* 2005). Such areas are characterized by an inadequate supply of feed, which is low in quality with low nitrogen and high fibre contents. Similarly, crop residues, which are other major sources of feed, are characterized by low nitrogen concentration, high fibre and low digestibility (Valarini & Possenti, 2006). Thus, these materials may be not sufficient to meet the maintenance and production nutritional requirements of the animal, which may increase the environmental footprint of livestock production from the system (Mendieta-Ariaca *et al.,* 2009). Therefore, supplementation with nitrogen and energy is essential to improve palatability, intake, and rumen fermentation and improve animal production (Ondiek *et al.,* 1999).

In current decades, the impact of global warming and continued uncontrolled release of greenhouse gasses (GHG) have negatively influenced livestock production and food security (FAO, 2013). According to Scholtz *et al.* (2014), global warming has direct influence on ambient temperature,which has a consequent effect on water supplies, the distribution of livestock species and breeds, their adaptability, the incidence and types of diseases, feed supplies, and overall food security. This is due to the changes associated with temperature itself, relative humidity, rainfall distribution in time and space, altered disease distribution, changes in the ecosystem and biome composition, woody species encroachment, and alien plant invasion (Linington, 1990; Scholtz *et al.,* 2011; Scholtz *et al.,* 2012).

Livestock production contributes a significant amount of GHG emissions worldwide, generating carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) throughout the production cycle. The global mean surface temperature increased by 0.6 ± 0.2°Cover the twentieth century, due primarily to increasing concentrations of GHG in the atmosphere (Forster *et al.,* 2007). Livestock contributes to total GHG emissions through land use and land-use change (2.5 Gt CO_2-eq); feed production, except C released from soil (0.4 Gt CO_2-

eq); enteric fermentation from ruminants (CH_4) and on-farm fossil fuel use (CO_2) (1.9 Gt CO_2-eq); manure management (2.2 Gt CO_2-eq); and processing and international transport (0.03 Gt CO_2-eq) (Steinfeld *et al.,* 2006). The contribution of livestock by respiration accounts for only a small part of GHG emissions, but other livestock-related activities play a much greater role.

Methane has a global warming potential roughly 25 times that of CO_2 over a 100-year time span and is estimated to contribute up to 20–30% of global warming (Solomon *et al.,* 2007). The big concern is its global atmospheric concentration,which increased from 715 ± 4 ppb in the 1750s to 1 799 ± 2 ppb in 2010 (Forster *et al.,* 2007; Kirschke *et al.,* 2013). This could increase mean surface temperatures by 2100 about 1.5 to 2°C (Collins *et al.,* 2013). This rate of increase has differed over time, most noticeably with global atmospheric CH_4 concentrations appearing to stabilize between 1999 and 2007; however, CH_4 concentrations are again rising (Kirschke *et al.,* 2013). In the developed world, they are expected to decline due to increased productivity, coupled with declining number of ruminants (Steinfeld, 2006). However, in the developing world and from the African continent they are expected to rise due to increases in animal numbers (Herrero *et al.,* 2008).

1.3. Ruminal carbohydrate fermentation and CH_4 production

1.3.1. Rumen environment

The rumen, the main site of enteric CH_4 production, contains a diverse and dense microbial population living symbiotically, and plays a significant role in the feed digestive processes of ruminant animals (McDonald *et al.,* 2011). The rumen has a stable and dynamic environment, which is well established to perform the function of bioconversion of feed into rumen fermentation products (Kamra, 2005). The microbial ecosystem comprises numerous populations of bacteria, anaerobic fungi, ciliate protozoa (Hobson 1989; Kamra 2005) and methanogen microbes (Cieslak *et al.,* 2013). These microbes play a crucial role through their involvement in fibre fermentation, either through direct attachment to feed particles and secreting fibre fermenting enzymes or indirectly by enhancing the attachment of other microbes to increase fibre digestion. According to Niwinski (2012), rumen microbes have highly complicated and diverse synergistic and antagonistic relationships among the classes of microbes. For example, populations of fibrolytic bacteria producing H_2 are positively

correlated to methanogens due to the inter-species hydrogen-transfer relationship in the rumen (Morgavi *et al.,* 2010). During this process, carbohydrates are fermented to produce volatile fatty acids (VFAs), energy, CH_4, CO_2 and heat. CO_2 and CH_4 are eliminated via the nose and mouth by belching and eructation, leading to loss of energy (Murray *et al.,* 1976). The relationship between fermenting species and H-utilizing microbes normally exists as a symbiotic function, which is called 'interspecies hydrogen transfer' (Miller, 1995). Thus, synthesis of CH_4 occurs because of the exchange of metabolites between H-producing microbes such as fibrolytic fungi and bacteria and H-consuming microbes such as methanogens (Kobayashi, 2010). This continuous production and removal of H facilitates continuous fibre fermentation in the rumen (Ushida *et al.,* 1997).

1.3.2. Methane production and associated loss of feed energy

Methane is produced by methanogenic archaea as a by-product of anaerobic fermentation of feed in the rumen. This production causes a significant loss of dietary energy (Bayat *et al.,* 2012). With normal rumen functioning, methanogenesis is essential for optimal rumen performance, because it prevents H accumulation that can lead to inhibition of dehydrogens involved in the oxidation of reduced co-factors (Martin *et al.,* 2009). Fermentation is an oxidative process in which co-factors such as NADH, FADH, NADPH have to be reduced to NAD^+, $NADP^+$, FAD^+ through dehydrogenation reactions by releasing H in the rumen (Martin *et al.,* 2009). As soon as reduced co-factors are produced, H is used by methanogens to reduce CO_2 by forming CH_4 according to the following equation:

$$\mathbf{CO_2 + 4H_2 = CH_4 + 2H_2O}$$ ---------- equation (1)

Methane is a source of feed energyloss to the animal (Waghorn *et al.,* 2002). It represents on average 10% of gross energy (GE) intake, which ranges from 7% to 17% of gross energy (GE), depending on diet characteristics (Woodward *et al.,* 2001; Robertson & Waghorn, 2002; Hristov *et al.,* 2013). Methane contains 892.6 KJ Mol^{-1} energy at 25°C and 101.3 kPa (Takahashi *et al.,* 1998, 2006). This amount of gross energy is lost instead of contributing to the total supply of energy for metabolism in the ruminant. In tropical ruminant production systems, this value might be higher due to poor-quality diets, which are often deficient in vital nutrients for optimal microbial growth in the rumen (Leng, 1991).

1.3.3. Relationship between chemical composition, digestion and CH_4 production

The amount of H produced during fermentation of feed is highly dependent on the quality of diet and the proportion of the different types of rumen microbes, because the pathways for VFA production differ in terms of H input/output (Fig 1.1). Other factors, such as pH, feeding strategy, animal species and environmental factors, also determine CH_4 production in the rumen (Kumar *et al.,* 2009).

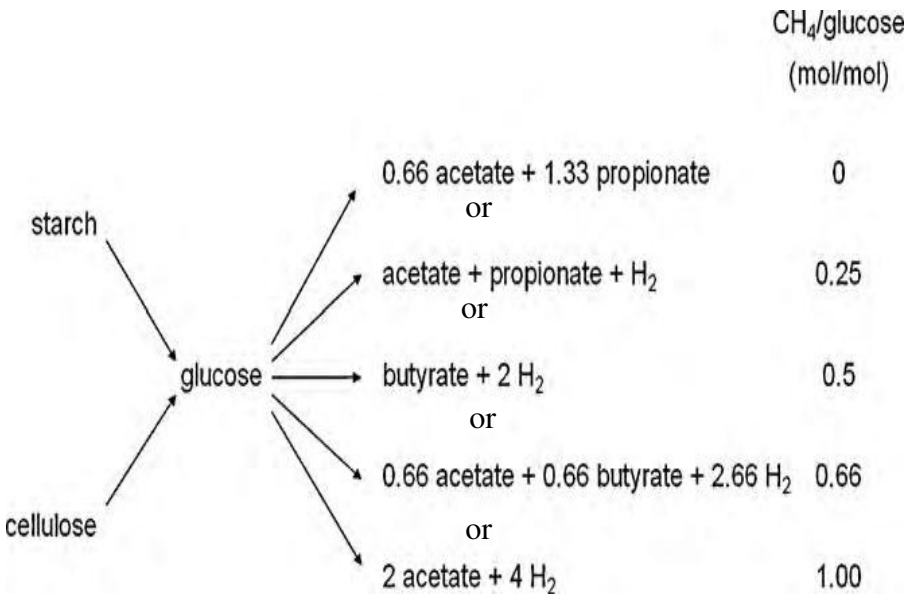

Figure 1.1 Possible pathways of cellulose and starch fermentation via glucose to acetate, propionate butyrate and hydrogen.For simplicity, CO_2, and H_2O are not shown (Jenssan *et al.,* 2010)

From these equations, shifting the ruminal fermentation patterns from acetate to propionate will significantly reduce both hydrogen and CH_4 production. Diets that have a high forage content result in an acetic-type fermentation, thus increasing CH_4 production, compared with concentrate-rich diets that result in a propionic type of fermentation (Rowlinson *et al.,* 2008; Kingston-Smith *et al.,* 2010). Thus, establishing a relationship between CH_4 emission and molar proportions of the various VFA could create an opportunity to better understand the ways to reduce enteric CH_4 emissions (Mirzaei-Aghsaghali *et al.,* 2011). In this regard, Jenssan *et al.* (2010) developed a model that predicts CH_4 production in relation to methanogenic growth rate, propionate formation, ruminal passage rate and pH. For example,

a large volume of CH_4 is produced per unit digested feed with low ruminal solid passage rate and less propionate formation, and vice versa, as described in Fig 1.2.

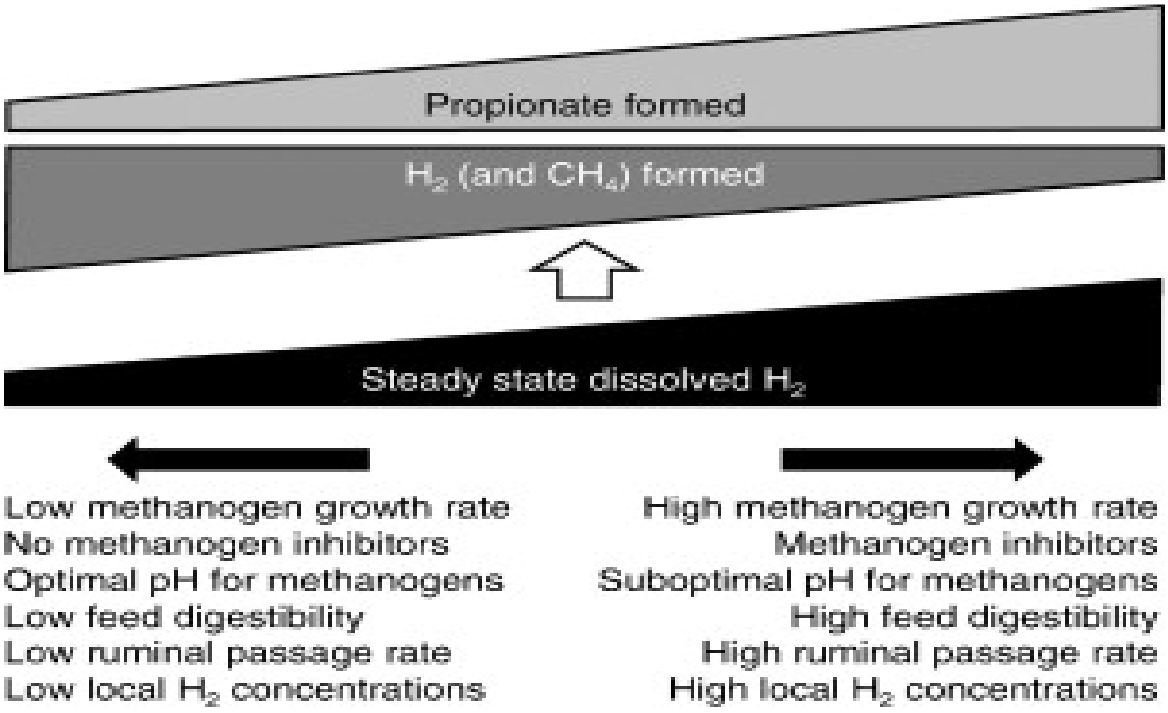

Figure 1.2 Diagram summarizing the model that considered methane production in association with methanogenic growth rate, priopionate formation, ruminal passage rate and pH (Jenssan *et al.,* 2010)

Forage plants vary according to species, variety, age at harvest and preservation. This allows for variation in composition that further affects fermentation and CH_4 production. Thus, forage quality significantly influences intake, digestibility and enteric CH_4 production (Sejian *et al.,* 2011). In the rumen, during fermentation, carbohydrate fractions vary with the amount of CH_4 yield, but the lowest come from sugars (Tamminga *et al.,* 2007). Digestion of cell wall components normally leads to higher losses than non-cell wall components (Johnson & Johnson, 1995). Methane production per unit of cellulose digested has been shown to be three times that of hemicellulose (Moe & Tyrell, 1979). Cellulose and hemicellulose ferment at a slower rate than non-structural carbohydrates, yielding more CH_4 per unit of substrate digested (McAllister *et al.,* 1996). This is because digestion of organic matter and production of VFA and CH_4 are dependent mainly on its structural composition. In addition, the relative proportion of cell types present in its tissues and the existence of factors restricting microbial access to walls determine its digestion (Van Soest, 1994). Hence carbohydrates vary greatly in these structures and consequently in CH_4 production.

The concentration of fibre (NDF and ADF) also rises with increasing maturity of the plants and this greatly limits fermentation and significantly augments the volume of CH_4produced. This is because fibre is a major component of the cell wall of forages and has a low energy and low digestibility co-efficient (Hatfield *et al.,* 1999; Azzaz *et al.,* 2012). In addition, the cell wall is an interwoven matrix of polymers that form a complex linkage and dynamic structure. This forms barriers against microbial invasion and limits their access to the digestible cell-wall components of plants, as well as digestibility (Krueger *et al.,* 2008; McDonald *et al.,* 2011). In this regard, lignin is the key compound that limits forage cell wall digestibility by microbes and the host animal enzymes (Jung & Allen, 1995). For example, feeding temperate grass is better than tropical grass, as the latter is 13% less digestible and more lignified (Minson, 1990). Likewise, feeding legumes or legume-grass forage mixture showed better fermentation and lower CH_4 production than grass alone (Johnson & Johnson, 1995). This is due to lower proportions of structural carbohydrates and faster passage rates in legumes compared withgrasses. On the other hand, grasses have higher cell wall (NDF) content than legumes, but NDF in grasses is generally more digestible than in legumes (Van Soest, 1994). Itwould be more practical to consider forage composition in the context of the whole ration in ruminant feeding with regard to digestibility and CH_4 production (FAO, 2013).

Feed intake is a critical factor for improving productivity, increasing feed efficiency and decreasing CH_4 production in ruminant feeds. Dietary NDF concentration is one of the important regulators of feed intake that determine energy yield, digesta flow and the so-called fill-limitation mechanism (McDonald *et al*., 2011). Increased intake of poor-quality feeds has little effect on CH_4 production, while increasing the digestibility of feed results in a depression in the amount of CH_4 produced per unit of feed consumed (Blaxter & Clapperton, 1965). As the digestibility of a feed increases, the amount of energy available to the animal rises, therefore the CH_4 emitted per kg of production, for example weight gain, decreases. Thus, increased digestibility of diets often means fewer CH_4 emissions per unit of production (Allard, 2009). However, with digestibility above 72%, the increasing effect on the emissions becomes marginal. Broderick *et al.* (2002) compared ryegrass silage with alfalfa silage as part of a total mixed ration of iso-nitrogenous and similar amount of NDF and reported that feed intake and milk production were much higher with the alfalfa silage diet, but with a relatively lower feed efficiency. This indicated that the major challenge with forage-based diets is improving diet quality in order to reduce CH_4, as the correlation between forage

quality and CH_4 emissions is sometimes low (Boadi *et al.,* 2004; Pinares-Patino *et al.,* 2007; Beauchemin *et al.,* 2008).

In extensive ruminant production from forage-based feeding systems, a substantial increase in CH_4 production was noted compared with grain-based feedlot systems (Pelletier *et al.,* 2010; Beauchemin *et al.,* 2011). This indicates that low fermentable feeds generally result in higher CH_4 production. Johnson and Johnson (1995) stated that as feed intake increases, the CH_4 yield decreases by about 1.6 percentage units per level of intake above maintenance. This is because of increasing intake that raises the fractional passage rate and lowers digestibility. The extent of decrease in digestibility depends on diet type and quality. A meta-analysis of 92 trials considering 497 diets showed that intake had a negative effect on diet digestibility in lactating dairy cows (Huhtanen *et al.,* 2009). The NRC (2001) model assumes that the decline in digestibility with level of feeding is a function of diet digestibility at maintenance intake. Another meta-analysis study by Sauvant and Giger-Reverdin (2009) concluded that OM digestibility decreases linearly with increasing feed intake and accompanied with a linear decrease in CH_4 yield.

In extensive grazing systems, where diets are of low to moderate digestibility, increased dry matter intake (DMI) is associated with increased CH_4 production (Hegarty *et al.,* 2010). In such conditions, CH_4 released per unit of additional intake is more than the CH_4 produced per unit intake of highly digestible feeds. On the contrary, CH_4 production per unit metabolizable energy (ME) intake is lowest for high-energy diets. In addition, small changes in energy intake result in corresponding minor changes in CH_4 output and large changes in animal performance. Many studies have attempted to determine the relationship between CH_4 production and GE intake using empirical prediction equations. These equations, however, do not fully describe changes in composition of the diet and have limited use in estimating the impact of varying nutritional strategies on CH_4 emissions (FAO, 2013). These challenges can be addressed by expressing CH_4 energy loss on a DE basis or per unit of animal product, which better reflects forage quality and other mitigation practices, such as grain or fat inclusion in ruminant diets (FAO, 2013).

1.4. Rumen modulators used to reduce CH_4 production

Enteric CH_4 production depends primarily on quantity and quality of the diet (Van Soest, 1994; Beauchemin *et al.,* 2008), the nature of fermented carbohydrates (Takahashi 2001; Santoso *et al.,* 2003), concentration of NDF and ADF (Hindrichsen *et al.,* 2003), the acetate to propionate ratio of fermented feeds (McAllister *et al.,* 1996) and the type and harvest stage of forage consumed by the animal (Arthington & Brown, 2005). If rumen fermentation patterns shift from acetate to more propionate in the total VFA production, net hydrogen and CH_4 production will be reduced. Thus, any viable strategy has to result in one or more of these goals:

1. Reduction of hydrogen production that should be achieved without impairing feed digestion.
2. Stimulation of hydrogen utilization towards pathways that produce other end products beneficial for the animal such as propionate production.
3. Inhibition of the methanogenic activity and its numbers. When using this strategy, it is important to suppress CH_4 producing micro-biota activities and proliferation without limiting rumen function.

Increased concentrate proportions in ruminant rations are generally associated with a reduction in CH_4 emission per unit of feed intake and per unit of animal product (Johnson & Johnson, 1995; Lovett *et al.,* 2003). For example, fermentation of a diet with 70% concentrate produced a 59% increase in ruminal propionate concentration and a 44 % drop in the A: Pratio in lactating dairy cows compared with a 50% concentrate diet (Agle *et al.,* 2010). Bannink *et al.* (2008) also reported that the fermentation of sugars and starch would shift rumen fermentation toward the production of propionate. In a relationship proposed by Sauvant *et al.* (2011), methane yield and A: P ratio in ruminal fluid showed a quadratic relationship in 23 experiments. These findings generally showed that higher inclusions of grain or starch content in ruminant diets lowered enteric CH_4 production. However, in most tropical and sub-tropical livestock production systems, ruminants receive only small quantities of concentrates owing to direct competition with human and monogastric animals and high costs of concentrates. In addition, previous research suggested that increased forage quality would reduce CH_4 emissions per unit of weight gain (Boland *et al.,* 2009; McGeough

et al., 2010) or per unit of animal product (Moss, 2000), owing to improvement in animal productivity. Many research reports have also shown potential for direct inhibition of methanogens through immunization of animals, and the use of plant polyphenols, bacteriophages and bacteriocins (Sliwinski *et al.,* 2002; Patra *et al.,* 2006; Goel *et al.,* 2008). Currently, indirect methods of redirecting hydrogen are also receiving a lot of scientific attention. These methods include the use of ionophores such as monensin, fatty acids/lipids, organic acids (Van Nevel & Demeyer, 1996; Ungerfeld *et al.,* 2005; Cottle *et al.,* 2011), nitrate and sulfate supplementation (Gutierrez-Banuelos *et al.,* 2007; Leng 2008; Brown *et al.,* 2011). Some of these strategies are discussed below in the context of ruminants consuming high forage diets.

1.4.1. Supplementation with concentrate-rich diets

Increasing the proportion of concentrate in the diet reduces enteric CH_4 production per unit of feed intake and animal product owing to conversion of high fermentable OM content to propionate (FAO, 2013). Increasing the proportion of concentrate in the diet of lactating dairy cows and beef cattles decreased CH_4linearly by 2.8 g CH_4 kg^{-1} DMI (Aguerre *et al.,* 2011; McGeough *et al.,* 2010). However, Moss *et al.* (1995) did not observe any relationship between dietary concentrate and CH_4 emission in sheep. In other studies, increasing the proportion of concentrate in the diets did not decrease CH_4 production (Beauchemin & McGinn, 2006; Popova *et al.,* 2011). On the contrary, some studies reported increased CH_4 production with increasing level of concentrate in the diets of ruminants (McGinn *et al.,* 2006; Doreau *et al.,* 2011). In these conditions, it is important to consider the type of concentrate, quality of forage, and the production and physiological status of the animal.

Concentrates tend to reduce rumen pH beyond the optimum range for methanogens and thus reduce CH_4 emissions. Protozoa and cellulolytic bacteria have poor biological activity at a low pH and this further decreases hydrogen production. Rowlinson *et al.* (2008) reported a positive correlation between cellulolytic bacteria and methanogens in the rumen of different animal species. Moreover, a high grain diet and the addition of soluble carbohydrates result in a shift in the fermentation pattern in the rumen and in an environment that is less conducive to methanogens. With a high grain diet, passage rates are increased, ruminal pH is lowered, and certain species of protozoa and methanogens may be eliminated or inhibited (Van Soest, 1982).

1.4.2. Supplementation with dietary lipids

Several studies have indicated the effectiveness of lipids in reducing enteric CH_4 emission (Eugene *et al.,* 2008; Eugene *et al.,* 2011; Grainger & Beauchemin, 2011; Rabiee *et al.,* 2012; Hristov *et al.,* 2013). The mitigation effect varies with concentration, chain length and degree of unsaturation of the fatty acids. It is important to consider the interactions between fatty acid and diet type (Johnson & Johnson, 1995; Eugène *et al.,* 2008). For example, in *in vitro* studies, it was revealed that medium-chain fatty acids such as lauric and myristic acids were more effective than long-chain or short-chain fatty acids (Dong *et al.,* 1997; Dohme *et al.,* 2000). The reduction in CH_4 due to lipid supplementation is attributed to an inhibitory effect of lipids on rumen microbial activity (Johnson & Johnson, 1995; Ivan *et al.,* 2004). Thus, inclusion beyond 5% had been reported to decrease fibre digestibility (Hristov *et al.,* 2013), decrease DM intake (Eugene *et al.,* 2011) and reduce OM fermentation (Johnson & Johnson, 1995; Ivan *et al.,* 2004). According to Grainger and Beauchemin (2011), inclusion levels of dietary fat of up to 8% are acceptable.This means that a 10 g kg^{-1} increase in dietary fat would decrease CH_4 yield by 1 g kg^{-1} DMI in cattle and 2.6 g kg^{-1} in sheep. The feasibility of using fats in ruminant diets is limited by affordability of oil products and the persistent effect of lipids as a mitigating agent (Woodward *et al.,* 2006). Long-term effects of dietary lipids have been studied, and many results have indicated that the fats have an inconsistent effect on fermentation parameters (Holter *et al.,* 1992; Grainger *et al.,* 2008, 2010; Grainger & Beauchemin, 2011). Therefore, the optimum level of lipid supplementation that does not affect rumen digestion and animal performance should be considered in ruminant diets (Beauchemin *et al.,* 2008; Martin *et al.,* 2010; Popova *et al.,* 2011).

1.4.3. Supplementation with plant tannins

Tannins are compounds with high molecular weight that have the capacity to form reversible and irreversible complexes with proteins, polysaccharides (cellulose, hemicellulose, and pectin), alkaloids, nucleic acids and minerals (Van Soest, 1994; Schofield *et al.,* 2001). They are synthesized naturally in nutritionally important forage trees, shrubs and legumes, fruits, cereals and grains in variable amounts. Tannins are categorized broadly into condensed or hydrolysed tannins. Hydrolysable tannins are made up of a carbohydrate core whose hydroxyl groups are esterified with phenolic acids. Condensed tannins (pro-antho-cyanidins) are non-branched polymers of flavonoid units and usually have a higher molecular weight

relative to hydrolysable tannins (Mueller-Harvey, 1999). Hydrolysable tannins can be hydrolysed and utilized by rumen microbes, while condensed tannins are resistant to hydrolysis.

Effects of tannins on methanogenesis

Hydrolysable and condensed tannins and their extracts have been shown to decrease CH_4 production under both *in vivo* and *in vitro* conditions (Patra *et al.,* 2006, 2011; Bhatta *et al.,* 2009; Grainger *et al.,* 2009; Jayanegara *et al.,* 2009; Ramirez-Restrepo *et al.,* 2009). The molecular weight is a key factor for its effect on digestive enzymes and microbes in the rumen. Low molecular weight tannins could be more effective inhibitors of microbes, including methanogens, compared with high molecular weight tannins (Patra *et al.,* 2006, 2011; Jayanegara *et al.,* 2011). This is because low molecular weight tannins form strong complexes with microbial enzymes, while high molecular weight tannins cannot penetrate to bacterial proteins, causing lower toxicity to methanogens (Field *et al.,* 1989). The anti-methanogenic effect of CT may be attributed to the direct inhibitory effect on methanogens, depending on the chemical structure of CT and methanogen species (Animut *et al.,* 2008; Patra, 2010). The anti-methanogenic activities of tannins may involve tannin action on functional proteins (enzymes) at accessible sites in or on methanogens (Field & Lettinga, 1987).

Most studies conducted so far support the anti-methanogenic effects of tannins in the rumen. The decrease in CH_4 production due to supplementation of tannins had been reported by many researchers (Woodward *et al.,* 2002; Hess *et al.,* 2003; Carulla *et al.,* 2005; Min *et al.,* 2005, 2006; Puchala *et al.,* 2005; Patra *et al.,* 2006, 2011; Animut *et al.,* 2008; Patra *et al.,* 2008; Bhatta *et al.,* 2009; Grainger *et al.,* 2009; Jayanegara *et al.,* 2009; Ramirez-Restrepo *et al.,* 2009). On the contrary, some researchers reported that tannins did not show any effect on methanogenesis or even enhanced CH_4 production in sheep (Sliwinski *et al.,* 2002). Such discrepancies could be the result of doses, types and sources of tannins and types of diets. However, several studies have indicated that tannins have anti-methanogenic activity, either by direct inhibition of methanogens or indirectly through inhibition of protozoa (Moss *et al.,* 2000; Kamara *et al.,* 2006; Animut *et al.,* 2008; Bhatta *et al.,* 2009; Jayanegara *et al.,* 2009). The effects of tannins on rumen feed fermentation, digestibility, and methane production were reviewed in Tables 1.1 and 1.2.

Table 1.1 Effect of tanninsor their extracts on rumen CH_4 production and fermentation parameters *in vitro*

Tannin sources	**Level of inclusion**	**Feed used in the study**	**Effect on CH_4 (decrease)**	**Effect on digestion and fermentation parameters**	**Sources**
Acacia angustissima	20% of substrate	Brachiaria grass	12.3%	Digestibility unaffected	Zeleke *et al.* (2006)
Acacia mangium	20% of substrate	Elephant grass	28.9%	Digestibility, TVFA & protozoa numbers unaffected	Hariadi & Santoso (2010)
Biophytum petersianum	20% of substrate	Elephant grass	25%	Digestibility, TVFA & protozoa numbers unaffected	Hariadi & Santoso (2010)
Castanea sativa	0.5 and 2.5 g kg^{-1} DM	Grass silage and hay: barley (77:23)	2.6-13.3%	Digestibility, TVFA, A : P, protozoa & total bacterial numbers unaffected	Sliwinski *et al.* (2002)
Chestnut tannins	1- 10%	Soybean	5.1- 33.3%	No information	Roth *et al.* (2002)
Emblica officinalis	0.5 ml per 0.2 g substrate	Wheat straw: concentrate (1:1)	20 and 27.7%	TVFA, A : P & digestibility unaffected, protozoa numbers decreased	Patra *et al.* (2006)
Jatropa curcas	20% of substrate	Elephant grass	22.4%	Digestibility, TVFA & protozoa numbers unaffected	Hariadi & Santoso (2010)
Lotus corniculatus	As sole diet	Itself	29.7%	TVFA unaffected, A : P increased	Tavendale *et al.* (2005)
Mimosa tannins	1- 10%	Soybean	7.7-30.8%	No information	Roth *et al.* (2002)

Table 1.1 (continued)

Tannin sources	**Level of inclusion**	**Feed used in the study**	**Effect on CH_4 (decrease)**	**Effect on digestion and fermentation parameters**	**Sources**
Populus deltoides	50.0-150 g kg^{-1}DM	Wheat straw: concentrate (1:1)	14.6-21.5%	TVFA, A : P & protozoal numbers unaffected	Patra *et al.* (2008)
Psidium guajava	20%	Elephant grass	18.4%	Digestibility & protozoal numbers decreased, TVFA unaffected	Hariadi & Santoso (2010)
Quebracho tannins	10 -20 g kg^{-1} DM	Wheat grass	24.6 -51.1%	Digestibility decreased	Min *et al.* (2006)
Quebracho tannins	5- 25% of substrates	Timothy hay: concentrate (65:35)	12.9-38.2%	Digestibility, TVFA & methanogen numbers decreased ; A : P unaffected	Bhatta *et al.* (2009)
Sesbania grandifora	20%	Elephant grass	9.2%	Digestibility, TVFA and protozoal numbers unaffected	Hariadi & Santoso (2010)
Sesbania sesban	20% of substrate	Brachiaria grass	37.4%	Digestibility unaffected	Zeleke *et al.* (2006)
Terminalia belerica seed pulp extracts	0.5 -30 ml of 0.2 g substrate	Wheat straw: concentrate (1:1)	4.4 - 27.7%	TVFA, digestibility, A : P and protozoal numbers unaffected	Patra *et al.* (2006)
Terminalia chebula seed pulp	0.33-1 g l^{-1} or 50-150 g kg^{-1} DM	Wheat straw: concentrate (1:1)	10.6-25.5%	TVFA, A : P and protozoal numbers unaffected	Patra *et al.* (2006)
Persea americana	20%	Elephant grass	11.8%	Digestibility, TVFA & protozoal numbers unaffected	Hariadi & Santoso (2010)

Table 1.2 Effect of tanninsor its extracts on rumen CH_4 production and fermentation parameters *in vivo*

Tannin sources	**Study condition/animals used**	**Level of inclusion**	**Feed used in the study**	**Effect on CH_4 (decrease)**	**Effects on other fermentation parameters**	**Sources or references**
Acacia Mearnsii	Sheep	41 g kg^{-1} diets (extract)	Mixture of ryegrass and lucerne (1:1)	9.9%	Digestibility, TVFA & total protozoa numbers unaffected, A : P decreased	Carulla *et al.* (2005)
Acacia Mearnsii	Cattle	8.6 and 14.6 g kg^{-1} DM	Grazing rye grass pasture with 4.5 kg grain	117.1 and 30%	Digestibility decreased	Grainger *et al.* (2009)
Hedysarum coronarium	Dairy cows	As sole feed	Rye grass pasture	2.35%	No information	Woodward *et al.* (2002)
Lespedeza cuneata	Goats	As sole feed	*L. cuneata*	51.4%	Digestibility & protozoa numbers decreased TVFA &A : P unaffected	Animut *et al.* (2008)
Lespedeza cuneata	Goats	As sole diet	In pasture of crabgrass/tall fescue	30.2%	TVFA & A : P unaffected	Puchala *et al.* (2005)
Lespedeza striata	Goats	33-100%	sorghum-sudangrass	32.9-58.4%	Digestibility & protozoal numbers decreased, TVFA &A : P unaffected	Animut *et al.* (2008)
Lotus pedunculatus	Sheep	As sole feed	In ryegrass & lucerne pasture	No effect		Woodward *et al.* (2001)
Quebracho tannins	Beef cattle	10-20 g kg^{-1} of DM	Barley silage, barley grain and rye grass mixture	No effect	No effect on digestibility; TVFA decreased; A : P decreased	Beauchemin *et al.* (2007)

Effects of tannins on feed intake, digestion and fermentation

The negative effects of high tannin concentration include reduced voluntary feed intake, reduced feed palatability, decreased digestion and development of conditioned aversions (Mueller-Harvey, 2006; Waghorn, 2008). Tannins affect intake by slowing down digestion and emptying the digestive tract, and stimulating the nervous system to inhibit further intake of feed. Loss of palatability could be a result of reactions between tannins and salivary muco-proteins, or a direct reaction with taste receptors, provoking an astringent sensation (McLeord, 1974). Consumption of low to moderate concentrations of tannins did not affect voluntary feed intake, while high tannin concentrations resulted in reduced intake (Barry & Duncan, 1984; Waghorn *et al.,* 1994).

Although many reports indicated negative effects of tannins, ruminants rely on tanniferous forages, which are usually high in N content, especially in tropical regions. The saliva of these ruminants is rich in proline protein, which binds to tannins, forming tannin-proline-rich protein complexes (Robbins *et al.,* 1987; Austin *et al.,* 1989; McArthur *et al.,* 1995; Foley *et al.,* 1999). The complexes are stable within a wide range of pH of the digestive tract, unlike other protein-tannin complexes (Hagerman & Butler, 1989). In addition, ruminants have developed various adaptive mechanisms against the effects of tannins (Robbins *et al.,* 1987; Hagerman *et al.,* 1992). Ruminants can benefit from dietary CT when the increases in protein flow from the rumen exceed the reduction in absorption of amino acids from the intestine (Waghorn, 1996). This is owing to the formation of tannin-proline-complexes, coupled with adaptive mechanisms developed by the microbes and the host animal. The ability of micro-organisms to degrade tannin-protein complexes is another important phenomenon that explains the utilization of tanniferous feeds by ruminants. There are *Streptococcus* species in the caecum (Osawa & Mitsuoka, 1990) and entero-bacteria in the alimentary tracts of koalas (Osawa, 1992) that are capable of degrading tannic-acid-proteincomplexes. Brooker *et al.* (1994) have also isolated *Streptococcus caprinus* from feral goats that browse tannin-rich *Acacia* species with similar activity. In such scenarios, microbes may develop adaptive mechanisms to become resistant to adverse effects of tannins (Smith *et al.,* 2005). For example, proteolytic bacteria that were initially sensitive to tannins were found to adapt after a short period of exposure by modifying their metabolism when tannin levels were not too high (Jones *et al.,* 1994; O'Donovan & Brooker, 2001).

Various findings have reported the effect of tannins on digestibility feeds. McSweeney *et al.* (1988) noted that CT might increase the intestinal digestibility of organic matter. However, other authors reported a negative effect of tannins on feed digestion and nutrient absorption from the small intestine (McNabb *et al.,* 1998; Silanikove *et al.,* 1994, 2001). The explanations for the anti-nutritive nature of tannins included poor solubility of tannin-protein complexes in the abomasum, formation of tannin-digestive-enzyme complexes or new tannin-dietary-protein complexes and changes in intestinal absorption, due to the interaction of tannins with intestinal mucosa (Silanikove *et al.,* 1994, 2001; McNabb *et al.,* 1998). Tannins affect degradation of proteins in the rumen owing to their high affinity with proteins, as the ruminal pH is conducive to the formation of tannin-protein complexes (McLeod, 1974). The reduction in protein degradation is associated with a lower production of ammonia-N and a greater non-ammonia nitrogen flow to the duodenum (Waghorn, 1996). The effect of tannins on protein degradation is a reduction in the immediately degradable fraction and fractional rate of degradation (Aharoni *et al.,* 1998; Frutos *et al.,* 2000; Hervás *et al.,* 2000). Though tannins exert their negative effects mainly on proteins, they have negative effects on carbohydrates, particularly hemicellulose, cellulose, starch and pectins (Chiquette *et al.,* 1988; Schofield *et al.,* 2001).

Application of tannins in ruminant feeding

Addition of quebracho tannins up to 1.5 g kg^{-1} BW in the diets of sheep did not affect feed intake, but decreased CH_4 production (Hervas *et al.,* 2003). Beauchemin *et al.* (2007) also reported that inclusion of quebracho CT up to 2% of DM had no influence on feed intake in cattle. On the other hand, inclusion of quebracho tannins at 8.93% of DM in a diet of sheep reduced feed intake (Vasta *et al.,* 2009). In the study of Carulla *et al.* (2005), feed intake in sheep was enhanced when an *Acacia Mearnsii* tannin extract was included in the diet, while CH_4 emissions were suppressed, which is an important aspect for the practical application of tannins in animal diets. It is generally suggested that condensed tannin concentrations of more than 5% in diets may have adverse effects on nutrient utilization and productivity of animals, though the response depends on the types of tannin (Waghorn, 2008). In other studies, digestibility was not affected and CH_4 production was reduced by inclusion of tannin extracts from *Terminalia belerica* (Patra *et al.,* 2006), *Acacia Mearnsii* (Carulla *et al.,* 2005), *Quebracho tannins* (Bhatta *et al.,* 2009), *Biophytum petersianum*, *Acacia magnum* and *Jatropa curcas* (Hariadi & Santoso, 2010) in the diet of ruminants. Other reported beneficial

effects of tannins are protection of protein from degradation in the rumen, thereby increasing metabolizable protein supply to the duodenum, preventing bloat and increasing conjugated linoleic acid concentrations in ruminant-derived foods (Mueller-Harver, 2006; Waghorn, 2008). However, tannins exert an anti-microbial action on microbial growth, including cellulolytic bacteria and fungi (Patra & Saxena, 2009), which may adversely affect fibre utilization. Inclusion of quebracho tannins at a dosage of 22.5 g kg^{-1} DM to lucerne hay decreased the fibre digestibility, whereas no effect was noted at dosages of 7.5 and 15 g CT kg^{-1} DM (Al-Dobaib, 2009). It has been proposed that higher concentrations of tannins in diets, which remain free after binding with proteins, may depress fibre digestion. This occurs through complexion with ligno-cellulose, thus preventing microbial digestion or by directly inhibiting cellulolytic micro-organisms and activities of fibrolytic enzymes or both.

The responses of tannins on concentrations of TVFA are not conclusive, with some researchers reporting no effect (Carulla *et al.,* 2005; Puchala *et al.,* 2005; Tavendale *et al.,* 2005; Patra *et al.,* 2006; Animut *et al.,* 2008), while others reported decreased concentrations (Min *et al.,* 2006; Beauchemin *et al.,* 2007; Grainger *et al.,* 2009), depending on dose and source (Bhatta *et al.,* 2009; Hariadi & Santoso, 2010). There is some evidence that a significant decrease in methanogenesis could be possible without a considerable reduction of TVFA concentration and digestibility (Carulla *et al.,* 2005; Bhatta *et al.,* 2009; Hariadi & Santoso, 2010), and such interventions needs to be tested further to refine the technology for practical application under field conditions.

1.4.4. Supplementation of exogenous fibrolytic enzymes

Exogenous fibrolytic enzymes

Enzymes are proteins with the ability to catalyse chemical reactions in biological systems. Digestive enzymes are one of the broad categories of enzymes that assist in breaking down nutrients in the digestive system of mammals. In the context of animal nutrition, enzymes can be endogenous (animal origin) and exogenous (microbial origin) and catalyse the degradative reactions of feedstuffs in order to release nutrients such as glucose for utilization by microorganisms or host animal itself.

It is widely known that ruminants cannot digest the major portion of fibre (Hatfield *et al.,* 1999); while pigs and poultry cannot digest 15-25% of feed they eat (Bedford & Partidge, 2010). Fibre has low energy and low digestibility coefficients (Hatfield *et al.,* 1999; Azzaz *et al.,* 2012) owing to an interwoven matrix of polymers of plant cell walls that form complex and dynamic structures. These dynamic structures are barriers against microbial invasion and limit their access to the more digestible intracellular fraction of cell wall networks of plants (Krueger *et al.,* 2008; McDonald *et al.,* 2011). Because of this, increasing the digestibility of fibrous diets has been a topic of research for many years. To avoid feed wastage and improve utilization efficiency, supplementing ruminant feeds with specific exogenous enzymes has been tested and has resulted in some positive outcomes. Supplementation of exogenous enzymes might improve the nutritional value of feeds (nutritional status of animals) by increasing the efficiency of digestion, and absorption and by reducing feed costs. The types of exogenous enzymes used can be available as fibre-fermenting enzymes, protein-degrading enzymes, starch-digesting enzymes and phytases (Bedford & Partidge, 2010). This study focused on exogenous fibre-fermenting enzymes (EFE).

Exogenous fibrolytic enzymes in animal nutrition

Plant cell walls typically consist of about 35-50% cellulose, 20-35% hemicelluloses and 10-25% lignin in the dry mass that had structural properties linked to their crystallinity (Sticklen, 2008). This structural complexity of hemicellulose obviously requires many enzymes for its digestion, thus the majority of fibre fractions are not readily digested in animal feeds. Digestion of feed particles requires hundreds of enzymes in animals (Hristov *et al.,* 1998). Therefore, exogenous fibrolytic enzymes (EFE) can be supplemented to enhance fibre digestion and to complement the rumen microbial system. Cellulases and xylanases are the most commonly used EFEs, which have recently received a wide range of potential applications, including in ruminant feeding. Thereare two major ruminant diet enzyme groups that breakdown the cellulose and xylans in plant cell wall components, respectively (Beauchemin *et al.,* 2003; Lynd *et al.,* 2005). Enzyme inclusion as an alternative way of improving digestibility in fibrous feeds has resulted in positive outcomes (Pinos-Rodríguez *et al.,* 2002; Beauchemin *et al.,* 2003; Nowak *et al.,* 2003; Adesogan *et al.,* 2007; Krueger *et al.,* 2008; Bala *et al.,* 2009; Azzaz *et al.,* 2012). Others have reported a negative or no effect (Bowman *et al.,* 2003; Vicini *et al.,* 2003; Baloyi, 2008). The positive effects of cellulase and xylanase enzyme inclusion in ruminant feeding are summarized in Tables 1.3 and 1.4.

In dairy and feedlot cattle, high-energy diets often result in a pH below 6.0 for much of the day, which is sub-optimal for efficient fibre digestion (Morgavi *et al.,* 2000). In this condition, fibre digestion is inhibited because of the depression of the ruminal cellulolytic bacteria. However, the optimum pH for most enzymes is lower than the optimum pH of the rumen. Therefore, its supplementation can positively influence fibre digestion under these conditions. There are many reports on the successful use of these enzymes to improve intake, digestibility (Beauchemin *et al.,* 1995; 2003; Lopez-Soto *et al.,* 2000) and milk production in dairy cattle (Beauchemin *et al.,* 1995*,* 2003; Tricarico *et al.,* 2008) and to increase intake, digestibility and weight gain in beef cattle (Beauchemin *et al.,* 1995, 2003; Murillo *et al.,* 2000; Alvarez *et al., 20*09). Their effectiveness has been demonstrated to increase intake, digestibility and weight gain in sheep (Pinos-Rodriguez *et al.,* 2002; Cruywagen & Goosen, 2004; Cruywagen & Van Zyl, 2008; Giraldo *et al.,* 2008) and to increase intake, digestibility and weight gain in goats (Cruywagen & Goosen, 2004; Bala *et al.,* 2009). In addition, increased digestibility of CP, NDF, ADF (Colombatto *et al.,* 2003; Eun & Beauchemin, 2007) and increased TVFA, acetate and *in vitro* gas volume have been reported with the use of enzymes in ruminant feeds (Eun &Beauchemin, 2007).

The positive nutritional effects achieved with the addition of enzymes in feed are proposed to be caused by several mechanisms. It is widely assumed that the ability of β-glucanases and xylanase to degrade plant cell walls leads to the release of nutrients from grain endosperm and aleurone layer cells (Hristov *et al.,* 1998). With the application of enzymes, the increase in cell wall degradation from enzyme-incubated feeds resulted in the rapid growth of the microbial population due to increased energy supply. This caused increases in ruminal bacteria numbers that could lead to increased microbial colonization of the feed particles (Alvarez *et al.,* 2009). Then the cell wall structure was gently eroded, allowing ruminal microbes to obtain earlier access to the fermentable substrate during the initial phase of digestion (Colombatto *et al.,* 2003). Once the structure of fibre had beenaltered by enzymes (Giraldo *et al.,* 2008) and microbial numbers increased, fermentation of feeds was enhanced due to the access of microbes to the potentially fermentable components of cell walls (Sutton *et al.,* 2003; Elwakeel *et al.,* 2007), thus shortening the lag phase (Yang *et al.,* 1999) and improving feed fermentation and digestion. Besides increases in fibre fermentability, enzymes improved kinetics of fermentation, digesta flow in the digestive system, nutrient absorption and bioavailability (Giraldo *et al.,* 2008). This would trigger increases in feed intake, weight gain and productivity. Although the mechanism of this improvement is not

clearly understood, improvement in the attachment of micro-organisms to the plant cell (Nsereko *et al.,* 2000; Wang *et al.,* 2001) or an alteration in the fibre structure due to the enzyme effects (Giraldo *et al.,* 2008), or a combination of these might shorten the lag time and could be possible reasons for the observed improvement.

Microbes would have better access to potentially ferment fibre when enzymes act on the structures of plant cell walls (Sutton *et al.,* 2003; Elwakeel *et al.,* 2007). In addition, the pre-incubation of feed samples with enzymes has enhanced the attachment of enzymes to the cell wall components and thus improved the fermentation of feeds. The positive effects of pre-feeding treatment were elaborated earlier by many researchers due to enzyme-substratepre-incubation interaction (McAllister *et al.,* 2001; Colombatto *et al.,* 2003; Elwakeel *et al.,* 2007; Krueger & Adesogan, 2008; Alvarez *et al.,* 2009; Moharrery *et al.,* 2009). According to Kung *et al.* (2000) and Yang *et al.* (2000), the formation of stable enzyme-feed complexes might increase the resistance of the enzymes to proteolysis and lengthen its residence during subsequent fermentation periods. As a result of a continuous effect of the enzymes on the fermentation of fibre of incubated feeds, there would be improvement in fermentable metabolizable energy and this could increase the flow of microbial-N and microbial colonization of the substrate, resulting in enhanced fermentation and fibre digestion. There is evidence that the mode of action of exogenous enzymes in ruminants is a combination of pre- and post-feeding effects (McAllister *et al.,* 2001; Colombatto *et al.,* 2003). In addition, there is evidence that EFE work in synergy with the microbial enzymes produced in the rumen; hence, the hydrolytic activity in the rumen is increased (Morgavi *et al.,* 2000).

Table 1.3 Effects of addition of cellulase and xylanase enzymes on feed fermentation in the rumen (*in vitro* results)

Declared primary activities	**Feed/Substrate type used**	**Effects reported**	**Sources**
Endo-glucanases, xylanases	Fresh low-quality lucerne hay	Improved fermentation, gas production and OMD, but its combination did not yield additional effects.	Eun & Beauchemin (2007)
Endo-glucanases, xylanases	Lucerne hay corn silage	Improved NDF fermentation and superior results were obtained with the optimum dose rate.	Eun *et al.* (2007)
Mixture of enzymes	300-700 g kg^{-1} DM forage	Increased DM fermentation, total VFA, acetate and propionate.	Giraldo *et al.* (2008)
Mixture of enzymes	Grass hay and concentrate	Increased acetate, butyrate and methane. Increased substrate DM and fibre disappearance.	Giraldo *et al.* (2007)
Cellulase, xylanase	Bahia grass hay	Increased DMD, decreased acetate and increased propionate. Decreased lag phase	Krueger & Adesogan (2008)
Cellulase	Grasses and legumes	Improved DMD and NDF OM digestibility. Increased 'a' and decreased 'b' values.	Moharrery *et al.* (2009)
Fibrozyme	Hay, straw	Increased VFA and acetate concentration.	Ranilla *et al.* (2008)

Table 1.4 Effect of addition of cellulase and xylanase enzymes on fermentation in the rumen (*in vivo* results)

Declared primary activity	**Feed/Substrate type used**	**Animals studied**	**Effects reported**	**Sources**
Xylanase,cellulase	High fibre diet	steers	Increased DM and CP fraction, no effect on fibre disappearance, DMI or feed conversion.	Alverez *et al.* (2009)
Xylananes, cellulase	Wheat middling and oat straw	steers	Increased disappearance of ADF (Wheat middling) and NDF and ADF(Oat straw)	Alvarez *et al.* (2009)
Cellulase, xylanase	Concentrate	goats	Increased digestibility of DM, OM, CP, NDF, and ADF. Improved bodyweight, but decreased feed intake.	Bala *et al.* (2009)
Cellulase, hemicellulase	High corn, roughage diets	cattle	Improved DMI and FCR. No effect reported for total tract digestibility.	Burroughs *et al.* (1960)
Enzyme cocktail	Roughage-based formulated diet	lambs	Improved bodyweight and feed conversion ratios. Improved feed conversion ratio's (FCR) , but no effect on DMI.	Cruywagen & Goosen (2004)
Enzyme cocktail	High and low forage diets	lambs	Improved bodyweight (BW) gains and FCR.	Cruywagen & Van Zyl (2008)
Endogluconase, xylanase	70 grass hay: 30 concentrate	sheep	Increased DM fermentation and rate of fermentation. Increased propionate and decreased A : P ratio	Giraldo *et al.* (2008)
Fibrozyme	Lucerne or rye grass-based feed	lambs	Increased apparent digestibility of CP, NDF and hemicellulose (lucerne). Increased total VFA concentration. Increased DMI, OMI and CP intake (both hays).Improved N balance (both hays).	Pinos-Rodriguez *et al.* (2002)
Fibrozyme	TMR (forage ratio 40-60)	sheep	Increased DM and NDF disappearance rates, increased soluble fraction (*a*) of DM. No effects on feed intake or N balance.	Pinos- Rodriguez *et al.* (2008)
Cellulase, xylanase	TMR (39% forage)	dairy	Decreased milk fat, increased digestibility of DM,OM,NDF,ADF and CP	Rode *et al.* (1999)
Cellulase, xylanase	Forage (55% forage)	dairy	Effects not reported	Schingoethe *et al.* (1999)
Cellulase, xylanase	TMR(55% forage)	dairy	Increased milk yield OM, and NDF fermentation	Yang *et al.* (1999)
Xylanase, endocellulase	TMR(45% forage)	dairy	Increased milk protein and increase DM fermentation	Beauchemin *et al.* (2000)
Cellulase, hemicellulase	Fresh forage	dairy	Increased milk yield, but decreased milk fat &proteins	Kung *et al.* (2000)
Xylanase	TMR (38% forage)	dairy	Increase milk yield, increased DM fermentation	Yang *et al.*(2000)
Cellulase, xylanase	Forage (50-65% forage))	dairy	No effect reported	Zheng *et al.* (2000)

Table 1.4 (Continued)

Declared primary activities	Feed/Substrate type used	Animals studied	Effects reported	Sources
Cellulase, xylanase	TMR(55% forage)	dairy	Increased milk fat % protein, increased DM,NDF and ADFfermentation	Bowman *et al.* (2002)
Cellulase, xylanase	TMR(45% forage)	dairy	No effect reported	Kung *et al.* (2002)
Xylanase, endoglucanase	TMR(55% forage)	dairy	Increased milk protein, decreased DM and OM fermentation	Sutton *et al.* (2003)
Xylanase, endoglucanase	TMR(43-57% forage)	dairy	No effect reported	Vicini *et al.* (2003)
Cellulase, phytase	TMR(37% forage)	dairy	Increased trend in digestibility	Knowlton *et al.* (2007)
Amylase, Xylanase	Forage (40%))	dairy	Increased DM % CP digestibility	Hristov *et al.* (2008)
Xylanase and endoglucanase	Pasture and concentrate supplement	dairy	No effect reported	Miller *et al.* (2008)
Cellulase, protease & amylase	TMR (70% Forage)	dairy	Increased DM, OM, NDF and ADF fermentation	Gado et al. (2009)
Cellulase	TMR (50-54%forage)	dairy	No effect reported	Bernard *et al.* (2010)
Cellulase, xylanase	TMR (50% forage)	dairy	No effect reported	Peters *et al.* (2010)
Xylanase, endoglucanase	TMR (52%forage)	dairy	No effect reported	Holtshausen *et al.* (2011)
Xylanase, endoglucanase	TMR(52-56% forage)	dairy	Increased DM, OM, NDF and ADF fermentation	Arriola *et al.* (2011)
Cellulase, xylanase	91-97%roughage	beef	Increase DMI and ADG	Beauchemin *et al.* (1995)
Cellulase, xylanase	TMR (70% basal diet)	beef	Increase DM, NDF and ADF digestibility	Lewis *et al.* (1996)
Cellulase, xylanase	Concentrate with 5% basal diet	beef	Increase ADF fermentation	Krause *et al.* (1998)
Cellulase, xylanase	Concentrate with 7.8% basal diet	beef	Increase ADG	Beauchemin *et al.* (1999)
Cellulase, xylanase	TMR(70-82.5% basal diet)	beef	Increase DMI, ADG and digestibility	Mcallister *et al.* (1999)
Xylanase,endoglucanase	TMR(20-65% basal diet)	beef	No effect reported	Zobell *et al.* (2000)
Xylanase, cellulase	Roughage mainly	beef	Increased ADG and feed conversion efficiency	Balci *et al.* (2007)
Cellulase, xylanase	Hay mainly	beef	Increase DMI, digestibility of DM,NDF and CP	Krueger *et al.* (2008)
Cellulase, xylanase	TMR (20-58% basal diet)	beef	No effect reported	Eun *et al.* (2009)

Factors to be considered in utilization of EFE in ruminant feeding

For EFE to be efficient and successful in improving the digestibility of feed, their ability to hydrolyse the plant cell wall components and the ability of the animal to utilize the resultant products efficiently are important factors. However, supplemented EFE should contain enzymatic activities that are limiting the rate of the hydrolysis reactions (Morgavi *et al.,* 2000). In ruminant feeding, application of EFE could be more effective if these conditions were considered:

i. ***Enzymes should be incubated with feeds before feeding***

According to most reports, pre-incubation of these enzymes with feed is important (Forwood *et al.,* 1990; Elwakeel *et al.,* 2007; Krueger & Adesogan, 2008). This is because they require an adsorption and binding time to the substrate to allow for protection against proteolytic breakdown in the rumen (Forwood *et al.,* 1990; Beauchemin *et al.,* 2003. Once it is bonded to the feed, the resultant stable enzyme-feed complex would be formed that could potentially degrade the relevant tissue in the rumen (Kung *et al.,* 2000). On the other hand, there are other reports that did not show any effect on digestibility or fermentation with pre-incubation (Alvarez *et al.,* 2009).

ii. ***The dose rate should be determined prior to feeding***

The efficiency of enzymes to digest fibre depends on the optimal dose rate (Eun *et al.,* 2007; Jalilvand *et al.,* 2008), type of feed (Pinos-Rodriguez *et al.,* 2002), and chemical composition of feed (White *et al.,* 1993), temperature, moisture content and pH (Colombatto *et al.,* 2007). The proportion of concentrate in a diet is another important factor. Therefore, it is vital to determine the dose of enzymes for the diets under consideration.

iii. ***Key enzymatic activity should be identified***

As many of the enzymes used in ruminant studies were developed for other applications, the key enzyme activities are likely to differ from those needed for fibre fermentation, making it is important to identify the key enzymatic activities (Wallace *et al.,* 2001; Eun & Beauchemin, 2007). Under current scenarios in which many

enzymes are manufactured and are proven to improve feed efficiency in animal nutrition, it is important to define the site of action or to match the feed to the enzyme (Hristov *et al.,* 1998; Beauchemin *et al.,* 2003).

1.4.5. Application of electron acceptors (nitrate, sulphate, fumarate)

Hydrogen gas produced during microbial fermentation of feed is used by methanogens to produce methane. Although other intermediate products of fermentation (such as formate) could be used by methanogens to synthesis methane in the rumen, H_2 is an important precursor (Hungate *et al.,* 1970). The total pool of H_2 in the rumen is small (about 0.1–50 µM, which is 0.014 to 6.8% of its maximal solubility, which indicates continuous utilization by methanogens). The rate at which H_2 is utilized, determines the rate of CH_4 formation and vice versa (Janssen *et al*., 2010). The efficiency of H_2 removal is postulated to increase the rate of fermentation by eliminating the inhibitory effect of H_2 on the microbial degradation of plant material (Wolin, 1979; McAllister & Newbold, 2008). However, before methane production can be lowered effectively, hydrogen in the rumen must be prevented from accumulating, as this will alter fermentation in the rumen, and affect digestibility of the feed (Mitsumori *et al.,* 2008). For example, it can be shifted towards propionate production via formate and lactate. It has been described that reducing the concentration of hydrogen and formate in the rumen is the most efficient means of decreasing methane production. In this regard, the use of fumarate, nitrates, sulfates and nitro-ethane as electron (hydrogen) receptors reveals promising methane mitigation agents and has received wide research as alternate electron acceptors in the hydrogen pathway (Leng, 2008).

According to Gerber *et al.* (2013), nitrates can be attractive in developing countries where forages contain negligible levels of nitrate and low crude protein content. This is to avoid associated toxicity in the animal (Leng, 2008). Nitrate toxicity occurs when animals consume large doses of nitrate, resulting in acute or chronic methemoglobinemia, which limits tissues' supply of oxygen in red blood cells, causing reduced feed intake and productivity (Allison & Reddy, 1984). However, this might occur in grazing situations and is associated with sudden

increases in nitrate intake from lush green pastures that are high in crude protein or with plants that accumulate nitrate due to high nitrogen fertilization (Wright & Davison, 1964; O'Donovan & Conway, 1968; Lovett *et al.,* 2004). In most cases of nitrate poisoning in the literature, the crude protein content of the feed was noted to be high, generally between 18 and 38%, resulting in consistently high rumen NH_3-N levels, often up to thrice the recommend levels for optimal microbial efficiency (Preston & Leng, 1985). Therefore, when nitrates are used, it is critical that animals should be properly adapted to avoid nitrite toxicity (Hristov *et al.,* 2013). Supplementing nitrate does not merely provide a source of microbial nitrogen, but serves as an alternative sink for hydrogen. Nitrate has been identified as an effective inhibitor of methanogenesis in all anaerobic fermentation systems such as the rumen ecosystem and anaerobic bio-digesters (Hungate, 1965; Allison *et al.,* 1981; Akunna *et al.,* 1993). This might be due to the conversion of nitrate to NH_3-N that is noted to be more energetically favourable than methane production. Thus, it can effectively compete with methanogenesis in the rumen, but only if an adequate nitrate concentration is available (Morgavi *et al.,* 2010). In addition, during this conversion of nitrate to NH_3-N, the process consumes or traps more electrons (8), thus out-competing methanogens for electrons (Leng, 2008; Leng & Preston, 2010). With such a conversion or assimilatory nitrate reduction, energy is conserved for microbial use instead of being lost in methane generation (Leng, 2008). Carbon dioxide can be replaced by dietary nitrate as an alternative electron acceptor to generate another reduced product (NH_3-N), which can be recycled in the rumen system (Leng, 2008).

$$\mathbf{NO_3^- + 4\ H_2 + 2\ H^+ \rightarrow NH_4^+ + 3\ H_2O}\text{----------equation (2)}$$

According to the above equation, 4mol of H_2 is required to produce 1 mol of CH_4; 1 mol of nitrate would trap 4 mol hydrogen to produce 1mol NH_3, reducing CH_4 production by 1 mol (Leng, 2008). It has been indicated that nitrate can reduce enteric methane production up to 50% from sheep (Sare *et al.,* 2004; Nolan *et al.,* 2010; Van Zijderveld *et al.,* 2010) and cattle (Van Zijderveld *et al.,* 2011; Hulshof *et al.,* 2012). In another study, nitrate supplementation increased N retention per unit of organic matter digested (28%), with reduction of methane by 43% in cattle (Sophal *et al.,* 2013). Moreover, Van Zijderveld *et al.* (2010) have reported 32% emission reduction for cattle and Nolan *et al.* (2010) 25% emission reduction in sheep. Inthapanya *et*

al.(2011) have also reported 32% reduction of methane per unit of substrate fermented in an *in vitro* related experiment with no decline in fermentation. Furthermore, it is important to consider that the adaptability of the rumen microbes to nitrate may be lost within a short time of nitrate withdrawal from the diet (Alaboudi & Jones, 1985), and intake following re-introduction must be gradual and regulated to prevent poisoning (Hristov *et al.,* 2013).

Adding sulfate to the diet of sheep reduced CH_4 production, but its potential effects on animal health are unclear. Other electron acceptors, such as fumaric, malic acids, and acrylate, might reduce CH_4 production when applied in large quantities, but most results indicate no mitigating effects, and its costs are likely to be prohibitive (Echard *et al.,* 2010; Hristov *et al.,* 2013).

1.4.6. The use of combinations of additives

The use of enzymes with other treatments, such as fumarate, has also given good results in digestibility and fermentation. It was reported that their inclusion with enzyme resulted in increased production of VFA (acetate and butyrate) and methane and cell wall digestibility (Lopez *et al.,* 1999; Garcia-Martines *et al.,* 2005; Giraldo *et al.,* 2007). Although the inclusion of fumarate was intended to reduce methane, neither these reports nor others have confirmed this to occur (Giraldo *et al.,* 2007). On the other hand, the inclusion of enzymes with nitrate sources could promote efficient fibre fermentation and methane production if the mixing results in an additive or complementary effect. This is a hypothesis that needs research investigation. Under tropical and sub-tropical conditions, there is limited information about the use of nitrate supplementation or a combination of enzyme with nitrate to improve fibre fermentation without increasing methane production per unit of fibre digested.

1.5. General objectives and specific objectives

The overall aim of this work is to test potential rumen modulators or feed additives that would improve fibre fermentation and reduce enteric methane production.

Specific objectives

1. To characterize the chemical composition, *in vitro* gas production and methane output of various tropical grass species and to relate methane production of grass species to theirchemical composition, digestibility and *in vitro* gas production attributes
2. To investigate the effect of tannin-rich browses on gas and CH_4 production, organic matter fermentation, ammonia-N and volatile fatty acid production, as well as studying the correlation of IVOMD and CH_4 with chemical and phenolic composition by incubating the samples with and without PEG (molecular weight, 6000)
3. To investigate the effects of substituting lucerne hay in a diet of Merino rams with three tannin-rich browses foliages on ruminal fermentation and methane emission.
4. To evaluate the effects of cellulase and xylanase enzymes at different levels of application on *in vitro* digestibility, rumen fermentation and methane production characteristic of *Eragrostsis curvula*, maize stover and a total mixed ration (TMR).
5. To investigate possible complementary effects of supplementation of commercial fibrolytic enzymes and nitrate on rumen fermentation and methane production of Merino rams.

1.6. Hypothesis of the experiment

In pursuit of the above objectives, the study aimed to test the following hypotheses:

1. Tropical perennial grasses of Kalahari Desert of South Africa did not vary in their chemical composition, *in vitro* ruminal fermentation, digestibility and methane production.
2. Tannin from different plants did not vary in their effect on *in vitro* fermentation, digestibility and methane production.
3. Lucerne hay in a total mixed ration of Merino rams can be replaced with *Acacia nilotica, Grewia flava* and *Monechma genistifolium* foliages without variation in ruminal fermentation and enteric methane production.

4. Cellulase and xylanase enzymes incubated at different levels with *Eragrostis curvula*, maize stover and a total mixed ration (TMR) did not cause variation in their *in vitro* digestibility, rumen fermentation and methane production of these feeds.
5. Supplementation of commercial fibrolytic enzymes mixture, nitrate and a mixture of enzymes-nitrate with a diet of Merino ram did not cause variation in rumen fermentation and reduce enteric methane production compared with control.

CHAPTER 2

In vitro fermentation, digestibility and methane production of sixteen tropical perennial grass species

2.1. Abstract

This study characterized sixteen tropical perennial grass species in terms of *in vitro* methane (CH_4) output and its relationship with their digestibility and rumen fermentation characteristics. Samples of *Anthephora argentea*, *Brachiaria ciliaris*, *Cenchrus ciliaris*, *Eragrostis trichophora*, *Panicum coloratum*, *Pogonarthria squarrosa*, *Setaria verticillata*, *Stipagrostis uniplumis*, *Schmidtia pappophoroides*, *Centropodia glauca*, *Stipagrostis obtusa*, *Aristida vestita*, *Tricholaena monachne*, *Stipagrostis ciliata*, *Cynodon dactylon* and *Digitaria eriantha* were collected, dried in a forced oven, ground and analysed for nutrient composition. *In vitro* gas production (GP) and organic matter digestibility (IVOMD) were determined using rumen fluid collected, strained and anaerobically prepared. A semi-automated system was used to measure gas production through *in vitro*incubation at 39°C. *Anthephora argentea* and *Stipagrostis ciliata* had the highest production of methane both in terms of g kg^{-1} digestible dry matter (IVDDM) and g kg^{-1} digestible organic matter (IVOMD). While incubation of *Cenchrus ciliaris, Setaria verticillata and Panicum coloratum* produced the lowest amount of methane when expressed in terms of g kg^{-1} DDM and g kg^{-1} DOM. Ash, ether extract (EE), non-fibrous carbohydrate (NFC), neutral detergent insoluble nitrogen (NDFN), acid detergent insoluble nitrogen (ADFN) and crude protein (CP) were negatively correlated with methane production. Methane production was positively correlated with NDF, ADF, cellulose and hemicellulose. This *in vitro* work indicates that methane emission by ruminants under tropical conditions could be reduced bybreeding and selection of perennial grass with increased N content.

Key word: digestibility, fermentation, methane, perennial grass, tropical livestock

2.2. Introduction

Enteric methane (CH_4) production in ruminants decreases its energy utilization efficiency and contributes to the global greenhouse gas effect. Methane is produced under anaerobic conditions by rumen microorganisms called methanogenic archaea, which gain energy by reducing CO_2 with H_2 to form CH_4 (Leng, 2008). Ruminal microbes convert major portions of carbohydrate and protein in feed to volatile fatty acids, microbial protein, CH_4 and CO_2. Enteric methane production depends primarily on quantity and quality of the diet (Van Soest, 1994; Beauchemin *et al.,* 2009), the nature of fermented carbohydrates (Santoso *et al.,* 2003), the concentration of NDF and ADF (Hindrichsen *et al.,* 2003), the acetate to propionate ratio of fermented feeds (McAllister *et al.,* 1996) and types and maturity stages of forage consumed by the animal (Arthington & Brown, 2005).

The mitigation of methane production from ruminants by altering the diet is an effective way to reduce enteric CH_4 production (Smith *et al.,* 2007; Singh *et al.,* 2012). Increased concentrate proportion in ruminant rations is generally associated with a reduction in CH_4 emission per unit of feed intake and per unit of animal product (Johnson & Johnson, 1995; Lovett *et al.,* 2003). However, in many tropical and sub-tropical livestock production systems, ruminants receive small quantities of concentratesdue to unavailability and cost. Therefore, under such systems, it is important to focus on tropical grassland forages and to design effective feed-based mitigation strategies. Previous research suggested that increased forage quality will reduce CH_4 emission per unit of weight gain (McGeough *et al.,* 2010) or per unit of animal product (Moss, 2000), due to improvement in animal productivity.

Characterization of tropical grasses and relating those attributes to potential CH_4 production is important for selection and improvement through breeding. Methane production is mainly related to the extent of organic matter digestion and profile of volatile fatty acid produced and fermented (McDonald *et al,* 2011). Thus, CH_4 output from a wide range of grass species can be studied using *in vitro* gas-production techniques as this method is not expensive and widely used (Tavendale *et al.,* 2005). For tropical grass species in Africa, little information is documented on their potential CH_4 outputs and their correlation with other nutrient constitutes. Therefore, this study was undertaken with the aim to generate information on grass species commonly found in the Kalahari Desert of southern Africa. The specific

objectives were i) to characterize the grass species in terms of chemical composition and related attributes; ii) to compare *in vitro* gas production and CH_4 output of various tropical grass species and iii) to relate CH_4 production of grass species to their chemical composition, digestibility and *in vitro* gas production attributes.

2.3. Materials and methods

2.3.1. Study area description

Grass samples used in this study were collected from Kalahari Dune Bushveld in the province of North West, South Africa. The site covers 2000 hectares with an altitude of about 900-1100 m above sea level (asl). It comprises fenced areas with paddocks where it was rotationally grazed with carrying capacity of 13.2 ha per tropical livestock unit (TLU). The land is used for livestock farming (96%), including beef cattle, sheep and goats. The soil is red, excessively drained sandy soils with high base status, and contains elevated concentrations of copper, which is bound in the soil in the form of the secondary copper hydroxyl mineral atacamite ($Cu_2(OH)_3Cl$) (Le Roux, 2013). The area has highly erratic rainfall that ranges from 150 mm to 350 mm; however, it barely exceeded 150 mm for the two years preceding the study and during the study period. The wettest months are usually from January to April and the temperature extremes ranges from winter low reaching -10.3°C to summer highs of up to 45.4°C (Rooyen, 2001).

The study area has sparsely scattered trees, mainly camel thorn *(Acacia erioloba),* false umbrella thorn *(Acacia luederitizii)* and shepherd's tree *(Boscia lehmanniana)*. The dominant herbaceous plants are perennial grasses rather than annual ones. The dominant perennial grasses include *Eragrostis species, Schmidtia species* and *Stipagrostis species.*

2.3.2. Grass sampling and chemical composition analysis

The study area was categorized as lightly grazed and heavily grazed sites according to grazing histories and condition of the rangeland. In each grazing site, eight (7.5 m long) transects were set up in the canopied and uncanopied sub-habitat. Data on floristic composition, total plant cover and individual species cover were collected using the point

quadrat method (Daget & Poissonet, 1971). Le Houe'rou's (1987) formula was used to determine the DM of above-ground parts of vegetation.

From each transect, four randomly selected sub-sites were taken. The herbaceous vegetation was cut at ground level using a 50 cm by 50 cm quadrat and immediately identified and categorized into perennial grasses, annuals, legumes and others. Perennial grasses were separated into species and put into paper bags for further study. Sampling was done when most pasture plants were fullygrown and flowering (important for identification). The perennial grass species common to both study sites were used to analyse for nutritive value, *in vitro* fermentation and methane production.

The grasses were kept under a shaded area until transported to the nutrition laboratory of University of Pretoria where they wereoven-dried (55°C for 48 h). For all *in vitro* studies and chemical composition analyses, the samples were ground to pass through a 1 mm sieve in a Willey mill (Arthur H. Thomas, Philadelphia, PA, USA). The samples were analysed for OM by ashing in a muffle furnace at 550°C (AOAC, 2002). Crude protein (CP) was measured according to the combustion method (AOAC, 2002) for nitrogen on a Leco FP-428 nitrogen and protein analyser (Leco Corporation, St. Joseph, MI, USA) and ether extract (fat) was done according to AOAC (2002) procedures. The neutral detergent fibre (NDF) and acid detergent fibre (ADF) contents were determined using an ANKOM$^{200/220}$ fibre analyser(ANKOM Technology, Fairport, NY) based on the methods described by Van Soest *et al.,* (1991). Sodium sulphite and heat-stable amylase were used in the analysis of NDF. Lignin (ADL) was determined by solubilization of cellulose with sulphuric acid in the ADF residue (Van Soest *et al.,* 1991). The N content of NDF and ADF (that is, neutral detergent insoluble N, NDFN, and acid detergent insoluble N: ADFN) were determined by the CP method referenced before and expressed exclusive of residual ash. The non-fibre carbohydrate (NFC) content of feeds were calculated by subtracting CP, NDF, fat, and ash from total DM (Sniffen *et al.,* 1992). Hemicellulose was estimated from the difference between NDF and ADF, while cellulose was estimated as the difference between ADF and ADL.

2.3.3. *In vitro* gas production measurement

Collection of rumen fluid from donor sheep

Rumen fluid was collected before the morning feeding from two ruminally cannulated Merino wethers fed lucerne hay *ad libitum*. Approximately 500 ml rumen fluid were collected from each donor animal, mixed, strained through four layers of cheesecloth and transferred to a pre-heated thermos flask. In the laboratory, the flasks were emptied into an industrial blender and simultaneously purged with CO_2 to maintain anaerobic conditions (Grant & Mertens, 1992). After blending, the rumen fluid was transferred into a large glass beaker that was kept inside a 39°C water bath purged with CO_2 and stirred continuously as recommended by Goering and Van Soest (1970). Thereafter, 15 ml rumen fluid wasadded to 25 ml buffer solution in the incubation bottles.

Buffer media preparation, sample incubation and gas measurement

The buffer solution, macro mineral solution and micro mineral solution were prepared in large quantities and utilized as required according to the procedure described by Goering and Van Soest (1970). The micro mineral solution was prepared with a slight modification in which $MgSO_4.7H_2O$ was replaced with $MgCl_2.6H_2O$ to reduce the amount of SO_4 in the medium, as suggested by Mould *et al.* (2005). It was stored in a dark glass bottle to maintain the quality of the solution. In the morning before the experiment commenced, appropriate amounts of distilled water, rumen buffer solution, macro and micro mineral solutions were mixed with the tryptose. Then 0.1% (wt/vol) resazun was added. Appropriate amounts of L-cysteine hydrochloride were weighed and added directly to the rest of the solution once all the chemicals had been dissolved. As soon as L-cysteine hydrochloride was added, the buffer solution was placed in a 39°C water bath and bubbled with CO_2. The serum bottles were then sealed with a rubber stopper and left at 39°C until the buffer solution was clear, which indicated that the solution was sufficiently reduced.

A semi-automated gas production system was used to measure gas production through *in vitro* incubation at 39°C, according to Theodorou *et al.* (1994). The system consists of a digital data tracker (Tracker 220 series indicators, Omega Engineering, Inc., Laval, QC, Canada) connected to a pressure transducer (PX4200-015GI from Omega Engineering, Inc.,

Laval, QC, Canada) with a needle on the tip. Approximately 400 mg of each grass feed sample were weighed into 150 ml serum bottles. About 40 ml rumen fluid + medium were added under a stream of CO_2 to each serum bottle, which was closed with a rubber stopper and crimp seal cap. A needle was inserted through the rubber stopper of each bottle for about five seconds to release small amounts of gas that might have built up since the starting point of incubation. All serum bottles were placed in the incubator and the rotary shaker was turned on at 120 rpm. Gas pressure was taken at 2, 4, 8, 12, 24, 32, 48, 54, and 72 hr. To quantify the gas production derived from the culture medium and the rumen inoculum, two blanks were included in every analysis. Two replicates were used in each run and four runs were executed for every grass sample included in this study. The pressure and gas volume were recorded at different times and were added to the values of the previous readings. Thus, the cumulative pressure and gas volume of the fermentation were obtained. Fermentation was terminated after 72 hr by removing serum bottles from the incubator and placing them on ice. Then the supernatants were immediately pipetted and stored at -20°C until analysed for NH_3-N (McDonald *et al.,* 1960) and VFA (Ottenstein & Bartley, 1971).

2.3.4. In vitro digestible organic matter determination

The *in vitro* organic matter digestibility (IVOMD) content was determined according to Tilley and Terry's (1963) method, as modified by Engels and Van der Merwe (1967). The method involved two digestion phases. During the first phase, feed samples (200 mg) were incubated in triplicate under anaerobic conditions with rumen liquor for 48 hr at 39°C with the inclusion of blanks and standards in every batch of incubation. This was followed by a 48 hr acid pepsin digestion phase at 39°C, under anaerobic conditions. Following the 96 hr incubation, the residual plant materials were collected and oven-dried at 105°C for 12 hrs. Ash content was determined by combustion at 550°C for 2 hr (Engels & Van der Merwe, 1967).

2.3.5. Methane production measurements

Methane production was measured from the duplicate bottles incubated with each grass sample at 2, 12, 24, and 48 hr. The methane concentration was determined using a gas chromatography (SRI 8610C Gas Chromatograph (GC) BTU Gas Analyzer GC System) equipped with a solenoid column packed with silica gel and a flame ionization detector

(FID). Gas production from each bottle was recorded and gas samples were immediately taken using a Hamilton syringe. The sampled gas was injected manually (pull and push method of sample injection) into a GC, which was already calibrated with standard CH_4 and CO_2. Two blanks were included to correct CH_4 produced from the inoculum in each run and two runs were executed for each sample. The measured methane concentration was related to the total gas measurement for each in order to estimate its concentration (Tavendale *et al.,* 2005), and subsequently converted to energy and mass values using 39.54 kJ l^{-1} CH_4 and 0.716 mg ml^{-1} CH_4 factors, respectively (Santoso *et al.,* 2007).

2.3.6. Calculations, statistical analysis and model

Non-fibrous carbohydrate (NFC) was calculated as NFC=100 - (CP+ Fat+ Ash+ (NDF - NDFN)).

Metabolizable energy (ME, MJ kg^{-1} DM) was estimated according to Menke and Steingass (1988) as: ME (MJ kg^{-1} DM) = 2.20 + 0.136 $IVGP_{24}$ (ml 0.5 g^{-1} DM) + 0.057 CP (% DM)
Methane production was calculated as:
g CH_4 g^{-1} digested DM = ((gas production 24 h×([CH4 24 h]) - gas produced blank24 h×[CH4 blank24 h]))/g digested DM according to Chaves *et al.* (2006).
The rate and extent of gas production was determined for each grass species by fitting gas production data to the non-linear equation: $y = b\ (1 - e^{-ct})$ (Ørskov & Mcdonald, 1979), where y = the gas production at time t; b = the slowly fermentable fraction (g kg^{-1} DM), and c = the rate (% h^{-1}) of fermentation of fraction b.

The experimental design used in this study was completely randomized. The data were statistically analyzed using the GLM option of SAS (2009) and differences among the means were determined using Tukey's test. The *in vitro* incubation times were used to fit non-linear regression models using the NLIN procedure (SAS, 2009). The following model was used:

$Yij = \mu + \alpha_i + \beta_j + e_{ijk}$, where

Yij is the response of the grass i observed in block j

μ overall mean of the grass

α_i is the effect of j^{th} block (run)

β_j is the effect of i^{th} grass

e_{ijk} is the associated random error

2.4. Results

2.4.1 Plant cover, density and dry matter yield

In the lightly grazed site, there was a significant difference ($p<0.05$) between the canopied and uncanopied sites in terms of total plant cover and DM yield. However, there was no difference ($p<0.05$) between the two sites at the heavily grazed site in terms of total plant cover and DM yield. The total plant cover and the DM yield values tended to be lower under a tree canopy (Table 2.1).

The mean perennial species cover (% DM) occurring in the study area is shown in Table 2.2 (only species presenting a cover higher than 0.5% were indicated). In the lightly grazed site, the perennial grass species cover showed significant differences ($p<0.05$) for *Aristida vestita*, *Schmidtia pappophoroides*, *Stipagrostis ciliata* and *Stipagrostis obtusa.*The highest cover for these species was recorded under the tree canopy and varied from 0.99% to 10.3% in the canopied sub-habitat. The most abundant species in both grazing sites were *Schmidtia pappophoroides, Stipagrostis ciliata, Stipagrostis obtusa* and *Stipagrostis uniplumis*.

Table 2.1 Mean total plant cover and dry matter of perennial species occurring under shrubs and tree canopies and in open areas and outside the study area

	Lightly grazed site			Heavily grazed site		
	Canopied	Uncanopied	Sub-habitat effect	Canopied	Uncanopied	Sub-habitat effect
Total plant cover (%)	66.8	51.0	*	14.0	13.2	N.S
DM yield (kg ha^{-1})	1200.0	1045.0	*	713.0	700.0	N.S
• Perennial grasses	721.0	658.0	*	480.0	479.0	N.S
• Annual grasses, legumes and others	479.0	387.0	*	233.0	221.0	N.S

Significantly different at *P < 0.05; N.S =not significant

2.4.2. Chemical composition

Table 2.3 summarizes the chemical composition of grass species used in this study. A significant ($p<0.05$) variation in terms of chemical composition was recorded for different grass species. The highest ash concentration was recorded for *Setaria verticillata* (147 g kg^{-1} DM), while the lowest value was recorded for *Panicum coloratum* (20 g kg^{-1} DM). The CP content in the grass species ranged between 20 and 126 g kg^{-1} DM. The highest CP content was recorded for *Pogonarthria squarrosa* (124 g kg^{-1} DM) and *Stipagrostis ciliata* (126 g kg^{-1} DM), while the lowest amount was observed in *Cynodon dactylon* and *Digitaria eriantha* (20 g kg^{-1} DM).

Cynodon dactylon had the highest value of NDFN (64 g kg^{-1} DM-NDF), while *Panicum coloratum* contained the lowest value (2.21g kg^{-1} DM). The highest value of ADFN was recorded in *Digitaria eriantha* (11 g kg^{-1} DM-ADN), while the lowest value was noted for *Anthephora argentea* (1.59 g kg^{-1} DM).

The NDF ranged from 564 to 827 g kg^{-1} DM, while ADF ranged from 332 to 572 g kg^{-1} DM. *Stipagrostis obtusa* had the highest value of NDF (827 g kg^{-1} DM) and ADF (572 g kg^{-1} DM), while *Setaria verticillata* had the lowest values of NDF (564 g kg^{-1} DM) and ADF (332 g k g^{-1} DM). *Centropodia glauca* had the highest ADL (118 g kg^{-1} DM) content, whereas the lowest value was recorded for *Cynodon dactylon* (52 g kg^{-1} DM). *Stipagrostis obtusa* had the highest cellulose (502 g kg^{-1} DM), while *Setaria verticillata* contained the lowest cellulose value (261 g kg^{-1} DM).

Table 2.2 Mean perennial grass species cover (% DM) occurring under tree canopies and in open areas in and outside the study area

Scientific name	Common names	Lightly grazed			Heavily grazed		
		Canopied	Uncanopied	Sub habitat effect	Canopied	Uncanopied	Sub habitat effect
Anthephora argentea	Silver wool grass	3.62	3.53	N.S	2.01	1.98	N.S
Aristida vestita	Herder silk grass	5.96	1.18	*	2.01	1.87	N.S
Brachiaria ciliaris	Blackfoot brachiaria	0.99	1.02	N.S	0.05	0.01	N.S
Centropodia glauca	Gha grass	4.33	4.29	N.S	1.45	1.12	N.S
Cynodon dactylon	Couch grass	1.36	0.09	N.S	0.08	0.0	N.S
Cenchrus ciliaris	Foxtail buffalo grass	0.58	0.78	N.S	0.05	0.00	N.S
Digitaria eriantha	Finger-grass	0.68	0.67	N.S	0.01	0.0	N.S
Eragrostis trichopophora	Hairy love grass	1.36	1.12	N.S	0.6	0.11	N.S
Panicum coloratum	Small buffalo grass	1.00	0.97	N.S	0.98	0.58	N.S
Pogonarthria squarrosa	Herringbone grass	1.26	0.60	N.S	0.65	0.45	N.S
Setaria verticillata	Bur bristle grass	2.68	2.48	N.S	0.25	0.01	N.S
Stipagrostis uniplumis	Silky bushman grass	10.3	9.95	N.S	3.2	3.09	N.S
Schmidtia pappophoroides	Kalahari sand quick	7.67	2.02	*	2.87	2.88	N.S
Stipagrotis ciliata	Long bushman grass	5.83	1.59	*	2.37	2.20	N.S
Stipagrostis obtusa	Small bushman grass	7.91	2.58	*	2.32	2.02	N.S
Tricholaena monachne	Blue-seed grass	2.98	2.05	N.S	0.12	0.10	N.S

Significantly different *P < 0.05; N.S= not significant

Table 2.3 Mean of chemical composition (g kg^{-1} DM) of tropical grass species used in the study

Scientific name	Chemical components									
	ash	OM	CP	EE	NDF	ADF	ADL	ADIN	NDIN	cellulose
Anthephora argentea	44.8^{h}	907.7^{bcd}	52.4^{bc}	17.4^{cde}	806.1^{bcd}	540.6^{b}	93.2^{d}	1.59^{o}	21.6^{e}	447.4^{b}
Aristida vestita	41.1^{i}	915.7 ^{abc}	38.2^{bc}	12.6^{cde}	787.7 ^{def}	490.2^{de}	60.1^{ij}	1.87^{l}	17.9^{g}	430.1^{bc}
Brachiaria ciliaris	47.3^{g}	904.5 ^{cde}	85.2^{b}	12.4^{def}	776.0^{f}	455.3^{f}	90.5^{de}	2.54^{h}	18.3^{fg}	364.8^{g}
Cenchrus ciliaris	76.9^{c}	873.9^{gh}	41.0^{bc}	19.5^{ab}	682.3^{i}	418.7^{g}	58.5^{ij}	3.28^{d}	15.4^{h}	360.2^{gh}
Centropodia glauca	76.3^{c}	882.8 ^{fgh}	32.7^{bc}	12.6^{cde}	749.5^{g}	457.2^{f}	117.8^{a}	2.84^{e}	20.9^{ef}	339.4^{ij}
Cynodon dactylon	105.2^{b}	830.0^{i}	20.4^{c}	23.7^{a}	638.9^{i}	356.3^{h}	51.6^{k}	5.36^{e}	63.7^{a}	298.3^{k}
Digitaria eriantha	63.0^{e}	864.4^{h}	20.4^{c}	10.9^{de}	800.1 ^{cde}	514.6^{c}	102.7^{c}	10.9^{a}	10.9^{l}	411.9^{cd}
Eragrostis trichopophora	33.1^{k}	919.7 ^{abc}	34.3^{bc}	9.47^{e}	794.9 ^{def}	477.7^{e}	67.9^{h}	2.34^{i}	21.0^{e}	409.8^{d}
Panicum coloratum	18.6^{n}	893.3 ^{def}	34.8^{bc}	11.3^{cde}	783.2 ^{ef}	488.6^{de}	111.3^{b}	2.25^{j}	2.21^{k}	377.3^{fg}
Pogonarthria squarrosa	29.5^{l}	922.0^{abc}	123.7^{a}	18.4^{abc}	795.4 ^{def}	500.0^{cd}	87.5^{ef}	2.57^{g}	21.6^{e}	412.5^{cd}
Setaria verticillata	146.5^{a}	806.3^{j}	45.8^{bc}	13.2^{cde}	563.8^{k}	331.8^{i}	70.6^{h}	6.98^{b}	61.8^{b}	261.2^{l}
Stipagrostis uniplumis	30.9^{m}	927.0^{a}	74.4^{abc}	21.0^{b}	823.0^{ab}	486.0^{de}	83.6^{g}	2.23^{g}	26.6^{d}	402.4^{de}
Schmidtia pappophoroides	65.7^{d}	887.3 ^{efg}	37.3^{bc}	10.5^{de}	704.8^{h}	407.1^{g}	62.6^{i}	2.74^{f}	47.2^{c}	344.5^{hi}
Stipagrostis obtusa	26.3^{n}	926.4^{ab}	34.5^{bc}	12.1^{cde}	827.2^{a}	571.6^{a}	70.1^{h}	1.76^{m}	17.2^{gh}	501.5^{a}
Stipagrotis ciliate	37.9^{j}	909.5 ^{abcd}	126.2^{a}	9.47^{e}	778.0^{f}	514.8^{c}	109.1^{b}	2.16^{k}	12.6^{i}	414.4^{cd}
Tricholaena monachne	51.9^{f}	878.8 ^{fgh}	39.1^{bc}	12.9^{cde}	756.0^{g}	443.5^{f}	57.6^{j}	1.63^{n}	6.98j	385.9^{ef}
SEM	*0.35*	*3.63*	*10.4*	*1.27*	*3.72*	*3.71*	*0.81*	*0.004*	*0.45*	*3.73*
P-value	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*

OM, Organic matter; CP, crude protein; EE, ether extract; NDF neutral detergent fibre; ADF, acid detergent fibre; ADL, acid detergent lignin; ADFN and NDFN, acid and neutral detergent insoluble nitrogen.Within a column, means followed by the same letter are not significantly different at p ≥0.05, SEM, standard error of the mean

2.4.3. *In vitro* gas production, Organic matter digestibility and volatile fatty acid production

The cumulative gas production patterns from the *in vitro* fermentation of the grass species are given in Figure 2.1. The total volume and pattern of gas production varied from species to species; however, the observed differences were not consistent for the different incubation times except for *Schmidtia pappophoroides,* which produced considerably the highest volume of gas during all incubation times. The lowest gas production was measured for *Panicum coloratam* at 2 and 48 hr, and *Pogonarthria squarrosa*at 8 to72 hr. Gas production in general was higher in these two grass species, compared with the other grasses.

The grass species showed high variability in terms of gas production parameters and constants (Table 2.4). A higher 'b' (gas production from the slowly fermentable organic matter) value was recorded for *Schmidtia pappophoroides* (174.3 ml g^{-1} DM), while the lowest b-value was recorded for *Panicum coloratum* (65.6ml g^{-1} DM). The potential gas production (PD) value for *Schmidtia pappophoroides* was the highest (88.3ml g^{-1} DM), while the lowest PD value (26.8 ml g^{-1} DM) was recorded for *Pogonarthria squarrosa.* The rate of gas production was the highest (0.057 ml h^{-1}) for *Eragrostis trichopophora* and the lowest (0.029 ml h^{-1}) for *Pogonarthria squarrosa*.

The total and individual short-chain VFA and ammonia-N concentrations are shown in Table 2.5. The total and individual short-chain VFA seemed to vary for perennial grasses. *Anthephora argentea* seemed to produce high amount of total VFA and individual fatty acids, while *Panicum coloratum* contained the lowest amount, except for isobutyric acid.

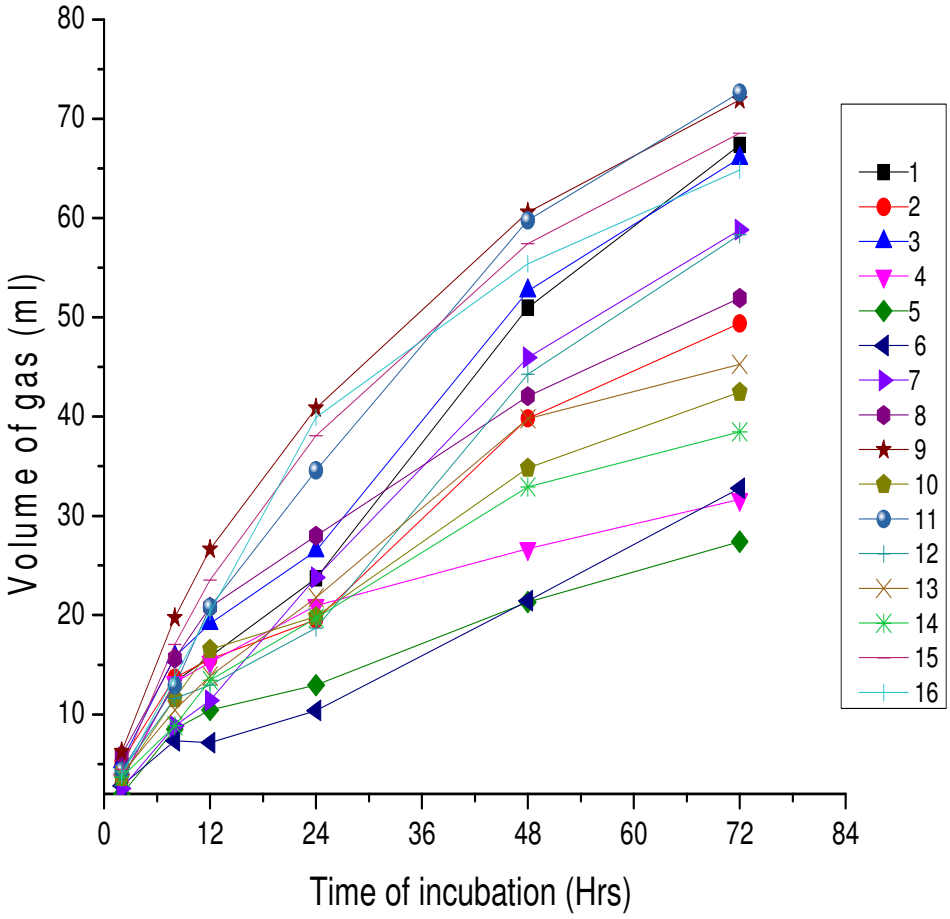

Figure 2.1 Gas production pattern of tropical grasses used in this study

1, *Anthephora argentea*; 2, *Brachiaria ciliaris*; 3, *Cenchrus ciliaris*; 4, *Eragrostis trichophora*; 5, *Panicum coloratum*; 6, *Pogonarthria squarrosa*; 7, *Setaria verticillata*; 8, *Stipagrostis uniplumis*; 9, *Schmidtia pappophoroides*; 10, *Centropodia glauca*; 11, *Stipagrostis obtusa*; 12, *Aristida vestita*; 13, *Tricholaena monachne*; 14, *Stipagrostis ciliata*; 15, *Cynodon dactylon*; 16, *Digitaria eriantha*.

Table 2.4 Gas production parameters of tropical perennial grasses used in the study

Scientific name	Gas production parameters		
	b	c	PD
Anthephora argentea	123.2^{f}	0.044^{e}	57.64^{f}
Aristida vestita	110.3^{i}	0.046^{d}	53.0^{h}
Brachiaria ciliaris	114.1^{g}	0.036^{j}	47.6^{j}
Cenchrus ciliaris	144.5^{e}	0.038^{i}	62.9^{e}
Centropodia glauca	99.0^{l}	0.040^{g}	44.0^{l}
Cynodon dactylon	150.1^{c}	0.047^{c}	72.5^{b}
Digitaria eriantha	145.2^{d}	0.046^{d}	69.3^{d}
Eragrostis trichopophora	75.8^{n}	0.057^{a}	40.3^{m}
Panicum coloratum	65.6^{p}	0.038^{i}	28.2^{o}
Pogonarthria squarrosa	72.5^{o}	0.029^{k}	26.8^{p}
Setaria verticillata	110.9^{i}	0.043^{f}	51.2^{i}
Stipagrostis uniplumis	112.8^{h}	0.051^{b}	56.9^{g}
Schimdtia pappophoroides	174.3^{a}	0.051^{b}	88.3^{a}
Stipagrostis obtusa	160.9^{b}	0.039^{h}	70.4^{c}
Stipagrotis ciliata	91.9^{m}	0.039^{h}	40.1^{n}
Tricholaena monachne	106.6^{k}	0.039^{h}	46.4^{k}
SEM	*0.006*	*0.001*	*0.006*
P-value	*<0.0001*	*<0.0001*	*<0.0001*

Units for *b* (slowly fermentable fraction) and PD (potential) are mL per g DM; units for *c* (rate of fermentation of fraction *b*) are mL h^{-1}. With columns, means followed by the same letter are not significantly different at p ≥0.05; SEM, standard error of mean

Table 2.5 Total and individual volatile fatty acid (mmol L^{-1}) production, acetate to propionate ratio (A : P), and NH_3–N (mg 100 ml^{-1}), in supernatant after 72 hr incubation of the grasses

Scientific name	NH_3-N	Volatile fatty acids						
		Acetic	Propionic	Iso butyric	Butyric	Valeric	TotalVFA	A : P
Anthephora argentea	7.97[m]	91.76	30.29	2.68	12.21	2.92	139.9	3.03
Aristida vestita	8.71[k]	52.35	22.99	1.81	6.79	2.04	85.97	2.42
Brachiaria ciliaris	15.3[d]	47.97	19.83	1.73	6.80	2.02	78.36	2.60
Cenchrus ciliaris	8.70[l]	56.34	21.68	1.63	6.17	2.37	88.19	3.26
Cynodon dactylon	10.6[h]	59.58	18.51	1.97	7.97	2.51	90.54	2.90
Centropodia glauca	15.4[c]	46.47	16.85	1.70	5.93	2.10	73.05	2.20
Digitaria eriantha	13.6[e]	48.55	18.81	1.47	5.67	1.74	76.24	2.86
Eragrostis trichopophora	11.4[f]	48.92	15.00	1.55	6.14	2.01	73.62	2.65
Panicum coloratum	19.5[a]	36.86	12.73	1.52	4.97	1.69	57.77	3.55
Pogonarthria squarrosa	5.31[p]	51.55	23.41	1.78	6.76	1.99	85.49	2.76
Setaria verticillata	9.18[j]	54.38	19.04	1.70	5.70	2.20	83.03	2.69
Stipagrostis uniplumis	18.3[b]	43.90	16.56	1.71	5.95	2.00	70.13	2.28
Schmidtia pappophoroides	6.66[n]	71.83	20.25	2.21	9.95	2.91	107.2	2.57
Stipagrostis obtusa	6.63[o]	48.22	17.94	1.55	5.13	1.70	74.54	2.79
Stipagrotis ciliata	9.39[j]	41.50	14.86	1.82	5.57	2.17	65.92	3.22
Tricholaena monachne	11.0[g]	52.18	20.31	1.60	6.97	1.90	82.96	2.58
SEM	*0.0001*							
P value	*<0.001*							

For NH_3-N, means followed by the same letter are not significantly different at $p \geq 0.05$; SEM, standard error of the mean

2.4.4. Methane production and its association with *in vitro* organic matter digestibility, volatile fatty acid and chemical composition

Methane production (ml g^{-1} DM) and its percentage concentration (v/v) in total gas differ (p<0.05) among the studied grass species and incubation periods (Table 2.6). Methane production (ml g^{-1} DM) and its concentration (%) from tested perennial grasses differed significantly (p<0.05) at various periods of incubation. The volume of CH_4 production (ml g^{-1} DM) was higher (p<0.05) for *Stipagrostis ciliata, Anthephora argentea* and *Aristida vestita* during the early incubation period (2 hr), while *Stipagrostis ciliata* and *Aristida vestita* produced significantly (p<0.05) higher volumes of methane during the 12-48 hr incubation period. The lowest volume of methane was recorded for *Stipagrostis uniplumis* during the early 2 hr period, while *Cenchrus ciliaris* and *Panicum coloratum* produced the lowest volume during 12-48 hr periods of incubation.

Methane production expressed in mass (g per DM and g per IVOMD) and lost energy (% ME) varied significantly (p<0.05) among the studied grasses (Table 2.7). *Cynodon dactylon* and *Tricholaena monachne* contained high amounts of ME (12.3 MJ kg^{-1} DM) and IVOMD (61%), while *Schmidtia pappophoroides* (6.21 MJ kg^{-1} DM) and *Stipagrostis ciliata* (40.7%) contained the lowest amounts of ME and IVOMD, respectively. The perennial grasses showed significant variation in their ME, IVOMD, and NH_3-N content.

Anthephora argentea and *Stipagrostis ciliata* produced the highest concentration of methane in terms of g kg^{-1} DM and g kg^{-1} IVOMD. *Cenchrus ciliaris*, *Setaria verticillata* and *Panicum coloratum* produced significantly (p<0.005) the lowest methane when expressed in terms of g kg^{-1} DM and g kg^{-1} IVOMD. Methane production, which was expressed in terms of eructated energy, was higher (p<0.05) from *Anthephora argentea* and *Stipagrostis ciliata,* and this ranged from 0.34% to 1.12% across the grass species.

Table 2.6 Percentage (%) and volumes (ml g^{-1} DM) of CH_4 production from the grasses

Species	2 hr		12 hr		24 hr		48 hr	
	%	ml	%	ml	%	ml	%	ml
Anthephora argentea	2.52^{cde}	0.81^{ab}	7.15^{b}	5.51^{a}	9.02^{a}	12.7^{a}	8.59^{e}	22.7^{b}
Aristida vestita	2.86^{ab}	0.79^{ab}	4.59^{i}	3.15^{i}	5.62^{h}	4.95^{k}	6.02^{g}	8.45^{h}
Brachiaria ciliaris	2.48^{cde}	0.63^{bcd}	5.88^{g}	3.58^{fg}	6.26^{g}	5.65^{j}	8.52^{e}	12.1^{g}
Cenchrus ciliaris	2.26^{efg}	0.18^{efg}	6.54^{d}	2.83^{j}	7.95^{cde}	4.38^{l}	8.16^{e}	7.68^{i}
Centropodia glauca	2.34^{def}	0.37^{def}	6.51^{d}	3.50^{gh}	7.63^{de}	6.14^{i}	9.46^{d}	14.4^{e}
Cynodon dactylon	2.146^{fg}	0.16^{fg}	5.33^{h}	3.22^{i}	4.60^{i}	4.50^{l}	5.16^{h}	7.10^{i}
Digitaria eriantha	1.03^{g}	0.05^{g}	6.36^{de}	3.66^{f}	7.47^{e}	6.53^{h}	9.85^{d}	11.77^{g}
Eragrostis trichopophora	2.578^{dc}	0.56^{bcd}	6.44^{d}	4.31^{d}	7.55^{e}	9.85^{c}	10.67^{c}	21.9^{bc}
Panicum coloratum	2.33^{def}	0.21^{efg}	6.03^{fg}	3.17^{i}	8.91^{a}	7.28^{g}	8.09^{e}	14.3^{e}
Pogonarthria squarrosa	2.51^{cde}	0.67^{bc}	7.96^{a}	4.91^{b}	8.34^{bc}	9.11^{d}	11.77^{a}	22.0^{b}
Setaria verticillata	2.39^{cdef}	0.19^{efg}	5.35^{h}	2.89^{j}	5.46^{h}	4.02^{m}	6.41^{g}	6.37^{j}
Stipagrostis uniplumis	2.01^{g}	0.01^{g}	6.20^{de}	4.18^{e}	6.90^{f}	7.61^{f}	7.37^{f}	13.3^{f}
Stipagrostis obtusa	2.44^{cdef}	0.47^{cde}	6.53^{d}	4.41^{c}	8.10^{cd}	9.68^{c}	8.33^{e}	19.1^{d}
Schmidtia pappophoroides	2.53^{cde}	0.46^{cde}	7.20^{b}	4.10^{e}	8.71^{ab}	8.08^{e}	11.06^{a}	19.7^{d}
Stipagrotis ciliata	3.10^{a}	1.01^{a}	6.84^{c}	4.86^{b}	9.18^{a}	11.70^{b}	11.33^{ab}	23.1^{a}
Tricholaena monachne	2.69^{bc}	0.68^{bc}	5.89^{g}	3.42^{h}	6.81^{f}	6.05^{i}	9.69^{d}	13.7^{ef}
SEM	0.093	0.009	0.078	0.032	0.161	0.103	0.190	0.250
P value	*<0.001*	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*

Within columns, means followed by the same letter are not significantly different at $p \geq 0.05$; SEM, standard error of the mean

Table 2.7 *In vitro* organic matter digestibility (IVOMD, g kg^{-1} DM), metabolizable energy (ME, MJ kg^{-1} DM), methane (CH_4) production (g kg^{-1} DM), and percentage of energy lost as CH_4 (% ME) from the grasses after 24 hr of incubation

Scientific name	IVOMD	ME	CH_4 (g kg^{-1} DM)	CH_4 (g kg^{-1} IVOMD)	Lost energy as methane
Anthephora argentea	572.9^{e}	11.7^{b}	12.7^{a}	15.6^{a}	2.24^{a}
Aristida vestita	548.8^{g}	9.56^{g}	4.95^{k}	7.73^{e}	1.65^{fg}
Brachiaria ciliaris	484.6^{j}	10.4^{f}	5.65^{j}	9.12^{d}	1.92^{def}
Cenchrus ciliaris	427.1^{l}	9.40^{h}	4.38^{l}	6.33^{f}	1.72^{gh}
Centropodia glauca	511.8^{h}	7.59^{k}	6.14^{i}	9.11^{d}	1.82^{def}
Cynodon dactylon	610.1^{a}	12.3^{a}	4.50^{l}	7.39^{e}	1.20^{h}
Digitaria eriantha	420.7^{m}	8.58^{i}	6.53^{h}	10.30^{c}	1.98^{bcdef}
Eragrostis trichopophora	557.9^{f}	11.4^{c}	9.85^{c}	12.7^{b}	1.16^{h}
Panicum coloratum	447.01^{k}	8.16^{j}	7.28^{g}	10.56^{c}	2.18^{abc}
Pogonarthria squarrosa	582.3^{c}	11.6^{b}	9.11^{d}	12.12^{b}	2.12^{abcd}
Schmidtia pappophoroides	407.9^{o}	6.12^{m}	4.02^{m}	10.60^{c}	1.86^{cdef}
Setaria verticillata	577.7^{d}	11.2^{d}	7.61^{f}	6.55^{f}	0.67^{i}
Stipagrostis ciliata	406.9^{n}	8.15^{j}	9.68^{c}	14.9^{a}	2.21^{ab}
Stipagrostis uniplumis	446.9^{k}	6.70^{l}	8.08^{e}	10.32^{c}	1.91^{def}
Stipagrostis obtusa	500.3^{i}	10.7^{e}	11.70^{b}	12.01^{b}	1.72^{ef}
Tricholaena monachne	604.7^{b}	12.2^{a}	6.05^{i}	9.88^{c}	1.31^{gh}
SEM	*0.004*	*0.06*	0.103	0.144	0.032
P value	*<0.0001*	*<0.0001*	0.001	0.001	0.001

Within columns, means followed by the same letter are not significantly different at $p \geq 0.05$; SEM: standard error of the mean

Methane production was negatively correlated with CP (-0.357), ash (-0.602*), ether extract (-0.299*), non-fibrous carbohydrate (NFC -0.635*), NDFN (-0.308*), and ADFN (-0.398*) of the grass species (Table 2.8). A significant positive correlation was also noted between methane production and NDF (0.652*), ADF (0.703*), ADL (0.371*), cellulose (0.658*) and hemicellulose (0.643*). There was also a positive correlation between methane production and acetate (0.307*), iso-butyrate (0.423*) and butyrate (0.323*).

Linear regression of methane production based on the studied parameters indicated that the equations based on NFC, NDF and ADF values can be regarded as better predictors of CH_4 production (ml g^{-1} DM) (Table 2.9).

Table 2.8 Pearson correlationbetween *in vitro* CH_4 production and chemical constituents of the studied grasses

Major feed components	CH_4	Fibre components	CH_4	Nitrogen component	CH_4	Fermentation characteristics	CH_4	Volatile fatty acids	CH_4
DM	*0.206*	*NDF*	*0.652**	*CP*	*0.357**	*GP_{24}*	*0.13*	*Total VFA*	*0.251*
ash	*0.602**	*ADF*	*0.703**	*NDFN*	*0.308**	*GP_{48}*	*0.20*	*Acetate*	*0.307**
OM	*0.218*	*ADL*	*0.371**	*ADFN*	*0.398**	*ME*	*0.15*	*Propionate*	*0.13*
EE	*0.299**	*NFC*	*0.635**			*IVOMD*	*0.03*	*Iso butyrate*	*0.413**
		Cellulose	*0.658**					*Butyrate*	*0.323**
								Valeric	*0.16*
								A : P	*0.223*

NDFN, ADFN: Neutral and acid detergent nitrogen; IVOMD, *in vitro* organic matter digestibility; Significant at *$P<0.05$

Table 2.9 Linear regression equation to predict CH_4 (g kg^{-1} DM) from chemical constitutes, ME, and IVOMD of the grasses

Equation	R^2	P
CH_4 = 8.05 - 0.048 Ash	*0.35*	<0.001
CH_4 = 7.95 - 0.1822 EE	*0.07*	0.039
CH_4 = 8.07 - 00171 EE -0.0475 Ash	0.33	<0.001
CH_4 = - 22.7 + 0.035 NDF + 0.0653 NDFN	*0.49*	<0.001
CH_4 = - 7.95 + 0.008 NDF + 0.0286 ADF + 0.08 ADL	*0.46*	<0.001
CH_4 = -6.19 + 0.00264 ADF - 0.213 ADFN	*0.51*	<0.001
CH_4 = -12.1 + 0.0232 NDF	*0.41*	<0.001
CH_4 = -8.08 + 0.0289 ADF	*0.48*	<0.001
CH_4 = 1.72 +0.0453 ADL	*0.11*	0.009
CH_4 = 3.99 - 0.0259 CP	*0.10*	0.013
CH_4 = 5.98 - 0.01 CP - 0.281 ADIN - 0.0304 NDFN	*0.22*	0.003
CH_4 = 7.30 - 0.356 ADFN - 0.0304 NDFN	*0.16*	0.007
CH_4 = 8.72 -0.0233 NFC	*0.39*	<0.001

EE: Ether extract; NDF and ADF: neutral and acid detergent fibre; NDFN and ADFN: neutral and acid detergent insoluble nitrogen; ADL: acid detergent lignin; CP: crude protein; NFC: non-fibre carbohydrate

2.5. Discussion

2.5.1. Plant cover and dry matter yield

The higher plant cover and DM yield for the canopied sub-habitat can be attributed to high soil fertility under trees, which might be associated with the accumulation of soil that swept away from uncanopied areas in arid regions (Abdallah *et al.,* 2008). Moreover, in such arid rangeland, trees provide better protection of herbaceous plants growing under cover. In addition, the higher plant cover under canopied areas has a positive effect on fertility and water balance of soil, which creates a better micro-climate for the growth of palatable perennial species with high water-use efficiency, OM decomposition and nutrient dynamics (Abule *et al.,* 2005; Snyman, 2005). The better performance in plant cover and DM yield from arid grassland has been reported by other researchers in tropical and sub-tropical conditions (Abule *et al.,* 2005; Snyman 2005; Abdallah *et al.,* 2008).

2.5.2. Chemical composition and gas production

The nutrient composition of the studied species was comparable with the values reported for South African feeds and forages by Bredon *et al.* (1987) in a similar season. However, the CP values of most species were low in terms of satisfying animal nutritional needs. The cell wall content (NDF, ADF and ADL) values were high for the studied grasses. The low CP and the high cell wall content observed in this study are due to seasonal fluctuation, which is caused partly by dynamics of the plant nutrients in seasons. In comparison with the wet season, when nitrogen is translocated to actively photosynthesizing tissues, resulting in a lower carbohydrate: nitrogen ratio, in dry season nutrients accumulate as lignin, cutin, and other phenolic plant defensive substances in the cell wall (Van Soest, 1994), and this influences the nutritive quality of forage. This generally results in low fermentation and organic matter digestibility and consequently in higher CH_4 emissions. Supplementation with concentrate is important to improve lower digestibility and enhance propionate production. It is possible for small-scale farmers of tropical and sub-tropical Africa to supplement ruminants with foliage from trees and shrubs, as it remains fairly constant during the early dry period and contains a reasonable amount of CP (14% and above) needed by ruminants for a medium level of production (Subba, 1999).

The differences in the volume of gas produced among grass species are due mainly to differences in the fermentable organic matter and fibre contents. These in turn affect the rate and extent of substrate fermentation to short-chain fatty acids, carbon dioxide and CH_4 (Blümmel & Becker, 1997). The amount of short-chain fatty acids produced is related to organic matter digestibility and energy content of feeds. However, CH_4 production is an energy loss, as the portion of animal feed converted to CH_4 would be lost through eructation (Getachew *et al.,* 2005).

2.5.3 Production of CH_4 and other fermentation end products

In ruminants, CH_4 is produced by rumen microbes through anaerobic fermentation of cell contents and cell-wall contents of feed. The variation in methane production among grass species in the present study may be attributed to their significant differences in chemical constituents such as CP, ether extract, ADF, NDF, ADL, NDFN, ADFN and NFC concentrations. Similarly, the differences in CH_4 production across incubation time were

attributed to the differences in the rate of fermentation of these feeds. Higher CH_4 production for *Anthephora argentea* and *Aristida vestita* grasses for most of the incubation times was due to its high cellwall contents (ADF, NDF, ADL, cellulose and hemi-cellulose). In addition, the efficiency expressed in ratio of CH_4 to total gas produced was comparatively the highest for these grasses. On the other hand, *Cenchrus ciliaris* and *Panicum coloratum* produced the lowest amount of CH_4 during 12, 24 and 48 hr incubation. This might be due to the low level of cell-wall content (ADF, NDF, ADL, cellulose and hemicellulose) and the A: P ratio. The concentration of methane production rose as the incubation time increased in this study. This might be because the more fermentable component of the fibre fraction was fermented at the early stage and produced less methane than the less fermentable component, which remained behind, and its fermentation produced more methane as fermentation time advanced. There is little information on tropical perennial grasses methane production under tropical conditions to compare with this result. It was assumed that the higher values in this report compared with temperate grasses (Getachew *et al.,* 2005) might be attributed to relatively higher levels of fibre and lignin (Van Soest, 1994) recorded for dry season harvested grasses (Table 2.1), and associated lower values of digestibility (13%) of most tropical grasses compared with temperate grasses (Minson, 1990).

Similar to this report, many findings showed that CH_4 production could be influenced by the contents and nature of cell wall composition (NDF, ADF, ADL, cellulose, and hemicellulose (Santoso *et al.,* 2003; Santhoso *et al.,* 2007; Singh *et al.,* 2012). This is because OM digestion, fermentation and production of short-chain volatile fatty acids of forages are dependent mainly on their structural factors. The relative proportion of cell types present in its tissues and the existence of factors restricting microbial access to walls determine digestion and the production of CH_4 (Van Soest, 1994). On the other hand, increasing protein in the diet is expected to decrease CH_4 emission due to the direct negative association of protein with methane (Table 2.7) and the replacement in the diet of methanogenic carbohydrate with protein (Pelchen & Peters, 1998).

2.5.4. Association between CH_4, *in vitro* organic matter digestibility, volatile fatty acid and chemical composition

In the present study, a significant association was revealed between CH_4 production and the various parameters. Similarly, many researchers have explained the relationship between the

quality of feed, expressed in terms of chemical constituents, and digestibility (CH_4 production) (Santoso *et al.,* 2003; Santoso *et al.,* 2007; Singh *et al.,* 2012). This is mainly because fermentability of feed to its end products is determined primarily by digestibility, which depends mainly on its composition. For example, VFA concentration and their relative proportion, which mainly affect CH_4 production, are influenced by the nature and fermentation of carbohydrates (Johnson & Johnson, 1995).

The positive correlation between CH_4 production and cell wall (NDF, ADF, cellulose and lignin) observed in this study is in agreement with previous reports (Moss, 2000; Singh *et al.,* 2012), while the negative correlation between CP and CH_4 production agrees with the findings of Moss (2000). Moreover, negative correlations of CH_4 production with energy and EE agree with previous findingsby Yan *et al.* (2009) and Ellis *et al.* (2007), respectively.

Our findings for a prediction equation of enteric CH_4 production with ADF and NDF had a R^2 = 0.48 (p<0.01) and R^2 = 0.41 (p<0.01), respectively, whereas equations using protein fractions and carbohydrate fractions had a R^2 = 0.51 (p<0.03) and 0.49 (p<0.01), respectively. This shows that carbohydrate and its fractions are a better estimate of *in vitro* CH_4 production from dry-season tropical perennial grasses. This is in agreement with a previous finding of Johnson and Johnson (1995,) which identified that carbohydrate fed to livestock had a major effect on CH_4 production, probably because of the effect on rumen pH and its microbial population. Similar to this finding, Santoso *et al.* (2007) and Singh *et al.* (2012) indicated that carbohydrate fractions (NDF and ADF) were better CH_4 predictors than feed components.

2.6. Conclusion

The results of the present study revealed that CH_4 production varied between grass species and indicated a potential for screening species for lower CH_4 production. The negative correlation between CH_4 production and CP indicated that screening and selecting perennial grass forages for higher CP or lower NDF and ADF content could mitigate CH_4 production by ruminants grazing tropical grasslands. Because most palatable perennial grasses produced low to medium CH_4, this might indicate that good-quality grasses reduce CH_4 emissions. Moreover, in formulating rations for ruminant animals, the use of grass with a lower CH_4 production might have the potential to mitigate CH_4 emission from animals receiving such rations.

CHAPTER 3

Effect of tannin and species variation on *in vitro* digestibility, gas and methane production of tropical browse plants

3.1. Abstract

Nineteen tanniferous browse plants were collected from South Africa to investigate their digestibility, gas production characteristics and methane (CH_4) production. Fresh samples were collected, dried in a forced oven, ground and analysed for their nutrient composition. *In vitro* gas production (GP) and organic matter digestibility (IVOMD) were determined using rumen fluid, which was collected, strained and prepared anaerobically. A semi- automated system was used to measure gas production by incubating the sample in a shaking incubator at 39°C. There was significant ($p<0.05$) variation in chemical composition of the browses. Crude protein (CP) content of the species ranged from 86.9 to 305.0 g kg^{-1} DM. The neutral detergent fibre (NDF) ranged from 292.8 to 517.5 g kg^{-1} DM, while acid detergent fibre (ADF) ranged from 273.3 to 495.1 g kg^{-1} DM. The total tannin (TT) concentration of the browses ranged between 13.4 to 172 g kg^{-1} DM, while hydrolysable tannin (HT) concentration ranged between 6.8 to 139 g kg^{-1} DM. The ash, ether extract (EE), non-fibrous carbohydrate (NFC), neutral detergent insoluble nitrogen (NDFN), acid detergent insoluble nitrogen (ADFN) and crude protein (CP) were negatively correlated with methane production. Methane production was positively correlated with NDF, ADF, cellulose and hemicellulose. Tannin decreased gas production, IVOMD, total VFA and methane production. The observed low methanogenic potential and substantial NH_3-N generation of some of the browses might be potentially useful as rumen manipulating agents. However, a systematic evaluation is needed to determine optimum levels of supplementation in a mixed diet in order to attain a maximal depressing effect on enteric CH_4 production with a minimal detrimental effect on rumen fermentation of a poor-quality roughage-based diet.

Key words: digestibility, gas production, methane, ammonia-N, volatile fatty acids

3.2. Introduction

Enteric methane is a greenhouse gas that causes significant losses of energy in ruminants and has been estimated to represent globally 2079 and 2344 Mt CO_2-eq $year^{-1}$ for 2010 and 2020, respectively (Hristov *et al.,* 2013). In South Africa, methane production has been estimated at 123, 803, 29.8 and 7.98 Gg annually from dairy cattle, extensive beef cattle, feedlot cattle and manure, respectively (Du Toit *et al.,* 2013). Enteric methane is produced mainly by methanogenic archaea during feed fermentation in the rumen (Leng, 2008). Therefore, in targeting CH_4 reduction, it is crucial to develop a strategy that decreases CH_4-producing micro-biota activities and proliferation without limiting rumen function.

Recently numerous reports have shown reduced enteric CH_4 due to inclusion of tannin-rich browses, because the tannins have anti-methanogenic activity, either by direct inhibition of methanogens or indirectly through inhibition of protozoa (Animut *et al.,* 2008; Hristov *et al.,* 2013). Tannins are polyphenolic compounds, which bind to protein, and can be used as chemical additives for protecting and decreasing ruminal fermentation of proteins in ruminant feeds (Makkar, 2003). Tannins are complex polymers with various linkages and bonds that vary among browse species and within parts of plants (Makkar, 2003; Patra & Saxena, 2011). This contributes to differences in degrees of polymerization and chemical structures that add to the differing biological properties (Patra & Saxena, 2011).

The huge diversity in tannin structures may explain their vast variable effects on methanogenesis and rumen function, depending on source, type and level of tannin (Mueller-Harvey, 2006; Patra *et al.,* 2011). Tannins from different plants exhibit variation in their effects at the same concentration, as evidenced by differences in magnitude of gas production and digestibility (Makkar, 2003, Guglielmelli *et al.,* 2011). This indicates that tannin from different plants might show different responses in digestibility and CH_4 production. Interestingly, most tropical browses contain reasonably high nitrogen and low fibre contents and thus can be considered supplemental feed for poor-quality roughages. However, the net improvement in digestibility is influenced greatly by the type and the level of phenolic compounds under tropical conditions where supplementation of nitrogen is critical. Characterization of tropical browse resources for fermentation and CH_4 production potential is important, especially if their inclusion is being considered in poor-quality feeds for better utilization for ruminant feeding. Thus this *in vitro* study was aimed at investigating the

effects of tannin-rich browseson gas and CH_4 production, IVOMD, NH_3-N and VFA production, as well as the correlation of IVOMD and CH_4 with chemical and phenolic composition by incubating the samples with and without poly ethylene glycol (PEG) (molecular weight, 6000).

3.3 Materials and methods

3.3.1. Sample collection, preparation and chemical analysis

Samples of nineteen tannin-rich browse plants (Table 3.1) were collected from Gauteng, South Africa, in the summer rainy season (December 2011–February 2012). The area is located at about 25°44'30" S, 28°15'30" E, at an elevation of about 1370 masl. It has two distinctive seasons: a dry season (March–September) and rainy season (October–February) with warm and humid condition in summer, while winter is dry, cold and sunny. This is a summer rainfall area with a mean precipitation of 674 mm and an average annual temperature of 17.3°C. The browses were harvested at the early vegetative stage. Fresh foliages (leaves and stems <3 mm diameter) were harvested from at least five randomly selected and tagged representative plants of each species. Samples from each species (up to 200 g) were dried at 55°C for 48 hr and ground to pass a 1 mm screen for subsequent chemical and *in vitro* analyses. Samples were analysed for DM, Ash, NDF, ADF, ADL, EE, NDFN, ADFN and CP according to standard methods described in detail in Chapter 2, section 3.2.

Total phenols (TP), total tannins (TT) and condensed tannins (CT) were determined according to the procedure described by Makkar (2003). Condensed tannins (CT) were determined by the butanol–HCl–iron method (Porter *et al*., 1986). Hydrolysable tannins (HT) were estimated as the difference between TT and CT (Singh *et al.,* 2005). Total phenols and tannins were expressed as tannic acid equivalent and CTs as leucocyanidin equivalent.

3.3.2. Measurement of gas production and *in vitro* organic matter digestibility

Collection of rumen fluid and preparation of buffer solution

The rumen fluid was collected from two rumen-cannulated Merino sheep fed lucernehay ad libitum. Sampling was done before the morning feeding. The hay contained 175 g kg^{-1} CP,

465 g kg^{-1} NDF and 8 MJ kg^{-1} DM ME. It was prepared and maintained anaerobically, being purged with CO_2 to maintain anaerobic conditions in the laboratory (Grant & Mertens, 1992). After blending, the rumen fluid was transferred to a large glass beaker inside a 39°C water bath, which was continuously purged with CO_2 and stirred as recommended by Goering and Van Soest (1970). The buffer solution, macro mineral solution and micro mineral solution were prepared in large volumes and utilized as needed, as described by Goering and Van Soest (1970).

Measurement of gas production and *invitro* organic matter digestibility

Gas production was determined as described by Theodorou *et al.* (1994). Approximately 400 mg of each browse feed sample, with or without 400 mg of polyethylene glycol (PEG) (MW, 6000, analytical grade sigma-aldrich), was weighed into a 120 ml serum bottle. Media preparation, incubation of samples with rumen fluid and media, gas pressure recording over incubation times, and sampling supernatants for NH_3-N and VFA were done according to procedures that were similar to those described in Chapter 2, section 3.3. The IVOMD content of the browses was determined according to the detailed method described in Chapter 2, section 3.4.

3.3.3. Methane measurements

Methane production was measured separately from duplicate bottles incubated for each browse species and gas sample were collected from each bottle at 2, 12, 24, and 48 hr. A procedure similar to that described in Chapter 2, section 3.5, was used to measure the methane production.

3.3.4. Calculations, statistical analysis and model

Metabolizable energy (ME, MJ kg^{-1} DM) was estimated according to Menke and Steingass (1988) as ME (MJ kg^{-1} DM) = 2.20 + 0.136 $IVGP_{24}$ (ml 0.5 g^{-1} DM) + 0.057 CP (% DM)

Rate and extent of gas production were determined for each feed by fitting gas production data to the non-linear equation: $y = a + b\ (1 - e^{-ct})$ (Ørskov and McDonald 1979), where y is the volume of GP at time t (ml); a is soluble component, b is the gas production from the

insoluble, but slowly fermentable/degradable fraction (ml); c is the rate of GP from insoluble fraction per hour.

Methane (mL) = Total gas produced (mL) × % methane in the sample

$$\text{Methane increase (\%)} = \frac{(CH_4 \text{ with PEG addition (ml)} - CH_4 \text{ without PEG addition (ml)})100}{CH_4 \text{ with PEG addition (ml)}}$$

Data were analyzed statistically using the GLM procedure of SAS (2009), regarding each incubation run as a block. The average of the two bottles was regarded as an observation from experimental unit. The differences among means were determined using Tukey's test. Pearson correlation (PROC CORR, SAS, 2009) analysis discovered significant relationships between the parameters used in the study. The following model was used:

$Yij = \mu + \alpha_i + \beta_j + e_{ijk}$, where

Yij is the response of the browse i observed in block j

μ overall mean of the browse

α_i is the effect of j^{th} block (run)

β_j is the effect of i^{th} browse

e_{ijk} is the associated random error.

3.4 Results

3.4.1. The composition of browses

The leaves of studied browses species differed significantly ($p<0.05$) in terms of their chemical composition (Table 3.1). The CP, NDFN, and ADFN values ranged between 86.9 to 305, 5.4 to 41.4, and 11.8 to 48.1 g kg^{-1} DM, respectively. The highest CP value was recorded for *Leucaena leucocephala*, while the lowest value was recorded for *Rhus lancea* and *Euphorbia tirucalli*. The highest NDFN content was found for *Melia azedarach*, while *Kirkia acuminata* had the highest value of ADFN. The lowest value of NDFN and ADFN was recorded in *Euphorbia tirucalli*. The NDF (g kg^{-1} DM) values ranged from 292.8 to 522.4 g^{-1} kg DM. The highest value was recorded in *Quercus rubica,* while the lowest value was recorded in *Morus alba,* whereas ADF (g kg^{-1} DM) values ranged from 271.6 in *Kirkia acuminata* to 485.1 in *Peltrophorum africanum.* The highest value of lignin was recorded for *Olea africanum,* whereas *Euphorbia tirucalli* contains the lowest value.

The phenolic compositions of the browse species were significantly different (p<0.05) (Table 3.2). The TP concentration ranged between 32.4 and 209.1 g kg^{-1} DM. The total tannin concentration of the browses ranged between 13.4 and 172 g kg^{-1} DM, while HT concentrations ranged between 6.8 and 139 g kg^{-1} DM. Among the browse species, *Rhus lancea* contained significantly (p<0.05) the highest concentration of TP, TT and HT, while *Peltrophorum africanum* had the highest concentration of TNT (total non-tannin) and CT. *Melia azedarach* contained significantly (p<0.05) the lowest concentrations of TP, TT, CT and HT.

3.4.2. In vitro gas production and fermentation products

The *in vitro* gas production of the browse species at different times of incubation with and without PEG is shown in Table 3.3. There was a significant (p<0.05) variation among browse species in terms of gas production. During the early period of incubation, *Melia azedarach* produced the highest volume of gas, whereas during later hours (after 12 hr) *Morus alba* produced the highest gas volume. *Rhus lancea* produced the lowest volume of gas during the early period, while the lowest volume of gas was recorded for *Combretum microphyllum* after 12 hours. Inclusion of PEG (the absence of tannins) significantly (p<0.05) improved gas production for all browses. For instance, the percentage increase due to the absence of tannins (with PEG) ranged from 1.0% in *Melia azedarach* and 121.2% in *Rhus lancea* (data not shown).

Table 3.1 Chemical composition of browse plants used in the study

Scientific name	Chemical components (g kg^{-1}DM)								
	Ash	OM	CP	NDFN	ADFN	ADF	NDF	ADL	EE
Acacia nilotica	90.8^{g}	836.8^{h}	153.9^{k}	23.9^{f}	22.8^{h}	410.3^{d}	517.5^{a}	153.2^{d}	26.3^{ijk}
Acacia sieberriana	72.4^{j}	804.3^{k}	285.4^{c}	33.4^{c}	32.2^{c}	364.7^{fg}	425.8^{d}	174.3^{c}	41.4^{b}
Combretum microphyllum	81.3^{i}	840.6^{gh}	165.1^{j}	30.0^{e}	26.0^{f}	437.8^{c}	498.2^{b}	88.2^{j}	26.2^{jk}
Combretum molle	62.7^{l}	847.8^{f}	130.8^{l}	19.4^{k}	17.8^{l}	430.5^{c}	498.6^{b}	67.8^{l}	26.7^{hij}
Euphorbia tirucalli	133.3a	767.9^{o}	87.8^{r}	11.8^{m}	5.4^{o}	367.4^{f}	377.9^{f}	66.1^{l}	28.1^{fgh}
Ficus religiosa	131.5b	797.2^{l}	126.6^{n}	19.2^{k}	19.4^{jk}	483.2^{b}	496.3^{b}	108.1^{g}	18.6^{m}
Ficus thronniggi	120.5c	781.6^{n}	277.8^{d}	23.8^{h}	15.1^{m}	336.9^{i}	415.6^{d}	155.1^{d}	29.4^{ef}
Kirkia acuminata	50.7^{q}	877.1^{d}	129.3^{m}	39.1^{b}	41.2^{a}	271.6^{k}	317.8^{h}	118.7^{f}	24.8^{kl}
Kirkia wilmsii	65.5^{k}	874.4^{d}	103.2^{p}	22.0^{i}	20.4^{i}	356.3^{g}	370.9^{f}	79.6^{k}	30.6^{e}
Lespedeza cuneata	58.0^{p}	885.8^{c}	214.8^{f}	23.0^{h}	19.8^{ij}	411.1^{d}	418.2^{d}	100.4^{h}	26.1^{jk}
Leucaena leucocephala	86.6^{h}	827.4^{i}	305.0^{a}	39.3^{b}	32.1^{C}	382.3^{e}	443.4^{c}	89.3^{j}	29.2^{efg}
Melia azedarach	112.7^{d}	821.6^{j}	298.7^{b}	48.1^{a}	39.4^{b}	322.9^{j}	375.4^{f}	95.0^{i}	30.1^{e}
Morus alba	120.4c	788.7^{m}	169.7^{i}	28.2^{f}	22.9^{h}	273.3^{k}	292.8^{j}	91.7^{ij}	40.3^{b}
Olea europaea	59.1^{0}	907.7^{a}	93.0^{q}	23.6^{h}	18.7^{k}	346.7^{h}	378.6^{f}	221.2^{b}	27.8^{ghi}
Olea africanum	61.7^{m}	907.2^{a}	111.9^{o}	30.2^{e}	26.4^{f}	389.3^{e}	397.5^{e}	230.9^{a}	24.3^{l}
Peltrophorum africanum	60.1^{n}	865.4^{e}	171.7^{h}	30.1^{e}	24.2^{g}	485.1^{a}	518.7^{a}	142.9^{e}	44.4^{a}
Quercus rubica	60.3^{n}	892.5^{b}	178.5^{g}	37.7^{c}	28.0^{d}	418.1^{d}	522.4^{a}	124.9^{f}	35.9^{c}
Rhus lancea	105.1e	845.3^{fg}	86.9^{r}	15.3^{l}	14.1^{n}	390.6^{e}	424.5^{d}	78.7^{k}	32.1^{d}
Ziziphus mucronata	96.9^{f}	840.4^{gh}	243.3^{e}	33.1^{d}	27.2^{e}	248.3^{l}	339.2^{g}	101.4^{h}	17.6^{m}
SEM	*0.303*	*1.83*	*0.429*	*0.266*	*0.253*	*3.23*	*3.63*	*1.80*	*0.50*
P value	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*

Means with different superscript (letters) across the column for each parameter are significantly different at $p<0.05$
OM-organic matter, CP-crude protein, ADF-acid detergent fibre, NDF-neutral detergent fibre, ADFN-The N content of ADF, NDFN-the N content of NDF, ADL-acid detergent lignin, EE-ether extract;
SEM, standard error of the mean

Table 3.2 Secondary compositions of browses

Scientific names	Total Phenol	Total NON Tannin (PVPP)	Total Tannin	Condensed tannin	Hydrolysable tannin
Acacia nilotica	167.5^{d}	54.5^{b}	113.0^{d}	48.8^{b}	64.2^{h}
Acacia sieberriana	113.4^{j}	17.2^{k}	96.1^{h}	37.8^{g}	58.3^{i}
Combretum microphyllum	134.9^{e}	19.8^{gh}	115.2^{c}	38.6^{f}	76.6^{e}
Combretum molle	171.7^{c}	36.9^{e}	134.7^{b}	47.7^{c}	87.0^{d}
Euphorbia tirucalli	100.0^{k}	18.7^{j}	81.3^{k}	5.22^{q}	76.1^{e}
Ficus religiosa	121.4^{h}	9.49^{o}	111.9^{e}	18.1^{k}	93.8^{b}
Ficus thronniggi	34.4^{q}	7.7^{p}	26.7^{o}	16.0^{l}	10.7^{p}
Kirkia acuminate	126.1^{g}	31.6^{f}	94.5^{i}	25.6^{i}	68.9^{g}
Kirkia wilmsii	130.7^{f}	15.2^{l}	115.5^{c}	43.4^{d}	72.1^{f}
Lespedeza cuneata	119.5^{i}	20.0^{g}	99.5^{g}	42.2^{e}	57.3^{j}
Leucaena leucocephala	99.2^{l}	11.3^{n}	87.9^{j}	18.3^{k}	69.6^{g}
Melia azedarach	32.4^{r}	19.0^{i}	13.4^{p}	6.54^{p}	6.8^{q}
Morus alba	59.1^{p}	11.8^{m}	47.3^{m}	11.1^{n}	36.2^{m}
Olea europaea	87.6^{n}	42.8^{d}	44.9^{n}	10.5^{o}	34.4^{n}
Olea africanum	88.5^{m}	43.2^{c}	45.2^{n}	12.1^{m}	33.2^{o}
Peltrophorum africanum	206.7^{b}	93.0^{a}	113.7^{d}	60.0^{a}	53.7^{k}
Quercus rubica	126.1^{g}	19.6^{h}	106.5^{f}	18.2^{k}	88.2^{c}
Rhus lancea	209.1^{a}	37.2^{e}	172.0^{a}	33.0^{h}	139.0^{a}
Ziziphus mucronata	79.6^{o}	11.4^{n}	68.2^{l}	21.8^{j}	46.4^{l}
SEM	*0.24*	*0.078*	*0.25*	*0.16*	*0.313*
Pvalue	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*

Means with different superscript (letters) across the column for each parameter are significantly different at p<0.05; PVPP: polyvinyl Polypyrrolidone; SEM: standard error of the mean; TPs and tannins were expressed as tannic acid equivalent and CTs as leucocyanidin equivalent

Table 3.3 Gas production (ml g^{-1} DM) of browse plants with or without PEG

Scientific names	Hrs of incubation							
	2		12		24		48	
	PEG(-)	PEG(+)	PEG(-)	PEG(+)	PEG(-)	PEG(+)	PEG(-)	PEG(+)
Acacia nilotica	31.5^{h}	45.7^{ef}	68.2^{g}	131.0^{f}	81.5^{j}	152.8^{g}	109.3^{i}	184.0^{j}
Acacia sieberriana	41.9^{d}	44.8^{f}	126.0^{d}	135.8^{e}	165.7^{d}	169.7^{e}	174.5^{c}	201.5^{h}
Combretum microphyllum	26.4^{i}	34.2^{i}	41.6^{l}	64.6^{m}	45.3^{n}	71.4^{n}	62.1^{m}	102.5^{q}
Combretum molle	31.1^{h}	45.8^{ef}	41.0^{l}	75.8^{l}	48.4^{n}	84.4^{m}	74.1^{l}	121.1^{o}
Euphorbia tirucalli	43.7^{c}	47.3^{de}	94.8^{e}	116.1^{g}	131.5^{e}	173.6^{d}	207.3^{d}	240.^{d}
Ficus religiosa	22.7^{i}	41.0^{g}	66.0^{g}	92.3^{i}	113.8^{h}	144.8^{h}	156.8^{f}	189.0^{i}
Ficus thronniggi	25.9^{g}	40.9^{h}	66.0^{c}	92.3^{c}	113.8^{c}	144.8^{bc}	156.7^{b}	188.9^{b}
Kirkia acuminata	40.0^{ef}	48.9^{cd}	46.3^{k}	75.5^{l}	54.7^{m}	91.1^{l}	86.6^{j}	124.2^{n}
Kirkia willmsii	40.8^{de}	45.6^{ef}	58.7^{i}	82.0^{jk}	72.2^{k}	97.7^{k}	108.2^{i}	137.4^{m}
Lespedeza cuneata	39.0^{f}	58.8^{b}	96.7^{e}	144.1^{d}	119.4^{f}	165.3^{f}	148.6^{g}	182.4^{j}
Leucaena leucocephala	45.2^{bc}	49.8^{c}	96.2^{e}	135.7^{e}	109.6^{i}	153.3^{g}	113.8^{h}	179.4^{k}
Melia azedarach	65.6^{a}	71.0^{a}	171.6^{a}	175.1^{a}	196.0^{b}	198.9^{c}	219.7^{c}	222.7^{e}
Morus alba	41.7^{de}	47.1^{de}	157.9^{b}	167.5^{b}	205.7^{a}	217.5^{a}	254.9^{a}	267.8^{a}
Olea europaea	34.7^{h}	40.5^{gh}	67.0^{f}	83.2^{j}	111.7f^{g}	141.5^{i}	177.2^{c}	205.0^{g}
Olea africanum	40.1^{ef}	40.5^{gh}	73.4^{f}	83.6^{j}	115.4^{gh}	142.5^{hi}	182.7^{c}	205.8^{g}
Peltrophorum africanum	25.1^{i}	35.0^{i}	53.4^{j}	80.1^{k}	591^{l}	118.9^{j}	79.2^{k}	108.2^{l}
Quercus rubica	32.4^{h}	40.5^{gh}	61.8^{h}	84.7^{j}	72.9^{k}	93.1^{l}	82.2^{k}	109.5^{p}
Rhus lancea	18.2^{j}	28.2^{j}	29.2^{m}	111.2^{h}	34.3^{o}	154.9^{g}	64.2^{m}	210.7^{f}
Ziziphus mucronata	46.0^{b}	40.2^{gh}	156.7^{b}	148.5^{c}	205.7^{a}	202.3^{b}	254.4^{a}	252.2^{b}
SEM	*0.57*	*0.58*	*1.083*	*1.0*	*1.25*	*0.98*	*1.3*	*1.0*
P value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Means with different superscript across the column for each parameter are significantly different at p<0.05; PEG: polyethylene glycol, PEG (-) denotes presence of tannin, PEG (+) denotes absence of tannin; SEM: standard error of the mean

Gas production

Morus alba had the highest (p<0.05) gas production from the slowly fermentable organic matter (b value) and potential gas production (PD) in the absence and presence of tannins (Table 3.4). The lowest (p<0.05) b value was recorded for *Combretum microphyllum* and the lowest PD was recorded in *Rhus lancea* in the absence of tannins. The rate of gas production (c) was the highest (9.4 % h^{-1}) in *Leucaena leucocephala* and the lowest (2.9 % h^{-1}) in *Rhus lancea*. The absence of tannins significantly improved the b and PD values of fermentation kinetics of browses.

Table 3.4 *In vitro* gas production characteristics of the browse plants with or without PEG

Scientific name	[1]b		[2]C		[3]PD	
	PEG(-)	PEG(+)	PEG(-)	PEG(+)	PEG(-)	PEG(+)
Acacia nilotica	115.0^{m}	180.6^{k}	0.068^{e}	0.087^{b}	66.3^{k}	114.5^{g}
Acacia sieberriana	160.4^{f}	183.3^{f}	0.093^{b}	0.079^{d}	123.0^{d}	130.8^{e}
Combretum microphyllum	82.3^{s}	121.9^{q}	0.045^{p}	0.050^{n}	38.0^{p}	60.8^{r}
Combretum molle	96.9^{r}	147.7^{p}	0.041^{r}	0.047^{p}	43.5^{o}	71.8^{p}
Euphorbia tirucalli	202.4^{e}	269.5^{d}	0.057^{i}	0.056^{k}	107.8^{f}	130.5^{e}
Ficus religiosa	186.0^{g}	154.5^{n}	0.050^{l}	0.058^{j}	342.8^{h}	83.0^{m}
Ficus thronniggi	185.9^{c}	154.4^{c}	0.049^{m}	0.060^{h}	116.8^{e}	149.3^{c}
Kirkia acuminata	108.5^{n}	149.2^{o}	0.041^{q}	0.050^{n}	49.0^{n}	75.0^{o}
Kirkia willmsii	120.1^{k}	158.2^{m}	0.051^{k}	0.052^{m}	61.3^{l}	80.3^{n}
Lespedeza cuneata	160.4^{j}	183.3^{j}	0.071^{d}	0.082^{c}	94.0^{g}	113.5^{h}
Leucaena leucocephala	115.3^{l}	190.8^{i}	0.094^{a}	0.072^{e}	75.3^{j}	112.5^{i}
Melia azedarach	235.5^{d}	235.8^{e}	0.062^{g}	0.064^{g}	130.3^{c}	132.3^{d}
Morus alba	276.8^{a}	290.3^{a}	0.064^{f}	0.066^{f}	155.3^{a}	164.5^{a}
Olea europaea	183.7^{h}	199.4^{h}	0.047^{o}	0.057^{k}	89.0^{i}	106.5^{k}
Olea africanum	181.7^{i}	201.0^{g}	0.052^{j}	0.057^{k}	93.0^{h}	107.0^{j}
Peltrophorum africanum	99.4^{o}	173.7^{l}	0.048^{n}	0.056^{l}	48.8^{n}	92.0^{l}
Quercus rubica	95.4^{q}	109.0^{r}	0.082^{c}	0.089^{a}	59.3^{m}	69.8^{q}
Rhus lancea	96.2^{p}	235.7^{e}	0.029^{s}	0.049^{o}	35.0^{q}	117.3f
Ziziphus mucronata	275.00^{b}	277.3^{b}	0.061^{h}	0.059^{i}	150.8^{b}	150.5^{b}
SEM	1.145	2.03	0.0035	0.004	0.375	0.60
P value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Means with different superscript (letters) across the column for each parameter are significantly differentat *P<0.05*; [1]b: gas production from the insoluble, but slowly fermentable fraction (ml);[2] C: the rate of GP from insoluble fraction per hour;[3]PD: potential gas production (ml);PEG-polyethylene glycol:PEG (-) denotes presence of tannin: PEG (+) denotes absence of tannin; SEM: standard error of mean

Volatile fatty acid and ammonia-N production

The production of total and individual VFAs varied among browses (Table 3.5). *Morus alba* produced the largest amount of total VFA and acetate. *Ziziphus mucronata* produced the highest amount of propionic and valeric acid. *Acacia sieberriana* produced the largest volume of isobutyric acid, while the highest A: P ratio was recorded for *Leucaena leucocephala*. The absence of tannin improved the production of individual and total VFA.

Table 3.5 Total and individual volatile fatty acid (mmol L^{-1}) production of the incubated browses with or without PEG

	Acetic		Propionic		Iso butyric		Butyric		Valeric		Total VFA	
Scientific name	PEG(-)	PEG(+)	PEG(-)	PEG(+)	PEG(-)	PEG(+)	PEG(-)	PEG(+)	PEG(-)	PEG(+)	PEG(-)	PEG(+)
Acacia nilotica	45.0	52.2	14.13	13.09	1.16	1.73	2.86	4.55	1.58	2.21	64.7	73.7
Acacia sieberriana	57.6	47.5	14.01	12.13	2.72	2.36	5.35	5.42	2.53	2.25	82.2	69.6
Combretum microphyllum	58.1	47.6	15.19	10.18	2.36	1.49	7.25	3.61	2.57	1.75	85.5	64.6
Combretum molle	43.4	48.8	9.12	9.66	1.09	1.25	2.70	2.69	1.40	1.50	57.7	63.9
Euphorbia tirucalli	72.4	76.9	16.26	16.89	1.89	2.12	8.53	9.28	2.41	2.57	101.5	107.8
Ficus religiosa	63.0	53.4	13.48	11.59	1.94	1.59	5.42	4.35	2.13	1.89	86.0	72.8
Ficus thronniggi	71.9	75.4	15.49	16.57	2.27	2.34	6.62	7.16	2.48	2.79	98.7	104.2
Lespedeza cuneata	46.7	61.6	11.67	15.09	1.51	2.36	3.47	5.84	2.12	2.97	65.5	87.9
Leucaena leucocephala	55.9	58.1	13.63	13.64	1.69	1.83	4.79	5.46	2.20	2.45	78.2	81.5
Kirkia acuminate	44.4	54.4	10.17	12.58	1.13	1.15	3.50	4.35	0.59	0.99	59.8	73.4
Kirkia wilmsii	53.2	62.9	12.87	14.23	0.92	1.50	3.79	4.68	0.85	1.68	71.7	85.0
Melia azedarach	58.1	63.0	15.19	16.18	2.36	2.41	7.25	7.96	2.57	2.85	85.5	92.4
Morus alba	72.5	73.7	16.80	17.42	2.33	2.43	7.37	7.85	2.51	2.70	101.5	104.1
Olea africanum	42.5	44.1	9.50	9.46	1.32	1.37	5.62	5.50	1.70	1.64	60.7	62.1
Olea europaea	53.5	61.3	11.68	12.9	1.66	1.87	7.30	7.82	2.09	2.26	76.3	86.2
Peltrophorum africanum	48.2	52.8	10.77	12.42	1.55	2.18	2.82	5.15	1.70	2.39	65.0	75.0
Quercus rubica	45.5	49.1	9.71	10.76	1.13	1.35	3.94	4.72	1.51	1.68	61.7	67.6
Rhus lancea	42.2	63.9	9.65	12.79	1.17	1.95	4.39	5.54	1.21	2.11	58.6	86.3
Ziziphus mucronata	69.8	67.8	18.52	18.17	2.18	2.17	7.75	7.66	2.60	2.47	100.8	98.3

PEG: polyethylene glycol, PEG (-) denotes presence of tannin, PEG (+) denotes absence of tannin

3.4.3. Production of CH_4 and digestibility of browses

The browses differed significantly (p<0.05) in methane production (ml g^{-1} DM) at different incubation periods (Table 3.6). The volume of CH_4 was significantly higher in *Melia azedarach* during the early incubation period (2-12 hrs) and in both *Ziziphus mucronata* and *Morus alba* during the 24-48 hr incubation periods. *Rhus lancea* produced the lowest volume of CH_4 during the entire incubation period. The absence of tannins significantly (p<0.05) increased the production of CH_4 during all incubation periods for all browses. Methane production expressed in mass (g kg^{-1} DM and g kg^{-1} IVOMD) and CH_4 expressed as lost metabolizable energy also varied significantly (p<0.05) among the studied browses (Table 3.7).

Morus alba and *Ziziphus mucronata* produced the highest amount of CH_4 expressed in mass of CH_4 production (g kg^{-1} DM of incubated feed), methane per digested organic matter (g kg^{-1} IVOMD), and lost energy as CH_4. On the other hand, high tannin-containing plants such as *Rhus lancea, Kirkia acuminata* and *Combretum microphyllum* produced the lowest values of CH_4 expressed in volumes, mass and wasted energy. The absence of tannin increased CH_4 production, which was depressed by tannins. The highest increment was recorded in *Rhus lancea*, while the lowest increment was observed for low CH_4- producing browses *(Ziziphus mucronata* and *Melia azedarach).* On the other hand, methane production per IVOMD decreased substantially for high tannin-containing browses in the absence of tannin, while a slight increment was observed for low tannin-containing browses.

The browses varied significantly (p<0.05) in their organic matter digestibility (IVOMD) and metabolizable energy (ME) content (Table 3.8). Browses with high tannin content were found to be low in digestibility. *Peltrophorum africanum* and *Rhus lancea* were the least digestible browses. There was a significant (p<0.05) increment in values of IVOMD and ME in the absence of tannin. The production of NH_3-N also differed (p<0.05) among browses. The highest NH_3-N concentration was recorded for *Morus alba*, while the lowest value was recorded for *Kirkia acuminata.* A pronounced improvement of NH_3-N concentration was obtained in the absence of tannin.

Table 3.6 Volume (ml g^{-1} DM) of CH_4 production from the browse plants with or without PEG

Scientific name	Time of incubation							
	2 hr		12 hr		24 hr		48 hr	
	PEG(-)	PEG(+)	PEG(-)	PEG(+)	PEG(-)	PEG(+)	PEG(-)	PEG(+)
Acacia nilotica	0.76^{h}	1.70^{f}	3.43^{m}	8.46^{j}	4.55^{k}	17.83^{e}	5.10^{ij}	19.46^{f}
Acacia sieberriana	1.15^{d}	1.50^{g}	9.93^{e}	11.15^{e}	15.33^{c}	17.27^{f}	16.41^{f}	21.66^{e}
Combretum microphyllum	0.078^{n}	0.95^{k}	2.08^{p}	4.31^{r}	2.13^{m}	4.64^{p}	2.93^{m}	7.53^{k}
Combretum molle	0.091^{m}	1.85^{l}	2.44^{o}	5.96^{n}	2.52^{o}	5.46^{o}	4.21^{k}	10.69^{i}
Euphorbia tirucalli	1.01^{e}	2.03^{c}	6.94^{f}	10.20^{h}	11.39^{d}	16.94^{g}	21.41^{c}	26.21^{c}
Ficus religiosa	0.28^{k}	1.04^{j}	4.23^{k}	7.71^{k}	8.91^{h}	14.11^{i}	13.2^{g}	18.18^{g}
Ficus thronniggi	1.51^{c}	1.90^{d}	11.94^{d}	13.47^{d}	18.45^{b}	20.28^{d}	26.11^{b}	28.25^{b}
Kirkia acuminate	0.15^{l}	0.94^{k}	0.77^{r}	3.91^{q}	1.65^{no}	4.61^{p}	3.56^{l}	8.07^{kj}
Kirkia wilmsii	0.30^{k}	0.89^{l}	2.75^{o}	5.10^{p}	3.23^{l}	5.93^{n}	5.20^{i}	9.25^{j}
Lespedeza cuneata	0.45^{i}	1.77^{e}	5.88^{h}	10.44^{fg}	10.72^{e}	17.16^{fg}	13.42^{g}	19.56^{f}
Leucaena leucocephala	2.08^{b}	2.36^{b}	6.30^{g}	10.3^{g}	7.99^{i}	13.23^{j}	8.86^{h}	21.69^{f}
Melia azedarach	3.12^{a}	3.54^{a}	15.10^{a}	16.84^{a}	18.37^{b}	20.82^{c}	21.17^{c}	24.39^{d}
Morus alba	0.40^{j}	0.95^{k}	13.61^{c}	14.98^{b}	20.67^{a}	22.26^{a}	29.1^{a}	30.35^{a}
Olea africanum	1.11^{d}	1.40^{h}	4.99^{i}	6.60^{l}	9.89^{f}	12.41^{k}	18.15^{d}	21.83^{e}
Olea europaea	0.95^{f}	1.26^{i}	4.47^{j}	6.56^{l}	9.51^{g}	12.31^{k}	17.51^{e}	21.74^{e}
Peltrophorum africanum	0.080^{n}	0.84^{l}	1.62^{q}	6.03^{m}	1.75^{n}	9.13^{l}	3.78^{l}	16.64^{h}
Quercus rubica	0.84^{g}	1.42^{h}	3.63^{l}	5.79^{o}	5.17^{j}	7.74^{m}	4.79^{j}	9.10^{j}
Rhus lancea	0.058^{o}	0.12^{l}	0.04^{s}	8.62i	0.10^{p}	14.45^{h}	2.21^{m}	22.58^{e}
Ziziphus mucronata	1.12^{d}	0.88^{l}	12.98^{b}	13.85^{c}	20.64^{a}	21.24^{b}	28.74^{a}	30.36^{a}
SEM	0.015	0.0087	0.0074	0.0194	0.056	0.093	0.135	0.397
P value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Means with different superscript (letters) across the column for each parameter are significantly different at P<0.05
PEG- Polyethylene glycol, PEG (-) denotes presence of tannin, PEG (+) denotes absence of tannin
SEM standard error of the mean

Table 3.7 Methane (CH_4) production in mass (g kg^{-1}DM, g kg^{-1}IVOMD) and percentage of energy lost as CH_4 (% ME) from the studied browse plants after 24 hours'incubation

Scientific name	Methane (g kg^{-1} DM)		Methane g kg^{-1} IVOMD		lost energy as CH_4	
	PEG(-)	(PEG+)	PEG(-)	(PEG+)	PEG(-)	(PEG+)
Acacia nilotica	3.26^{m}	11.0^{e}	7.77^{l}	14.9^{e}	1.29^{l}	2.41^{j}
Acacia sieberriana	6.66^{h}	10.9^{f}	9.59^{j}	13.0^{i}	2.62^{e}	2.68^{f}
Combretum microphyllum	1.53^{p}	3.19^{r}	3.07^{q}	4.64^{r}	0.72^{r}	1.13^{s}
Combretum molle	1.80^{o}	3.91^{p}	3.98^{o}	5.18^{p}	0.76^{q}	1.33^{r}
Euphorbia tirucalli	7.09^{e}	11.4^{d}	13.0^{a}	15.3^{d}	2.08^{f}	2.74^{e}
Ficus religiosa	6.38^{i}	10.1^{i}	10.2^{f}	12.9^{j}	1.80^{i}	2.29^{k}
Ficus thronniggi	7.48^{c}	11.8^{c}	11.0^{d}	15.7^{c}	3.04^{d}	3.16^{c}
Kirkia acuminate	1.18^{r}	3.30^{q}	2.29^{r}	4.03^{s}	0.86^{p}	1.44^{q}
Kirkia wilmsii	2.31^{n}	4.25^{o}	4.86^{n}	5.11^{q}	1.14^{n}	1.54^{o}
Lespedeza cuneata	6.24^{j}	10.1^{h}	9.69^{i}	13.4^{g}	1.89^{g}	2.61^{g}
Leucaena leucocephala	4.29^{l}	9.47^{j}	6.30^{m}	10.8^{l}	1.73^{k}	2.43^{i}
Melia azedarach	7.28^{d}	11.5^{c}	11.7^{c}	14.8^{f}	3.10^{c}	3.14^{d}
Morus alba	9.59^{a}	12.5^{a}	12.6^{b}	17.2^{a}	3.25^{a}	3.44^{a}
Olea africanum	7.08^{k}	8.89^{k}	9.87^{g}	10.7^{m}	1.82^{h}	2.25^{l}
Olea europaea	6.81^{g}	8.81^{l}	9.73^{h}	11.2^{k}	1.76^{j}	2.24^{m}
Peltrophorum africanum	1.25^{q}	6.54^{m}	3.76^{p}	10.1^{n}	0.93^{o}	1.77^{n}
Quercus rubica	5.13^{k}	5.54^{n}	8.92^{k}	7.34^{o}	1.15^{m}	1.47^{p}
Rhus lancea	0.07^{s}	10.4^{g}	0.22^{s}	13.3^{h}	0.54^{s}	2.45^{h}
Ziziphus mucronata	8.56^{b}	12.0^{b}	10.9^{e}	16.6^{b}	3.25^{b}	3.20^{b}
SEM	0.067	0.09	0.086	0.067	*0.02*	*0.019*
P value	<0.001	<0.001	<0.001	<0.001	*<0.001*	*<0.001*

Means with different superscript (letters) across the column for each parameter are significantly differentat p<0.05
PEG: polyethylene glycol, PEG(-) denotes presence of tannin, PEG(+) denotes absence of tannin
SEM standard error of the mean

Table 3.8 *In vitro* organic matter digestibility (IVOMD, g kg^{-1}DM), metabolizable energy (ME, MJ kg^{-1} DM) and NH_3-N (mg $100ml^{-1}$) from the browse plants with and without PEG

Scientific name	IVOMD		NH_3-N		ME	
	PEG(-)	(PEG+)	PEG(-)	(PEG+)	PEG(-)	(PEG+)
Acacia nilotica	419.0^{q}	758.5^{n}	13.5^{ih}	18.9^{d}	10.2^{f}	23.4^{j}
Acacia sieberriana	694.6^{g}	838.2^{e}	16.8^{ef}	17.9^{e}	11.2^{e}	41.3^{c}
Combretum microphyllum	496.7^{m}	688.8^{r}	15.7^{f}	17.9^{e}	6.66^{o}	24.8^{i}
Combretum molle	453.9^{p}	755.3^{d}	17.2^{de}	17.9^{e}	7.07^{n}	20.2^{k}
Euphorbia tirucalli	479.2^{n}	792.5^{j}	18.5^{c}	18.9^{d}	9.02^{g}	14.4^{q}
Ficus religiosa	584.3^{j}	785.5^{l}	20.1^{b}	20.8^{b}	7.94^{i}	19.6^{m}
Ficus thronniggi	776.2^{c}	835.3^{f}	20.4^{b}	21.0^{b}	11.9^{c}	40.3^{d}
Kirkia acuminate	515.8^{l}	819.3^{i}	3.88^{l}	9.10^{i}	7.04^{n}	19.9^{l}
Kirkia wilmsii	475.8^{o}	830.6^{g}	10.4^{k}	$13.\ 9^{g}$	7.25^{m}	16.4^{o}
Lespedeza cuneata	644.2^{i}	757.6^{o}	16.2^{f}	17.9^{e}	11.3^{e}	31.7^{f}
Leucaena leucocephala	681.0^{h}	877.0^{d}	11.9^{j}	13.2^{h}	11.3^{e}	43.8^{a}
Melia azedarach	746.1^{d}	882.5^{c}	18.2^{c}	19.6^{c}	13.4^{a}	43.2^{b}
Morus alba	805.7^{b}	929.0^{a}	21.3^{a}	22.3^{a}	12.3^{b}	25.7^{h}
Olea europaea	700.1^{f}	789.9^{j}	14.1^{g}	15.9^{f}	7.39^{l}	15.0^{p}
Olea africanum	717.3^{e}	830.5^{h}	13.7^{h}	15.4^{fg}	7.39^{l}	17.6^{n}
Peltrophorum africanum	333.3^{r}	647.5^{s}	14.3^{g}	18.0^{e}	7.53^{k}	25.7^{h}
Quercus rubica	575.4^{k}	754.8^{q}	13.6^{ih}	15.4^{fg}	7.82^{j}	26.7^{g}
Rhus lancea	324.0^{s}	776.0^{m}	12.2^{j}	18.2^{de}	8.74^{h}	14.2^{r}
Ziziphus mucronata	894.3^{a}	919.0^{b}	18.1^{c}	18.8^{d}	11.7^{d}	35.6^{e}
SEM	*0.190*	*0.21*	*0.2106*	*0.158*	*0.01*	*0.01*
P value	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*

Means with different superscript (letters) across the column for each parameter are significantly different at $p<0.05$
PEG: polyethylene glycol, PEG(-) denotes presence of tannin, PEG(+) denotes absence of tannin
SEM standard error of the mean

3.4.4. Correlation of IVOMD and CH_4 with chemical and phenolic composition of browse plants

The correlation coefficient values for IVOMD with chemical composition, gas production, phenolic compounds, short-chain VFA and NH_3-N of browse species are presented in Table 3.9. Significantly (p<0.001) positive correlations were observed between IVOMD and nitrogen content, gas production (Gp_{24} and Gp_{48}), ME and total and individual VFA. There was a significant (p<0.001) and negative correlation of IVOMD with fibre component (ADF) and the phenolic compounds (TP, TT, CT and HT).

Correlation coefficients (r) between chemical composition and phenolic compounds with CH_4 production parameters of studied browse plants are shown in Table 3.10. Methane production at 24 hr showed a similar pattern of correlation with chemical and phenolic composition of browses. However, the relative increase in CH_4 production in absence of tannins showed an opposite trend with the 24 hr CH_4 production. Methane production showed a significantly (p<0.001) negative correlation with phenolic compounds (TP, TT, CT and HT), and a positive correlation with nitrogen and fibre components. An increase in CH_4 production, in absence of tannins had a positive correlation with phenolic compounds and a negative correlation with the fibre components and CP.

Table 3.9 Correlation coefficients (r) between *in vitro* fermentation parameters with the chemical composition and phenolic compounds of the browse plants

	$IVGP_{24}$	$IVGP_{48}$	NH_3-N	VFA	IVOMD	ME
Chemical composition						
Ash	0.501***	0.523***	0.654***	0.732***	0.297*	0.488***
EE	-0.102	-0.112	0.027	0.351*	0.0458	0.121
CP	0.588***	0.362**	0.292*	0.352*	0.372**	0.767***
NDF	-0.350*	-0.414***	-0.417***	-0.138	-0.427	-0.247*
ADF	-0.605***	-0.619***	-0.453***	0.012	-0.619***	-0.456***
ADL	-0.620***	-0.698***	-0.182	-0.100	-0.326*	-0.124
Secondary compounds						
TP	-0.833***	-0.825***	-0.43***	-0.676***	-0.641***	-0.417***
TT	-0.782***	-0.739***	-0.329*	-0.566***	-0.630***	-0.514***
CT	-0.566***	-0.632***	-0.366**	-0.545***	-0.702***	-0.301*
HT	-0.706***	-0.691***	-0.324*	-0.423**	-0.439***	-0.499***

IVGP: *in vitro* gas production; VFA: volatile fatty acid; IVOMD: *in vitro* organic matter digestibility; ME: metabolizable energy; EE: ether extract; CP: crude protein; NDF: neutral detergent fibre; ADF: acid detergent fibre; ADL: acid detergent lignin; TP: total phenol; TT: total tannin; CT: condensed tannin; HT: hydrolysable tannin * p<0.05; ** p<0.01; *** p<0.001.

Table 3.10 Correlation coefficients (r) between chemical composition and phenolic compounds and methane production parameters of thebrowse plants

Predictor	Methane 24 hr	Methane increase
Chemical composition		
CP	0.36*	-0.221
NDF	0.453***	-0.653***
ADF	0.845***	-0.449**
ADL	0.426**	-0.299*
Secondary compounds		
TP	-0.827***	0.808***
TT	-0.774***	0.725***
CT	-0.685***	0.617***
HT	-0.631***	0.604***

CP: crude protein; NDF: neutral detergent fibre; ADF: acid detergent fibre; ADL: acid detergent lignin; TP: total phenol; TT: total tannin; CT: condensed tannin; HT: hydrolysable tannin
* p<0.05; ** p<0.01; *** p<0.001

3.5. Discussion

The nutrient composition of the browse plants was comparable with a range of values reported for southern African species (Tefera *et al.,* 2008) and tropical browses reported by various researchers (Soliva *et al.,* 2008) in a similar season. However, there was a slight deviation for some of the species and this may be due to variation in site and within species. The means of secondary plant compounds obtained in this study were within the ranges of values reported for other tropical browses (Mueller-Harvey, 2006). There was little information on secondary compounds for most browses used in this study to make individual comparisons. In addition, large variations within secondary compounds, even for the same species, can be due to the assay method and differences in ecotypes, seasons, ecological zones, soil types, and age of the plants (Mueller-Harvey, 2006).

Feed fermentation in the rumen and digestibility determine nutritive values of browse and these are further influenced by chemical and phenolic compositions (Blummel & Becker, 1997; Getachew *et al*., 2005). High gas production indicates more rumen fermentation to support rapid rumen microbial growth. In the current study, the main factors affecting gas production and IVOMD of browse were their fibre and tannin contents. The differences in fibre among the browses could be due to their differences in chemical structure of cell walls

(Tiemann *et al.,* 2008; Patra & Saxena, 2009). In the present study, tannins showed a depressing effect on the fermentability and digestibility of browses. *Morus alba* was the most fermentable and digestible browse, which could be associated with the low ADF, ADL and phenolic compounds. On the other hand, *Rhus lancea* was the least fermentable and digestible browse, which could be due to the negative influence of high total tannin and phenolic compounds. However, for some species such as *Acacia nilotica*, the effects of ADL and tannin contents cannot be separated. This is because *Acacia nilotica* contains substantially high amount of tannin and ADL. In agreement with the current findings, studies on various tropical browses showed negative effects of plant phenolic compounds on their fermentation and digestion (Guglielmelli *et al.,* 2011; Jayanegara *et al.,* 2011; Sebata *et al.,* 2011). These negative effects could be related to the formation of tannin–carbohydrate and tannin–protein complexes, which are less degradable, or to toxicity to rumen microbes (Bhatta *et al.,* 2009).

The high PD and b for *Morus alba* could be linked to the high volume of gas produced and their low fibre concentrations, since the PD and b were found to be negatively correlated with fibre components. The relative slow rate of gas production (c) from high tannin-containing browses might be due to the presence of high levels of plant secondary compounds, which are known to be toxic to rumen bacteria (Bhatta *et al.,* 2009).

Some studies found that a lower concentration of tannin (20 g^{-1} kg) had little or no impact on gas production and total VFA concentration (Getachew *et al.,* 2008). However, in the current study, the lower concentration of tannin *(Melia azedarach, Ficus thronniggi, Olea europaea* and *Olea africanum)* influenced fermentation and digestibility negatively. The decrease in fermentation and digestibility with increasing levels of tannin might be due to their continuous effect during fermentation and digestibility. Tan *et al.* (2011) reported similar effects with increasing tannin level supplementation onfermentation and digestibility.

The increase in NH_3-N and total VFA with the inclusion of PEG during incubation indicated that the depression of fermentation and digestion of organic matter by tannin content. The observed increase in IVOMD and volume of gas production with inclusion of PEG supports this. The effect of tannin on NH_3-N production might be due to the formation of tannin-protein complexes, which could reduce degradation of protein to NH_3-N (Bhatta *et al.,* 2009). This finding is consistent with those of other researchers (Mueller-Harvey, 2006; Patra

&Sexena, 2011). The absence of tannin resulted in a substantial increase in total and individual VFA production. This finding was consistent with those of other researchers (Getachew *et al.,* 2008; Singh *et al.,* 2012). There is general agreement that dietary tannins supplied at adequate concentrations reduce ruminal NH_3-N and branched-chain VFA concentrations *in vitro* (Bhatta *et al.,* 2009; Getachew *et al.,* 2008; Singh *et al.,* 2012) and *in vivo* (Getachew *et al.,* 2008). This is because NH_3-N is a product of amino acid deamination, while iso-butyrate and iso-valerate are products of breakdown of the carbon skeleton of amino acids during rumen fermentation.

Enteric CH_4 production depends primarily on the quantity and quality of the diet, as it affects the rate of ruminal digestion and passage (Beauchemin *et al.,* 2008). Production of CH_4 is a sink for hydrogen in the rumen during the process of utilization of feed energy. However, with the fermentation in the rumen of tannin-rich plants, their bacteriocidal and bacteriostatic effects on the rumen microbes, and inactivation of their enzymes suppress fermentation and this could result in a decrease of CH_4 production. For *Melia azedarach* and *Ziziphus mucronata,* the volume of methane was apparently high, even with the inclusion of PEG, which might be due their low tannin content that did not sufficiently depress methanogenic activities compared with high tannin-containing browses. In this study, browses with higher phenolic contents generally produced lower CH_4, regardless of their CP, NDF, ADF and lignin contents.

When PEG was included in browse samples, CH_4 production increased, which might be due to increased digestibility and change in VFAs. For instance, in the current study for some of the browses *(Peltrophorum africanum, Ziziphus mucronata, Ficus thronniggi, Morus alba, Ficus religiosa, Combretum microphyllum, Quercus rubica,* and *Acacia sieberriana).* PEG inclusion resulted in decreased acetate to propionate ratio (data not shown), though that had not resulted in a decreased CH_4 production, while for the other species (with PEG), the acetate shifted fermentation resulted in increased CH_4 production. Considering an increased ratio of CH_4: VFA, when tannin was bound for all browses, it is clear that tannins suppressed CH_4 production irrespective of its effect on digestibility and pattern of VFA production. This is perhaps partly due to the impact of the tannins on rumen methanogens, which had a more pronounced effect than their effect on substrate fermentation, as reported by Hassanat and Benchaar (2013). The decrease in CH_4 production with the inclusion of tannin in ruminant diets was also reported by several researchers (Bhatta *et al.,* 2009; Sebata & Ndlovu, 2011;

Singh *et al.,* 2012). Jayanegara *et al.* (2011) conducted a meta-analysis and concluded that increasing tannin concentrations consistently suppressed CH_4 production under *in vitro* and *in vivo* conditions. The impact of tannins on CH_4 production varies with tannin chemical structure (plant origin), as well as tannin concentration. Tannins acted as toxicants to methanogens, reduced acetate and butyrate production (i.e. reduced fibre fermentation) or caused a decline in OM digestibility (Beauchemin *et al.,* 2008; Patra & Sexena, 2011). Unlike Krueger *et al.,* (2010), who reported that low tannin concentrations (HT) had no or little impact on acetate, propionate or butyrate proportions, the present study revealed significant effects of tannins on these parameters.

The observed difference in methanogenic property in *Olea europaea* species and large variation among the browse species offer an opportunity to select species with a low methanogenic potential when fed to ruminants. Variations in methanogenic properties in and among browses have been reported by other researchers (Soliva *et al.,* 2008). Most of these tanniferous browses are considered medicinal plants and this may be attributed to their presumed antimicrobial properties. This property is desirable to reduce the efficiency of microbial protein synthesis by predation of bacteria and digestion of large amounts of ruminal bacteria. Hence, the reduction in protozoal counts was often found to be associated with a lower methanogenesis (Bhatta *et al.,* 2009).

3.6. Conclusion

Most experimental plants tested in the present study contained more than 170 g kg^{-1} of CP and using it as supplements to low CP tropical diets could increase digestibility and reduce methane. It was also observed that highly fermentable and digestible browses such as *Morus alba* and *Melia azedarach* with lower tannin concentrations could be included in roughage diets to increase fermentation and digestibility and reduce methane production. They might also provide extra ruminal NH_3-N necessary for maximal microbial protein production**.** However, a systematic evaluation is needed to determine optimum levels of supplementation in a mixed diet in order to attain a maximal depressing effect on enteric CH_4 production with a minimal detrimental effect on rumen fermentation of a poor-quality roughage-based diet.

CHAPTER 4

Effect of selected browse species foliage supplementation on digestibility and methane emission of sheep

4.1. Abstract

This study investigated the effects of inclusion of three tannin-rich browse foliages or browses in the diets of sheep on methane mitigation and rumen fermentation characteristics. In a 4 by 4 Latin square design, the foliage of three browse species *(Acacia nilotica, Grewia flava* and *Monechma genistifolium)* was used to replace lucerne hay (control treatment) in a total mixed ration diet (TMR). Each experimental period lasted for 25 days, with 14 days of adaptation, 7 days of data collection and a 4-day stay of the sheep in open-circuit methane respiration chambers to measure methane production. There were no significant differences with feed intake, digestibility and total volatile fatty acid production (VFA). However, the fermentability of neutral detergent fibre (NDF), acid detergent fibre (ADF) and nitrogen (N) balance of *Grewia flava* and *Acacia nilotica* diets were reduced due to the inclusion of browses in the diet. Addition of the browses to the diets of sheep led to a net N balance, though the values were lower than those on the control diet. The inclusion of the browses reduced methane emission per day and per unit of feed intake up to 39% relative to the control diet. Methane reduction was not associated with a shift in VFA, which suggests that the reduction might be due to depression of methanogenesis and fibre fermentation. The foliages of the three browse species *Monechma genistifolium*, *Acacia nilotica* and *Grewia flava* are good sources of nitrogen in the diet of ruminants and could be regarded as an alternative option to be included for the benefit of reducing enteric methane emission from ruminants in tropical livestock systems.

Keywords: *Acacia nilotica,* digestibility, feed fermentation *Grewia flava,* lucerne hay, methane, *Monechma genistifolium,*

4.2. Introduction

Livestock production, which is an integral component of agriculture, is currently faced with climate change challenges globally. This is in addition to feed shortages in developing and least developing countries. Domesticated ruminant animals have been criticized for contributing to the emission of global warming gases into the atmosphere. Enteric methane is produced in the rumen by methanogenic archaea through the process of methanogenesis and its production decreases the energy utilization efficiency of ruminants. Its production depends primarily on the quantity and quality of diets (Van Soest, 1994; Beauchemin *et al.,* 2008), the acetate to propionate ratio of fermented feeds (McAllister *et al*., 1996), and the activity and number of methanogenic archaea (Janssen *et al.,* 2010). Methanogenesis is a predominant hydrogen (H) removal pathway in the rumen system, which avoids hydrogen accumulation for normal anaerobic fermentation activities. Thus, providing a competitive alternative pathway or suppressing the activity and growth of methanogenic microbes offers good prospects for reducing enteric methane emission in ruminants. In this regard, tannins were reported to suppress the activity and growth of methanogens effectively (Animut *et al.,* 2008; Jayanegara *et al.,* 2009; Patra *et al.,* 2011). Some studies also showed that tannins influence methanogenesis by reducing the numbers of protozoa (Animut *et al.,* 2008; Patra, 2010).

In most regions of Africa, most trees and shrubs contain relatively high crude protein (CP) content and the foliage from these browses might present alternative options for farmers to improve the protein supply in the diets of ruminants (Kaitho *et al.,* 1996; Tolera *et al.,* 1997). The foliage from these trees and shrubs also contains variable amounts of tannins, which may have beneficial or detrimental effects on ruminants, depending on the type, amount consumed, structure and molecular weight and the physiology of the animals (Hagerman & Butler, 1991). Ruminants can benefit from dietary tannins when the increases in protein flow from the rumen exceed the reduction in the absorption of amino acids from the intestine (Waghorn, 1996). However, tannins at high concentrations may adversely affect intake, digestion and rumen fermentation. But some ruminants (e.g. goats) might utilize tannin-rich plants better due to higher concentrations of proline in their saliva, which form tannin-proline complexes that might deter the negative effect of tannin on ruminal degradation of dietary protein or enzyme activities in the rumen (McArthur *et al.,* 1995). This might overcome the negative effects of tannins on palatability and intake, and improve digestion of tannin-rich

feeds (Austin *et al.,* 1989; McArthur *et al.,* 1995). Thus, it is important to consider a proper level of tannin inclusion that would decrease methane without affecting feed digestion.

Acacia nilotica, *Grewia flava* and *Monechma genistifolium* are among the most common utilized browses in African livestock production systems. These browses showed satisfactory nutritional value and anti-methanogenic effects in an *in vitro* trial suggesting their potential use as supplements to low- quality tropical feeds (Theart *et al.,* 2014). Feeding these browses alone might reduce intake and digestibility, but if they are mixed with poor-quality roughages or included in TMR, this might benefit the animal. This strategy is well suited to small-scale farmers, especially in the dry season when hay and crop residue are the only roughage options. Under small-scale farming conditions, herbaceous legumes such as lucerne are not easy to establish and maintain, as the plant requires favourable well-drained soils, and need high seed and establishment costs. However, these browses are characterized by medium to high tannin contents (~50 g kg^{-1}DM) and are easily accessible in the tropical African conditions in arid to humid areas. This study aimed to investigate the effects of replacing lucerne hay with foliage from these browses in a diet for Merino sheep and to determine the effects on ruminal fermentation and methane emission by these animals.

4.3. Materials and methods

4.3.1. Experimental location and ethic approval

This study was conducted at the University of Pretoria Experimental Farm from January to June 2014. It was approved by the University of Pretoria Animal Ethics Committee with the number EC086-12.The area is located at 25°44’30” S, 28°15’30” E, at an elevation of 1370 m above sea level. The area receives an average rainfall of 674 mm per annum and has an average annual temperature of 17.3°C. It has two distinctive seasons: a dry cool and sunny season from March to September and a warm and humid rainy season from October to February.

4.3.2. Experimental diets

The experimental browses plants were collected from the Kalahari (S 26° 46.610'E 22° 34.557') in the Northern Cape between March and April 2012, when they were at medium

maturity vegetative stage. The area is characterized by highly erratic rainfall, which ranges from 150 to 350 mm. It has an altitude of 900-1100 m, with red and excessively drained sandy and highly basic soils. The leaves were collected at the same stage of maturity, air dried in a shaded area, and transported to the University of Pretoria Experimental Farm. Medium-quality lucerne was purchased and included in the diet as control (negligible amount of tannin).

The chemical compositions of browses are indicated on Table 4.1 and experimental treatment formulation and their chemical composition are indicated on Table 4.2. The formulated rations were intended to contain 500 g^{-1} kg DM roughage feed and 500 g^{-1} kg DM concentrate. The Lucerne hay (control) and browses (treatments) made up 60% of the roughage component of the experimental diet. The experimental diets were formulated according to the nutritional requirements and recommendations of the National Research Council (2001) to satisfy the maintenance requirements of adult rams (60 kg). To facilitate intake and to limit selection, all feeds were milled uniformly after mixing all ingredients.

4.3.3. Experimental design, procedures and description of open-circuit respiration chambers

Four male merino rams with an initial bodyweight (BW) of 60 (±3) kg were allocated to the four dietary treatments in a 4 × 4 Latin square design. Each experimental period lasted for 25 days, consisting of 14 days of adaptation to the experimental diet, followed by 7 days of intake and digestibility measures in metabolic cages and 4 days of quantitative measurement of methane gaseous exchange in open-circuit respiration chambers.

During intake, digestibility, and sample collection periods, rams were kept in individual metabolic crates (190cm x 180cm x 90 cm) arranged side by side and allowing visual contact. Faecal bags were fitted to rams before they were placed in the metabolic cages and afterwards urine pans were fitted to the cages. Rams were weighed before feeding in the morning, at the beginning of each experimental period, then twice a week during the adaptation periods to adjust the daily DM supply, and at the start of the digestibility periods, before entering the cages. During the cage trial, no weighing took place. Diets were offered twice daily at 08:30 and 17:30 and water was freely available. For digestibility, total feed intake and total faecal output were measured. Feed intake was determined daily for each ram

by recording the weight of the feed offered minus the orts. Feed and orts samples were taken daily for each ram during the collection periods to obtain a representative sample out of seven days for each treatment and frozen at -22 °C until further analysis in the laboratory. A representative sample of the voided faeces was taken daily for each ram, mixed and pooled over the seven-day collection period (Köster *et al.,* 1996; Olson *et al.,* 1999). Two separate sub-samples of faeces were taken. One sub-sample was used to determine DM content by drying at 105°C for 12 hours and the other sub-sample was dried at 55 °C for 48 hr for nutrient analysis. Urine was collected in urine pans containing 20 ml 10% sulphuric acid (H_2SO_4) to keep the pH below 4 and prevent the escape of ammonia. The urine was diluted with water up to 4000 ml. A sub-sample (50 ml) was then taken from each ram's diluted urine, to obtain a representative sample for each day (Chen and Gomes, 1992). The urine samples of the seven-day collection period were pooled to obtain the representative samples for each of the four treatments. Rumen fluid samples were collected at four-hour intervals a day for three days. The rumen fluid was extracted manually. Representative samples of the rumen fluid were collected at various locations within the rumen in a 500 ml plastic container and pH was determined. The filtrate was subdivided for NH_3-N and VFA analysis and preserved as follows: 30 ml of rumen fluid preserved with 5 ml 50% H_2SO_4 for rumen NH_3-N (Broderick & Kang 1980); and 20 ml of rumen fluid was preserved with 4 ml of 25% H_3PO_4 for VFA. All samples were stored at -20°C until analysis in the laboratory. The NH_3-N and VFA samples were pooled separately over the collection period to obtain a representative sample for each ram. During analysis of VFAs, composite samples were made for each day and analysed accordingly. The pH was measured with an electrode pH meter (Mettler Toledo) after it had been calibrated with pH 4 and pH 7 buffer solutions.

During the quantification of methane in the chambers, the rams were kept in metabolic cages of 180 cm x 180 cm x 150 cm arranged sidebyside in two rows (each row having three chambers) to allow the rams to have visual contact. The chambers were constructed of 25 mm x 25 mm powder-coated steel frames to give an approximate volume of 5 m^3. The chamber boxes consisted of a roof, three sides and a bisectional front door. The sides were covered with 1.0 mm thick UV-resistant clear flexible polyvinyl chloride (PVC) sheet. The chamber doors opened onto a common space that ran between the two rows. The bottoms of the two doors had air gaps of 330 mm to allow air into the chamber. The air gap also facilitated movement of air in and out of the chamber as a safety mechanism in the event of power failure, when the fans failed to extract air out of the chamber.

The respiration chamber airflow in and out was reliant on negative pressure created in the system. It was achieved by the extraction of air by high-speed fans placed at the exhaust of each chamber. A stream of ambient air was drawn from incoming air at the open section of the front doors. The air was circulated naturally in each chamber and then extracted by the fan through a 101 mm diameter hole at the back centre of the roof section. Air exiting the fan was connected to apipe containing two 90-degree bends prior to being vented to the outside of the shed through plastic tubing. A sub-sample of air leaving the chambers was continually with drawn from half way down the section of straight pipe through a 6 mm diameter plastic pipe. A variable speed controller for the fan was fixed at the top of each chamber in order to regulate air flow.

Each of the sample lines was connected to a manifold fitted with solenoid valves, which automatically connects air samples from each chambers to a gas analyser at a predetermined intervals. Prior to the flow of air via the manifold, each sample line passes through a desiccant (silica) and a membrane filter to remove moisture content and particulates that would affect the analyser. Recovery of methane from the chambers was quantified over a 24 hr period before the animals were brought to the chamber and after methane measurement from the animals was deterred. In order to work out the methane production from the rams average methane concentration was calculated for each chamber and ambient line using an Excel spreadsheet. Airflow was calculated from the mean airflow readings from the anemometer, which has been pre-set at regular intervals to calculate air volume from air velocity measurements and automatically recorded on the computer. The mean methane concentration from each chamber was corrected for mean ambient methane concentration and total methane emissions were calculated based on the total air volume multiplied by the net methane concentration from each chamber.

Feeding troughs were placed manually in each chamber. During methane quantification, rams were moved into the chambers prior to the morning feeding, and remained in the chambers for four-day (4 x 24 h) periods. Rams were fed the experimental diet in two equal portions at 08:30 and 17:30 h daily and refusals were recorded once in a day before the morning feeding. The rams had free access to water throughout the day. Everyday the rams were let out of the chamber between 8:00 and 9:00, during which time the chambers, water buckets and feeding troughs were cleaned, feed leftovers were all weighed and daily allowances were measured and filled into the troughs.

4.3.4. Laboratory analyses

Feeds offered, refusals and faeces were dried at 55°C for 48 h and ground to pass a 1-mm screen for chemical analyses. Samples were analysed for DM, total ash, NDF, ADF, ADL and CP with methods similar to those detailed in Chapter 2, section 3.2. Determination of total tannins (TT) and condensed tannins (CT) was done according to similar procedures described by Makkar (2003) and detailed in Chapter 3, section 3.1. The analyses of VFA andrumen NH_3-N were also carried out with methods similar to those described in Chapter 2, section 3.3.

4.3.5. Methane measuring

The methane chambers were connected to a gas analyser. The analyser (ADC MGA3000) was fitted with a non-dispersive infrared detector for methane and a range of other gases and high capacitance aluminium oxide to detect moisture (ADC Gas Analysis Ltd. Unit 35 Huddleston Industrial Centre, UK). Every four minutesthe analyser auto zeroed itself with oxygen-free nitrogen. Calibration of the analyser was done automatically using a 150 ppm span gas.

4.3.6. Calculations, statistical analysis and model

Data were analysed using the generalized linear model (GLM) procedures of SAS (version 9.1.3; 2009; SAS, Cary, NC, USA) with diet, animal and experimental periods as sources of variation. The following model was used:

$Yijk = \mu + t_i + a_j + p_k + e_{ijk}$, where

t_i is the effect of i^{th} treatment (diets)

a_j is the effect of j^{th}animal

p_k is the effect of the k^{th}period

e_{ijk} is the associated random error.

All multiple comparisons among means were done using the Tukey test.

4.4. Results

Browses contain more fibre (NDF, ADF and ADL) than lucerne hay (Table 4.1). *Monechma genistifolium* contained less CP and tannin concentration than *Acacia nilotica* and *Grewia flava. Grewia flava* contained high tannin and ADF concentrations, but low ADL concentration compared with *Acacia nilotica* and *Monechma genistifolium.*

Table 4.1 Chemical composition (g kg^{-1} DM) of browses used to replace lucerne hay in experimental diets

	Forages			
	Lucerne hay	*Monechma genistifolium*	*Acacia nilotica*	*Grewia flava*
Organic matter	825^{c}	872^{a}	840^{b}	841^{b}
Crude protein	161^{b}	84^{d}	169^{a}	140^{c}
NDF	416^{d}	484^{a}	456^{b}	444^{c}
ADF	228^{d}	394^{b}	292^{c}	400^{a}
ADL	76.9^{d}	216^{a}	225^{b}	205^{c}
Hemicellulose[1]	88^{a}	90^{a}	65^{b}	44^{c}
Cellulose[2]	252^{a}	129^{d}	168^{c}	194^{b}
Gross energy (MJ kg^{-1} DM)	18.6^{ab}	16.8^{ab}	19.1^{a}	18.1^{b}
Total tannin	0. 0^{d}	28.2^{c}	145^{b}	171^{a}
Hydrolysable	0. 0^{d}	14.4^{c}	79.9^{b}	90.2^{a}
Condensed	0. 0^{d}	13.8^{c}	67.2^{b}	81.1^{a}

Mean values in the same row without common superscript are significantly different at p<0.05
MJ^{-1} kg DM: megajoules of energy per kilogram of dry matter; NDF: neutral detergent fibre;
ADF: acid detergent fibre; ADL: acid detergent lignin; NFC: non-fibrous carbohydrate

The chemical composition of experimental treatments is indicated in Table 4.2. The formulated diets did not differ significantly ($p \geq 0.05$) with regard to crude protein (CP) and gross energy (GE) as intended. The fibre and tannins concentration differed among the formulated diets due to variation in nutrient composition of browses, as the browses replaced lectern on an iso mass basis. The NDF concentration was significantly ($p<0.05$) lower in the control diet, while it did not differ significantly ($p<0.05$) among the other treatments. Significantly ($p<0.05$) higher concentrations of total, hydrolysable and condensed tannin were detected in *Grewia flava* diet, while tannins were not detected in the control diet.

Table 4.2 Ingredients (g kg^{-1} DM) and chemical composition (g kg^{-1} DM) of experimental diets

Parameters	Experimental treatments			
	T_1	T_2	T_3	T_4
Ingredients used				
Medicago sativa hay	300.0	-	-	-
Monechma genistifolium		300.0	-	-
Acacia nilotica	-	-	300.0	-
Grewia flava	-	-	-	300.0
Eragrostis curvula hay	200.0	200.0	200.0	200.0
Hominy chop	344.0	300.5	331.8	355.0
Wheat bran	112.5	96.5	108.9	116.5
Sunflower oil cake	18.5	78.0	34.3	3.5
Limestone	10.0	10.0	10.0	10.0
Salt	5.0	5.0	5.0	5.0
Sodium bicarbonate	5.0	5.0	5.0	5.0
Vitamin premix	5.0	5.0	5.0	5.0
Chemical composition				
Organic matter	839(14.5)^{b}	872(12.3)^{a}	845(13.9)^{b}	845(17.4)^{b}
Crude protein	116(11.2)^{a}	119(8.9)^{a}	117(9.8)^{a}	119(9.9)^{a}
NDF	366(13.1)^{b}	379(10.0)^{a}	377(8.9)^{a}	376(10.1)^{a}
ADF	216(10.1)^{c}	248(8.1)^{a}	232(4.9)^{b}	240(3.8)^{a}
ADL	42.8(5.0)^{c}	86.5(8.7)^{a}	86.3(4.3)^{a}	82.8(3.7)^{b}
Hemicellulose[1]	151 (4.4)^{a}	146(4.7)^{b}	145(4.9)^{b}	136(4.2)^{c}
Cellulose[2]	172(4.8)^{a}	144(4.9)^{c}	145(4.4)^{c}	158(4.2)^{b}
Gross energy (MJ kg^{-1} DM)	16.7(4.1)^{ab}	18.5(2.9)^{a}	18.8(3.3)^{a}	18.5(3.7)^{a}
Total tannin	nd[3]	8.46(0.1)^{c}	44.3(0.1)^{b}	51.4(0.3)^{a}
Hydrolysable	nd	4.32(0.3)^{b}	24.0(0.2)^{a}	27.1(0.3)^{a}
Condensed	nd	4.14(0.2)^{c}	20.3 (0.1)^{b}	24.3(0.09)^{a}

Mean values in the same row without common superscript are significantly different at $p<0.05$
GE: gross energy; NDF: neutral detergent fibre; ADF: acid detergent fibre; ADL: acid detergent lignin; NFC: non-fibrous carbohydrate. Values in brackets are standard error.
[1]Calculated as difference between NDF and ADF:[2] Calculated as difference between ADF and ADL:[3]nd not detected.
T_1 (lucerne ration): T_2 (*Monechma genistifolium* ration): T_3 (*Acacia nilotica* ration): T_4 (*Grewia flava* ration)

The average bodyweight of the sheep (BW), diet intake and digestibility did not differ among treatments ($p \geq 0.05$) (Table 4.3). Replacing lucerne with *Monechma genistifolium, Acacia nilotica, Grewia flava* in the diet increased tannin intake of diets by 0.80, 4.20 and 4.8 g kg^{-1} DM, respectively. The ratio of extractable to bound CT was lowest in *Monechma*

genistifolium among tannin-containing diets. DM and OM intake did not differ among diets ($p \geq 0.05$).

Although OM digestibility did not differ among diets ($p \geq 0.05$), CP, NDF, and ADF digestibility were affected ($p < 0.05$) by high tannin-containing TRB, i.e. *Acacia nilotica* and *Grewia flava*. In contrast, *Monechma genistifolium* diet did not have ($p \geq 0.05$) any negative effect on nutrient digestibility compared with lucerne diet.

Table 4.3 Intake and digestibility in Merino rams fed with lucerne hay, *Monechma genistifolium, Acacia nilotica* and *Grewia flava* diets (diets n=4)

	Experimental treatments					
	T_1	T_2	T_3	T_4	SE	Diet effect
BW (kg)	60.3	60.0	59.2	59.8	1.17	NS
DM intake ($gday^{-1}$)	1700	1690	1690	1680	0.09	NS
OM intake ($g\ day^{-1}$)	1340	1330	1330	1320	0.07	NS
Intake per $BW^{0.75}$ ($g\ kg^{-1}$ DM, day)						
DM	64.9	64.1	63.9	63.0	1.13	NS
OM	54.9	54.6	54.2	53.9	1.06	NS
TT	0.00^{d}	0.40^{c}	1.60^{b}	2.10^{a}	0.05	*
HT	0.00^{d}	0.22^{c}	1.11^{a}	0.90^{b}	0.03	*
CT	0.00^{d}	0.23^{c}	0.99^{a}	0.76^{b}	0.01	*
Digestibility ($g\ kg^{-1}$, intake)						
OM	610	608	605	604	7.38	NS
CP	443^{a}	440^{ab}	427^{c}	410^{d}	6.37	*
NDF	470^{a}	466^{ab}	409^{c}	399^{d}	4.24	*
ADF	389^{a}	384^{ab}	359^{b}	287^{c}	4.11	*

Mean values in the same row without common superscript are significantly different at $p < 0.05$; SE, standard error; NS non-significant
NDF: neutral detergent fibre; ADF: acid detergent fibre; ADL: acid detergent lignin
$BW^{0.75}$ metabolic bodyweight
T_1 (lucerne ration): T_2 (*Monechma genistifolium* ration): T_3 (*Acacia nilotica* ration): T_4 (*Grewia flava* ration)

The effect of replacing lucerne hay with the browses on rumen pH, NH_3-N, total and individual volatile fatty acids are shown in Table 4.4. Ruminal fluid pH did not differ ($p \geq 0.05$) among treatments; the mean pH was 6.99. Ruminal NH_3-N concentration was high in the lucerne ration and significantly ($p < 0.05$) decreased by the inclusion of the browses, except *Monechma genistifolium.*

The total concentration of VFA varied between 93 and 109 mmol 100 ml^{-1} and did not differ ($p \geq 0.05$) among diets, except for the *Grewia flava* diet. However, the proportions of individual VFA differed significantly ($p<0.05$) between treatments. Higher proportions of acetic acid, propionic acid, isobutyric and valeric acid were observed in the lucerne ration.

Table 4.4 Ruminal pH, NH_3-N concentration (mg 100 ml^{-1}), total and individual VFA concentration (mmol L^{-1}) in Merino rams fed lucerne hay, *Monechma genistifolium, Acacia nilotica* and *Grewia flava* diets (diets n=4)

	Experimental treatments					Diet effect
	T_1	T_2	T_3	T_4	SE	
Rumen pH	6.9	6.98	6.99	6.99	0.005	NS
Rumen **NH_3-N**	6.05^{a}	6.00^{a}	5.88^{b}	5.58^{c}	0.12	
Total VFA	108.8	104.7	106.7	92.9	10.0	NS
Acetic acid	61.9^{ab}	61.4^{ab}	62.3^{a}	50.5^{c}	1.00	*
Propionic acid	23.2^{a}	21.3^{b}	20.6^{bc}	21.2^{b}	1.11	*
Iso-butyric acid	6.20^{a}	4.86^{b}	2.60^{d}	3.80^{c}	1.0	*
Butyric acid	12.9^{b}	13.1^{b}	16.3^{a}	12.8^{b}	1.70	*
Valeric acid	4.7	4.3	5.0	4.7	1.27	NS
A : P	2.67^{c}	2.86^{b}	3.02^{a}	2.38^{d}	0.15	*

Mean values in the same row without common superscript are significantly different at $p<0.05$;
NS: non-significant; SE: standard error; VFA: volatile fatty acid
T_1 (lucerne ration): T_2 (*Monechma genistifolium* ration): T_3 (*Acacia nilotica* ration): T_4 (*Grewia flava* ration)

Daily N intake and losses differed ($p<0.05$) among treatments (Table 4.5). Replacing lucerne with *Acacia nilotica* and *Grewia flava* decreased ($p<0.05$) N intake and increased N losses in faeces. In contrast, N intake in rams fed *Monechma genistifolium* did not differ from thelucerne diet. Faecal N was significantly ($p<0.05$) higher for the *Grewia flava* in comparison with the lucerne-fed sheep. However, replacing lucerne with browses did not significantly affect urinary nitrogen; however, there was a decreasing trend as the concentration of tannin increased. Moreover, all diets induced a positive nitrogen balance (N was retained) with a higher retention for the lucerne ration ($p<0.05$).

Table 4.5 Nitrogen balance in Merino rams fed lucerne hay, *Monechma genistifolium, Acacia nilotica* and *Grewia flava* diets (diets n=4)

Nitrogen balance (g kg^{-1} intake)	Experimental treatments					Diet
	T_1	T_2	T_3	T_4	SE	effect
Intake	32.5^{a}	31.6^{a}	29.7^{b}	30.4^{b}	0.7	**
Faeces	10.1^{cd}	10.5^{c}	11.0^{b}	12.0^{a}	0.4	*
Urine	10.5	9.8	9.8	9.6	0.7	NS
Balance	11.9^{a}	11.3^{b}	9.0^{c}	8.6^{d}	0.2	***

Mean values in the same row without common superscript are significantly different at $p<0.05$;
NS: non-significant; SE: standard error
T_1 (lucerne ration): T_2 (*Monechma genistifolium* ration): T_3 (*Acacia nilotica*ration): T_4 (*Grewia flava*ration)

Daily CH_4 production was lower in browse-containing diets compared with lucerne (Table 4.6). A similar trend was observed for energy loss through methane (kJ kg^{-1} $BW^{0.75}$ day^{-1}), methane production adjusted for metabolic BW ($BW^{0.75}$), and methane production per DM intake. Sheep that received the *Grewia flava* diet emitted the lowest methane amount.Daily methane emission was reduced by 14.5, 29.8 and 39.2% with replacement of lucerne hay in the diets of sheep with *Monechma genistifolium, Acacia nilotica* and *Grewia flava,* respectively. Likewise, the daily energy loss of sheep was reduced by 12.1, 26.9 and 37.4 % by including *Monechma genistifolium, Acacia nilotica* and *Grewia flava,* respectively in their diets. Methane production expressed per unit of digested OM and NDF of the lucerne hay diet showed significantly high values compared with the tannin-containing diets. The methane conversion rate (energy lost via methane as proportion of estimated GE intake) decreased ($p<0.01$) with increasing dietary concentration of tannins in the diet and was clearly lower at the high dietary tannin level than with the lucerne ration.

Table 4.6 Methane emissions of Merino rams fed lucerne hay, *Monechma genistifolium, Acacia nilotica* and *Grewia flava* diets (diets n=4)

Methane production	Experimental treatments				SE
	T_1	T_2	T_3	T_4	
l day^{-1}	32.8^{a}	28.5^{b}	23.0^{c}	19.8^{d}	1.14
l kg^{-1} $BW^{0.75}$ per day	1.50^{a}	1.32^{b}	1.08^{c}	0.93^{d}	0.05
kJ kg^{-1} $BW^{0.75}$ per day	60.03^{a}	52.7^{b}	43.1^{c}	37.1^{d}	2.00
l kg^{-1} DM intake	21.9^{a}	20.4^{b}	19.5^{c}	18.2^{d}	0.31
l kg^{-1} OM digested	25.2^{a}	23.8^{b}	23.2^{bc}	22.0^{c}	0.26
l kg^{-1} NDF digested	67.0^{a}	64.0^{b}	54.1^{c}	48.2^{d}	1.73
kJ MJ^{-1} GE intake	141^{a}	127^{b}	99.3^{c}	86.9^{d}	4.89
l $TVFA^{-1}$	0.30^{a}	0.27^{b}	0.18^{d}	0.24^{c}	0.01

Mean values in the same row without common superscript are significantly different at $p<0.05$;
NS: non-significant; SE: standard error;
$BW^{0.75}$: metabolic; BW:bodyweight
T_1 (lucerne ration): T_2 (*Monechma genistifolium* ration): T_3 (*Acacia nilotica*ration): T_4 (*Grewia flava*ration)

4.5. Discussion

4.5.1. Chemical composition and intake

The tannin-rich browses used for diet formulation in the present study are commonly utilized in tropical ruminant livestock production systems. Previous studies indicated that these plants (or browses) have considerable inter- and intra-species variation in their chemical composition, including CT content with differences observed in *in vitro* fermentation, digestibility and methane production (Theart *et al.,* 2014). The plants were collected from the Kalahari Dune Bushveld in South Africa, where plants are adapted to extensive sunlight, drought and poor acidic soils. These conditions increase lignification and fibre concentration in browses (Van Soest, 1994). Thus the higher fibre content in browse diets compared with the lucerne diet was due to the higher fibre content of *Acacia nilotica* and *Grewia flava* and *Monechma genistifolium*. The three browse species used in the study generally have CP values of more than 120 g kg^{-1} DM and can be used as a supplemental feed for low-quality diets, especially under small-scale farming conditions of Africa (Tolera *et al.,* 1997; FAO, 2007).

In the current study, feed intake was not affected by browse, although at the beginning of the adaptation period rams tended to have a reduced DM intake. This might be due to the astringent effects of tannin, and the animals might have adapted to some extent during the adaptation period. Reports indicated that tannins exerted negative effects on voluntary intake in ruminants if their concentration was higher than 5% in the diets (McNaughton, 1987). Many reports in agreement with this study report that low levels of tannins (CT) did not decrease voluntary feed intake of sheep (Barry & Duncan, 1984; Waghorn *et al.,* 1994). However, it was well proven that voluntary feed intake is generally reduced by high CT level (Mueller-Harvey, 2006; Waghorn, 2008).

4.5.2. Feed digestibility and N balance

In the current study the total organic matter fermentation was not affected by inclusion of browses. This was reflected in the absence of differences in the concentration of total VFAs in the rumen. The moderate 30% inclusion rate of TRP may explain this absence of negative effects. The final concentration of condensed tannin in diets containing *Monechma genistifolium*, *Acacia nilotica and Grewia flava* was 0.41, 2.30, and 2.43%, respectively. These concentrations are below the level known to negatively affect feed digestibility or digestion. However, this concentration affected fibre digestibility except for the *Monechma genistifolium* diet. This might indicate that a concentration of condensed tannin of about 2.4% in these two plants is high enough to irreversibly complex the digestible component of fibre, as suggested by Mullar-Harvey (2006). Moreover, the structure of condensed tannins might be different in *Acacia nilotica*and *Grewia flava* compared with *Monechma genistifolium*. It should also be noted that browses were collected from a desert area where soil is acidic and water is a primary limitation, and such stress can increase lignification and relocation of N and soluble carbohydrates to inaccessible sites for microbial utilization (Van Soest, 1994). However, the decrease in fibre digestibility was lower than that reported for sheep fed CT-containing diets (Abreu *et al.,* 2004; Tiemann *et al.,* 2008).

In the current study, the value obtained for three browse diets for NH_3-N concentration was lower than the control (lucerne hay). This might be due to the high digestibility of lucerne compared with the three browse foliages. The decrease in ruminal NH_3-N concentration in the browse diets indicates that ruminal degradation of dietary proteins was affected. The concomitant increase in faecal N losses suggests that tannins affected post-ruminal

degradation or absorption of proteins or both. The decrease in NH_3-N in this study was inconsistent with other studies that included tannins in sheep diets (Abreu *et al.,* 2004; Hess *et al.,* 2003; Carulla *et al.,* 2005; Tiemann *et al.,* 2008). Interestingly, in this study the values obtained for N balance were comparatively higher; N loss through urine and faeces was lower; and NH_3-N was in similar range to these reports. It was widely reported that tannins (CT) reduced ruminal protein degradation by complexing proteins and making them less degradable by ruminal microbes (Mueller-Harvey, 2006). This is due to the formation of tannin–protein and tannin–fibre complexes that are resistant to degradation. On the other hand, the lower N retention might indicate that the tannin -protein complexes were irreversible, and not digested and absorbed in high quantities in the lower gut (Barry &Duncan, 1984). The formed complex passes to the lower tract. Therefore, for animals, what matters most is whether the plant protein that was protected from ruminal degradation was digested and absorbed in the small intestine. Such studies are critical and need to be addressed. However, for all browse diets, the level of ruminally available ammonia-N (>2 mg dL^{-1}) in this study was not limiting for microbial fermentation in the rumen and this suggests that these browses could be appropriate N sources for small-holder farmers and a potential mitigation option for sheep. The reduced urinary losses imply that tannins might decrease ammonia-N emission from urine, as reported by Sliwinski *et al.* (2002).

During ruminal fermentation, high levels of tannins in the diet may decrease the availability of rumen degradable nitrogen to the rumen microbes, and this in turn affects optimal growth and multiplication of fibre-fermenting bacteria, which might reduce VFA production in the rumen (Patra *et al.,* 2006, 2011). In the current study, total VFA was not affected, as the amount of condensed tannins was too low to influence the overall OM fermentation of diets. However, isobutyric acid concentration decreased when tannin concentration increased. This might be due to decreases in AA catabolism in the rumen in the presence of CT because the protein was protected from ruminal de-amination. The decrease in acetic acid might be due to reduced fermentation of fibre by forming tannin–fibre complexes that resist microbial attack. Similar to this study, the decrease in acetate and isobutyric with the inclusion of CT-rich legumes in the diet of sheep was reported by others (Carulla *et al.,* 2005, Tiemann *et al.,* 2008; Bhatta *et al.,* 2009). The documented responses for the effects of CT on molar proportions of VFA are inconsistent (Waghorn *et al.,* 1994; Makkar, 2003, Carulla *et al.,* 2005, Tiemann *et al.,* 2008), as some of the authors reported higher molar proportions of

propionate when tannins were included in diets (Waghorn *et al.,* 1994; Makkar, 2003, Carulla *et al.,* 2005, Tiemann *et al.,* 2008)

4.5.3. Methane production

The inclusion of TRP in the diets of rams influenced their enteric CH_4 production and reduced energy lossvia CH_4 as a proportion of GE intake. The methane amount found in the current study was in the range of values reported by other researchers for sheep (Pelchen & Peters, 1998). The daily methane production from the lucerne ration averaged 32.8 l per sheep, and was significantly reduced by the inclusion of *Monechma genistifolium*, *Acacia nilotica* and *Grewia flava* by 13.0, 29.8, and 39.3%, respectively. This indicated that the reduction in methane concentration was partly a function of the level of tannin concentration in the diet. It might also be partly associated with the increased effect of tannin on methanogenic and ruminal microbes. Moreover, the ratio of CH_4 to NDF digested and ratio of CH_4 to TVFA when compared with the lucerne diet was reduced due to the inclusion of TRP. The decrease in CH_4 was not attributed to change in molar proportion of acetate or propionate, but it could be the effect of tannins on methanogens. Many studies revealed that, depending on type and dose, tannins influence methanogenesis by directly inhibiting the growth of methanogens as well as that of protozoa (Animut *et al.,* 2008; Bhatta *et al.,* 2009; Patra, 2010). Likewise Carulla *et al.* (2005) suggested that inhibition of methanogens by CT was primarily the result of suppressed fibre fermentation that limits H_2 derived from synthesis of acetate and butyrate. Similarly, ruminal fibre fermentability in sheep fed a diet containing CT was reduced as compared with PEG containing diet fed group (Barry *et al.,* 1986).This may involve tannin action on functional proteins (enzymes) located at accessible sites in or on the methanogens (Field & Lettinga, 1987).

4.6. Conclusion

In this study, inclusion of the *Monechma genistifolium*, *Acacia nilotica* and *Grewia flava*did not decrease intake, OM degradation, rumen pH, TVFA when included at 30% inclusion level in rations of sheep. However, crude protein degradation and fibre fermentation were reduced due to the inclusion of the browses, except in *Monechma genistifolium* foliage. Replacing lucerne hay with *Monechma genistifolium* significantly decreased CH_4 emission in sheep. Among the browses, *Monechma genistifolium* was found to be effective in reducing

methane emission with better N balance. However, for *Acacia nilotica* and *Grewia flava,*a lower level of inclusion has to be studied if N balance and CH_4 reduction are the primary targets. Although fibre fermentation was slightly affected at 30% inclusion level for these two browses, they can still be used as supplemental feed for small ruminants in order to overcome the negative effect of low N on ruminal fermentation. This is because the level of ruminally available ammonia-N was not limiting for microbial fermentation in the rumen of sheep fed *Monechma genistifolium*, *Acacia nilotica* and *Grewia flava*. In addition, utilisation of such browses may directly enhance the metabolic protein supply to the animal.

CHAPTER 5

In vitro fermentation, digestibility and methane production of two roughages and a total mixed ration as influenced by cellulase and xylanase enzyme application levels

5.1. Abstract

This study aimed to evaluate the effects of cellulase and xylanase enzymes on *in vitro* ruminal fermentation and methane (CH_4) production of *Eragrostis curvula hay*, maize stover and a total mixed ration (TMR). The feed samples were pre-treated for 24 hr with the two fibrolytic enzymes at seven levels of application. Then the samples were incubated for 2, 12, 24, 48 and 72 hr in an *in vitro* batch culture with buffer and rumen fluid. Gas production was measured with a pressure transducer connected to a data tracker, while CH_4 gas was analysed witha gas chromatograph, which was calibrated with standard CH_4 and CO_2 gases. Increasing the level of enzyme application resulted in increased gas volume, total VFA production, DM and NDF fermentation and methane. The observed linear increase in percentage and volume of methane production with increasing level of enzyme application might be due to increased OM fermentation that resulted in a shift in VFA production towards acetate. Considering the efficiency of DM and NDF fermentation improvement and production of associated VFA with levels of enzymes, the use of 1 mg g^{-1} DM of enzyme could be a good option for the feeds. However, the addition of these enzymes could not decrease methane production. Thus, there is a need to consider simultaneous use of other hydrogen sinks to directly capture extra H^+ produced as the result of more acetate-oriented fermentation associated with the use of cellulase and xylanase enzymes.

Keywords: digestibility, fibrolytic enzymes, gas production, methane production

5.2. Introduction

In tropical and subtropical farming systems, forage plants are the major sources of energy for ruminants. The fibre is a major component of the forage dry matter (DM). The fibre generally has a low energy and low digestibility co-efficiency (Hatfield *et al.,* 1999; Azzaz *et al.,* 2012) since the plant cell wall is an interwoven matrix of polymers that form complex and dynamic structures. These structures are barriers against microbial invasion and limit their access to the digestible cell wall networks of plants (Krueger *et al.,* 2008; McDonald *et al.,* 2011). Moreover, the rumen environment affects fibre digestion (McDonald *et al.,* 2011).

Plant cell walls typically consist of about 35-50% cellulose, 20-35% hemicelluloses, and 10-25% lignin in the dry mass (Sticklen, 2008). Over past decades, various chemical treatment options have significantly improved cell wall digestibility (McDonald *et al.,* 2011). But, despite all these efforts, more than 50% of the fibre fraction is still not readily digested. Therefore, the efficient utilization of fibrous feeds in ruminant production systems is still limited (Hatfield *et al.,* 1999).

The inclusion of exogenous fibrolytic enzymes (EFE) as an alternative way of improving fibre digestibility in fibrous feeds has resulted in positive outcomes (Adesogan *et al.,* 2007; Krueger *et al.,* 2008; Azzaz *et al.,* 2012). Cellulase and xylanase are two major ruminant diet enzyme groups that break down the cellulose and xylans, respectively, in plant cell walls (Beauchemin *et al.,* 2003; Lynd *et al.,* 2005). Many authors have reported an increase in the digestion of fibre and improved animal performances (Adesogan *et al.,* 2007; Bala *et al.,* 2009). However, others reported either a negative or no effect at all (Bowman *et al.,* 2003; Vicini *et al.,* 2003; Baloyi, 2008). Although improvement in digestibility and animal performances due to enzyme supplementations was reported, there is limited information about their effect on methane production. In the few studies where methane production was measured, the effects were not consistent (McGinn *et al.,* 2004; Eun & Beauchemin, 2007; Chung *et al.,* 2012).

The activities of enzymes vary with the proportion of concentrate in the diet (Giraldo *et al.,* 2008), enzyme doses (Jalilvand *et al.,* 2008), rumen pH (Yang *et al.,* 2002), moisture content of the feed (Wang *et al,* 2002), and methods of supplementation (Krueger *et al.,* 2008). Moreover, the optimal level of inclusion is dependent on the diet under consideration. Thus

the optimum rate of inclusion of a given enzyme preparation for different feeds needs to be determined. The relationship between enzyme activity and forage utilization could in turn help to explain and determine the conditions most likely to result in positive responses of animals. The aim of this study was to evaluate the effect of fibrolytic cellulase and xylanase enzymes on rumen digestibility, fermentation characteristics and methane production of *Eragrostis curvula*, maize stover and a total mixed ration (TMR) *in vitro*.

5.3. Materials and methods

The cellulase and xylanase enzymes used in the study (Dyadic International Inc., Florida, USA) were concentrated liquids of acid cellulase (E.C. 3.2.1.4) and acid-neutral endo-1, 4-β-D-xylanase (E.C. 3.2.1.8), respectively. Cellulase and xylanase were produced by the fermentation of non-GMO*Trichoderma longibrachiatum* (formerly *Trichoderma reesei*). *Eragrostis curvula*, maize stover and a formulated total mixed ration (TMR) were used as test feeds. The TMR contains hominy chop (26.8%), wheat bran (7.9%), *Eragrostis* hay (14.85%), alfalfa hay (14.85%), sunflower oil cake (21.8%), soya meal cake (3.96%), molasses (6.94%), limestone (1%), dicalcium phosphate (0.5%), salt (0.5%), sodium bicarbonate (0.5%) and a premix (0.4%). *Eragrostis curvula*, maize stover and TMR were treated at 0, 0.5, 1, 2, 3, 4 and 5 mg g^{-1} DM with cellulose and at 0, 0.25, 0.5, 1, 2, 3, 4 mg g^{-1} DM with xylanase.

5.3.1. Feed sample collection, preparation and chemical analysis

Samples of test feeds were dried at 55°C for 48 hr in a forced draft oven and ground with a Wiley Mill fitted with a 1 mm screen and analysed for DM, ash, CP, EE, NDF, ADF, NDFN, ADFN and ADL according to methods similar to those described in Chapter 2 in section 3.2.

5.3.2. Enzyme assay

Enzyme activities were determined with single polysaccharides as a substrate in triplicate with the inclusion of a blank. Xylanase activity was assayed with 1% (w/v) and birchwood xylan as a substrate according to the procedure described by Bailey *et al.* (1992). Endo-glucanase and exo-glucanase enzyme activities were assayed according to the method described by Wood and Bhat (1988). The enzymes were studied at a range of pH levels (4-

6.6). One unit of activity was defined as the amount of enzyme required for releasing 1µmol equivalent of glucose or xylose per minute per gram of enzyme under the conditions of the assay.

5.3.3. *In vitro* gas production measurement

Collection of rumen fluid from donor sheep

The rumen fluid was collected before the morning feeding from two ruminally cannulated Merino wethers fed on ad libitum amounts of lucerne hay. The details of procedures to collect the rumen fluid were given in Chapter 2 in section 3.3.

Reducing buffer solution

The rumen buffer solution, macro mineral solution and micro mineral solution were prepared according to the procedure described by Goering and Van Soest (1970) in large quantities and utilized as needed. The micro mineral solution was stored in a dark glass bottle in order to maintain the quality of the solution. In the morning, before the commencement of the experiment, the appropriate amounts of distilled water, rumen buffer solution, macro and micro mineral solutions were mixed with the tryptose and prepared 0.1% (wt/vol) resazurin. The enzyme solution was prepared based on the required dose rate(s) for specific experimental treatments in order to deliver the desired amount of enzyme in a 1 ml aliquot. Appropriate amounts of L-cysteine hydrochloride and sodium sulphide were weighed and added directly to the rest of the solution once all chemicals had dissolved. As soon as the reducing agent was added, the buffer solution was placed in a 39°C water bath and bubbled with CO_2. The serum bottles were then sealed with a rubber stopper and left at 39°C until the buffer solution was clear, indicating that the solution was sufficiently reduced.

Measurement of gas production

A semi-automated system was used to measure gas production through *in vitro* incubation at 39°C, according to Theodorou *et al.* (1994). The system consisted of a digital data tracker (tracker 220 series indicators, Omega Engineering, Inc., Laval, QC, Canada) connected to a pressure transducer (PX4200-015GI from Omega Engineering, Inc., Laval, QC, Canada) with

a needle at the tip. About 500 mg of each feed sample was weighed into 150 ml serum bottles, and 1 ml of the appropriate enzyme treatment was pipetted directly into the substrate and incubated for 24 hr. After that, 42 ml of rumen fluid + medium was added under a stream of CO_2 to each of the serum bottles, which closed with rubber stoppers and crimp seal caps. A needle was inserted through the rubber stopper of each serum bottle for about five seconds to release the small amount of gas that might have built up and to create a starting point for incubation. All serum bottles were returned to the incubator, and the rotary shaker was turned on at 120 rpm. Gas pressure was taken at 2, 4, 8, 12, 16, 24, 32, 48, 54 and 72 hr incubation. To quantify the gas production derived from the culture medium and the ruminal inoculum, two blanks were included in every analysis. Two replicates and four runs were executed for every treatment. The pressure and volume values of each reading time were registered, and added to the values of the previous readings. Thus, the cumulative pressure and volume of the fermentation gases were obtained. Fermentation was terminated after 72 hr by removing serum bottles from the incubator and placing them on ice. Supernatants were taken immediately, pipetted and stored at -20°C until analysed for NH_3-N (McDonald *et al.,* 1960) and VFA (Ottenstein & Bartley, 1971)

5.3.4. *In vitro* fermentation

To evaluate *in vitro* NDF fermentation at 48 hr of fermentation, ruminal fluid samples and DM residuals were collected from two bottles per treatment. All of the serum bottle contents were transferred into gush crucibles and with a vacuum filter system. The fluid was filtered and dried in an oven at 55°C for 48 hr, after which DM disappearance was determined. The blank corrected sample weight was referred to as apparent undegradable DM, and the degradability was calculated as the ratio of degradable DM to that of substrate DM incubated. About 0.250 mg of dried samples were transferred into ANKOM filter bags for NDF determination and later estimation of the NDF disappearance. Total fermentable DM and NDF were derived from the difference between the weight of DM, NDF incubated and DM, NDF residues as indicated by formulas described in the manufacturer's manual.

5.3.5. Methane production measurements

Methane production was measured separately from duplicate bottles incubated for each incubated test feed sample at 2, 12, 24, and 48 hr. The details of procedures followed to measure methane production were described in Chapter 2 under section 3.5.

5.3.6. Calculations, statistical analysis and model

Glucose and xylose equivalents (mg) = a + bx
Where x is the absorbance obtained after correction for the enzyme and the substrate blanks.

Metabolizable energy (ME, MJ kg^{-1} DM) were estimated according to Menke and Steingass (1988) as ME (MJ kg^{-1} DM) = 2.20 + 0.136 $IVGP_{24}$ (ml 0.5 g^{-1} DM) + 0.057 CP (% DM)
Methane production was calculated as
g CH_4/g digested DM = ((gas production 24 h×([CH_4 24 h]) - gas produced blank 24 h×[CH_4 blank24 h]))/g digested DM according to Chaves *et al.,* (2006).

Rate and extent of gas production were determined for each feed by fitting gas production data to the non-linear equation $y = b\ (1 - e^{-ct})$ (Ørskov & Mcdonald, 1979), where y = the gas production at time t; b = the slowly fermentable fraction (g kg^{-1} DM), and c = the rate (% h^{-1}) of fermentation of fraction b.

The experimental design used in this study was completely randomized. The data were statistically analysed with the GLM option of SAS (2009), and differences among means were determined with Tukey's test. *In vitro* incubation times were used to fit non-linear regression models with the NLIN procedure (SAS, 2009). The following model was used:

$Yij = \mu + \alpha_i + \beta_j + e_{ijk}$, where

Yij is the response of the treatment i observed in block j

μ overall mean of the treatment

α_i is the additive effect of j^{th} block (run)

β_j is the additive effect of i^{th} treatment

e_{ijk} is the associated random error.

5.4. Results

5.4.1. Chemical composition

The nutrient compositions of test feeds are shown in Table 5.1. The TMR contained high crude protein (CP) and lower cell wall content (ADF, NDF, ADL and cellulose) among the three test feeds. High proportions of ADF and ADL were recorded for maize stover, while *Eragrostis curvula* contained high values of NDF and cellulose. The recorded CP value was low for roughages feeds, but the lowest value was recorded in maize stover. Maize stover contained low amounts of ADFN, and NDFN, while TMR contained high proportions of ADFN and NDFN.

Table 5.1 Chemical composition of test feeds (mean±SE) treated with commercial cellulase and xylanaseenzymesat seven levels

Chemical components	Test feeds composition (g kg^{-1} DM)		
	Eragrostis curvula	Maize stover	TMR
DM	945.1±8.4^{a}	922.5±0.5^{b}	938.6±0.05^{a}
Ash	37.5±0.4^{b}	22.8±0.1^{c}	74.8±0.26^{a}
OM	907.6±0.4^{a}	901.4±8.2^{a}	863.8±0.24^{b}
EE	10.8±0.2^{b}	8.71±0.18^{c}	59.9±1.4^{a}
CP	31.1±0.03^{b}	20.5±0.18^{c}	196.9±0.47^{a}
ADF	502.7±2.3^{b}	521.9±3.9^{a}	202.9±4.04^{c}
NDF	844.5±3.2^{a}	811.5±17.7^{b}	296.0±6.06^{c}
ADL	76.3±1.32^{b}	108.7±1.5^{a}	34.9±0.40^{c}
ADFN	9.78±0.3^{b}	2.70±0.1^{c}	116.4±0.4^{a}
NDFN	14.2±0.3^{b}	10.4±0.1^{c}	148.4±0.04^{a}
Cellulose	426.4±1.32^{a}	413.24±2^{b}	168.0±4.4^{c}
ME(MJ kg^{-1} DM)	7.9±0.03^{b}	8.5±0.01^{b}	14.2±0.03^{a}

Means with different letters (superscripts) within a row differ significantly at $p<0.05$

5.4.2. Enzyme activity

The enzyme activity profiles determined at different pH levels (temperature of 39°C) are presented in Table 5. 2. High enzyme activity was observed at a pH of 4.8, while enzyme

activity declined as pH increased for both enzymes. The lowest activity was recorded at high pH (6.6).

Table5.2 Activities of two fibrolytic enzymes used in the study and amount of released sugar (Uml^{-1}, mean±SE) at different pH from test substrate

pH	Cellulase activity		Hemicellulase activity (xylanase assay)
	Endo-glucanase assay	*Exo-glucanase assay*	
4	4232.2 ± 24.4^{b}	3 ± 0.24^{b}	1677.2 ± 11.0^{d}
4.8	4484.3 ± 32.8^{a}	4.3 ± 0.12^{a}	2497.6 ± 9.2^{a}
5.8	3373.6 ± 29.2^{c}	1.5 ± 0.5^{c}	1831.5 ± 10.2^{b}
6	2141.9 ± 28.2^{d}	1.0 ± 0.2^{d}	1737.2 ± 11.8^{c}
6.6	756.1 ± 35^{e}	nd	635.7 ± 13^{e}

Means with different letters (superscripts) within a column differ significantly at $p<0.05$ for each enzymes, nd-not detected

5.4.3. *In vitro* gas production

The effects of the application of different levels of cellulase and xylanase enzymes on the cumulative gas production pattern of the three test feeds are shown in Table 5.3. The addition of cellulase and xylanase enzymes increased the cumulative gas produced from the feeds during incubation periods. Cumulative gas production increased with increasing levels of enzyme application at quadratic functions for all test feeds.

The potential extent of gas production (b), the gas production rate (c) and the effective gas production (EGP) estimated or calculated based on gas production data for the three test feeds were different at the different application levels of cellulase and xylanase enzymes (Table 5.4). For all test feeds, the potential extent of gas production (b) increased with increasing levels of enzyme application, and the highest b values were obtained at an application level of 5 mg g^{-1} DM cellulase and 4 mg g^{-1} DM xylanase enzymes. For the three test feeds, application of cellulase and xylanase improved the gas production rate (c) when compared with the control. However, no clear pattern of improvement was observed with increased levels of applications for all feeds.

Table 5.3 Gas production (ml g^{-1} DM) of *Eragrostis curvula* hay, maize stover and TMR treated with cellulase and xylanase enzymes products at seven levels

Enzymes and levels	Gas production at different time intervals														
	Eragrostis curvula					Maize stover					Total mixed ration (TMR)				
	2	12	24	48	72	2	12	24	48	72	2	12	24	48	72
Cellulase															
0	2.32^{b}	5.30^{f}	13.0.^{g}	43.8^{g}	67.6^{g}	2.32^{c}	9.18^{c}	17.1e	49.4^{f}	74.8^{f}	4.30^{e}	52.2^{f}	49.8^{g}	112.4^{g}	134.0^{g}
0.5	2.42^{b}	7.08^{e}	14.8^{f}	45.6^{f}	69.0^{f}	3.52^{ab}	10.3^{c}	18.1^{ed}	50.6^{f}	75.6^{f}	5.62^{d}	53.8^{e}	91.4^{f}	117.4^{f}	140.6^{f}
1	2.10^{b}	9.24^{d}	18.6^{e}	54.6^{e}	79.4^{e}	3.18^{b}	1.12^{c}	19.8^{d}	53.8^{e}	80.4^{e}	7.30^{b}	57.2^{d}	96.4^{e}	125.6^{e}	150.6^{e}
2	3.66^{a}	11.8^{c}	24.8^{d}	33.8^{d}	92.2^{d}	3.64^{ab}	14.4^{b}	27.2^{c}	66.4^{d}	96.4^{d}	6.54^{c}	58.0^{c}	99.0^{d}	131.6^{d}	158.2^{d}
3	3.60^{a}	11.9^{c}	18.6^{c}	65.4^{c}	95.4^{c}	3.74^{ab}	17.8^{a}	25.6^{bc}	76.4^{c}	106.4^{c}	5.66^{d}	58.8^{c}	101.4^{c}	136.6^{c}	164.8^{c}
4	3.56^{b}	13.5^{b}	23.1^{b}	72.2^{b}	102.0^{b}	3.70^{ab}	19.9^{a}	29.6^{a}	81.6^{b}	113.2^{b}	7.32^{b}	62.2^{b}	106.6^{b}	147.4^{b}	175.6^{b}
5	3.58^{b}	16.8^{a}	29.8^{a}	82.2^{a}	120.4^{a}	4.28^{a}	19.9^{a}	31.8^{a}	84.4^{a}	122.6^{a}	9.98^{a}	66.4^{a}	112.4^{a}	157.4^{a}	188.2^{a}
SEM	*1.06*	*2.64*	*2.84*	*4.61*	*6.27*	*0.65*	*1.63*	*2.01*	*4.94*	*6.25*	*0.93*	*1.61*	*2.66*	*5.31*	*6.34*
Linear(P)	*<0.04*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.05*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.03*	*<0.001*	*<0.001*	*<0.001*	*<0.001*
Quadratic(P)	*<0.02*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.02*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.02*	*<0.001*	*<0.001*	*<0.001*	*<0.001*
Xylanase															
0	3.32^{d}	5.30^{f}	13.0^{e}	43.8^{f}	67.6^{f}	3.32^{d}	9.18^{e}	17.2^{de}	49.4^{f}	74.8^{e}	4.30^{c}	52.2^{e}	89.8^{f}	112.4^{f}	134.0^{f}
0.25	2.42^{d}	7.18^{e}	13.1^{e}	44.0^{f}	67.8^{f}	3.58^{c}	10.9^{d}	17.2^{de}	47.8^{f}	75.2^{e}	5.66^{b}	52.2^{e}	89.8^{f}	112.6^{f}	134.2^{f}
0.5	2.56^{d}	7.24^{e}	13.3^{e}	45.8^{e}	69.2^{e}	30.4^{c}	10.5^{de}	16.6^{e}	49.2^{f}	76.8^{e}	5.66^{b}	53.8^{d}	91.4^{e}	117.4^{e}	140.8^{e}
1	3.66^{c}	8.66^{d}	16.4^{d}	52.2^{d}	78.8^{d}	5.34^{b}	10.5^{ed}	18.3^{d}	54.2^{e}	87.4^{d}	7.32^{b}	56.2^{c}	96.6^{d}	123.8^{d}	150.6^{d}
2	3.66^{c}	11.14^{c}	20.6^{c}	59.6^{c}	86.4^{c}	4.36^{bc}	13.2^{c}	22.6^{c}	61.6^{d}	98.4^{c}	5.83^{b}	57.2^{c}	98.2^{c}	130.8^{c}	157.4^{c}
3	4.96^{b}	13.6^{b}	24.8^{b}	67.2^{b}	93.8^{b}	5.20^{b}	15.4^{b}	26.6^{b}	69.2^{b}	107.4^{b}	7.32^{b}	60.4^{b}	103.32^{b}	138.2^{b}	166.4^{b}
4	6.24^{a}	20.4^{a}	38.2^{a}	93.8^{a}	125.4^{a}	6.86^{a}	21.6^{a}	39.2^{a}	95.2^{a}	120.8^{a}	7.32^{b}	62.4^{a}	106.6^{a}	147.4^{a}	177.2^{a}
SEM	*0.57*	*1.65*	*3.08*	*6.04*	*6.99*	1.25	*1.45*	*2.8*	*7.26*	*7.23*	*0.95*	*1.31*	*2.37*	*5.03*	*6.3*
Linear(P)	*<0.04*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.05*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.03*	*<0.001*	*<0.001*	*<0.001*	*<0.001*
Quadratic (P)	*<0.02*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.02*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.02*	*<0.001*	*<0.001*	*<0.001*	*<0.001*

Means with different letters (superscripts) within a column differ significantly at p<0.05for each enzyme; SEM, standard error of the mean

Table 5.4 Kinetics of gas production (ml g^{-1} DM) of *Eragrostis curvula* hay, maize stover and TMR treated with cellulase and xylanase enzyme at seven levels

Enzyme and levels of application		*Eragrostis curvula*			Maize stover			Total mixed ration(TMR)		
		b	c	EGP	b	C	EGP	b	c	EGP
Cellulase	0	26.4^{g}	0.010^{f}	4.38^{g}	31.4^{f}	0.012^{f}	6.20^{f}	82.8^{g}	0.037^{b}	35.1^{g}
	0.5	28.8^{f}	0.010^{f}	4.80^{f}	37.8^{d}	0.029^{a}	13.8^{b}	81.8^{f}	0.039^{a}	36.6^{f}
	1	32.8^{e}	0.010^{e}	5.50^{e}	36.2^{e}	0.028^{b}	13.1^{e}	87.4^{e}	0.036^{c}	36.8^{e}
	2	40.2^{d}	0.016^{b}	9.78^{c}	41.0^{c}	0.013^{f}	8.58^{f}	92.8^{d}	0.033^{d}	37.4^{d}
	3	39.4^{c}	0.019^{a}	11.2^{a}	50.4^{b}	0.027^{c}	17.6^{a}	103.8^{c}	0.032^{e}	40.4^{c}
	4	42.2^{b}	0.0126^{c}	8.48^{d}	60.4^{a}	0.015^{d}	13.6^{c}	104.0^{b}	0.032^{f}	40.8^{b}
	5	56.8^{a}	0.0109^{d}	11.1^{b}	60.4^{a}	0.014^{e}	13.2^{d}	121.2^{a}	0.031^{g}	46.6^{a}
	SEM	0.29	0.0005	0.186	0.778	0.001	0.250	0.259	0.004	0.15
	Linear(P)	*<0.001*	*0.16*	*<0.001*	*<0.001*	*0.16*	*<0.001*	*<0.001*	*<0.01*	*<0.001*
	Quadratic(P)	*<0.001*	*0.23*	*<0.001*	*<0.001*	*0.08*	*<0.001*	*<0.001*	*<0.01*	*<0.001*
Xylanase	0	26.4^{g}	0.010^{f}	4.20^{g}	30.2^{f}	0.010^{b}	6.19^{e}	82.2^{d}	0.037^{b}	36.6^{d}
	0.25	27.0^{f}	0.011^{e}	4.74^{f}	30.2^{f}	0.010^{b}	5.04^{g}	82.4^{d}	0.037^{b}	36.6^{d}
	0.5	27.8^{e}	0.011^{e}	5.14^{e}	34.2^{e}	0.010^{b}	5.88^{f}	82.2^{d}	0.039^{a}	35.1^{f}
	1	33.2^{d}	0.020^{d}	9.50^{d}	35.6^{d}	0.011^{a}	6.28^{d}	86.2^{c}	0.036^{c}	36.0^{e}
	2	35.6^{c}	0.026^{b}	12.2^{b}	42.8^{c}	0.010^{b}	7.12^{c}	98.8^{b}	0.03^{d}	37.4^{c}
	3	41.2^{b}	0.021^{c}	12.1^{c}	45.8^{b}	0.010^{b}	6.66^{b}	98.8^{b}	0.03^{d}	38.4^{a}
	4	57.6^{a}	0.029^{a}	20.2^{a}	66.2^{a}	0.011^{a}	5.96^{a}	104.6^{a}	0.03^{d}	39.8^{b}
	SEM	0.10	0.001	0.001	0.58	0.0001	0.149	0.647	0.0002	0.01
	Linear(P)	*<0.001*	*0.1*	*<0.001*	*<0.001*	*0.19*	*<0.001*	*<0.001*	*<0.02*	*<0.001*
	Quadratic(P)	*<0.001*	*0.12*	*<0.001*	*<0.001*	*0.13*	*<0.001*	*<0.001*	*<0.02*	*<0.001*

Means with different letters (superscripts) within a column differ significantly at $p<0.05$, for each enzyme; b: gas production from the insoluble but slowly fermentable fraction (ml); [2]C: the rate of GP from insoluble fraction per hour; [3]EGD: effective gas production; SEM: standard error of mean

5.4.4. Volatile fatty acids and ammonia-N profiles

The ammonia-N and VFA profiles recorded for cellulase and xylanase treatments are shown in Tables 5.5 and 5.6. Generally, for all test feeds, acetate and total VFA concentration seemed to be higher for the enzyme-treated samples compared with the control. Among the enzyme-treated feeds, the production of acetate and total VFA showed a tendency of improvement with increasing enzyme level. Propionate production improved with increasing levels of cellulase enzyme for *Eragrostis curvula* and maize stover. In the case of the TMR, propionate production seemed to decrease with increasing levels of the two enzymes. There seemed to be an increase of isobutyric and butyric acid production, while no clear pattern was observed for valeric acid production in all the three test feeds. The acetate to propionate ratio (A: P) increased with increasing level of both enzymes for *Eragrostis curvula* and TMR test feeds. The NH_3-N production from the roughage test feeds seemed to be unaffected by application of cellulase and xylanase enzymes, while higher concentration of NH_3-N was recorded for the TMR test feed due to cellulase and xylanase application.

5.4.5. In vitro NDF fermentation

The NDF disappearance of the three test feeds treated with different levels of cellulase and xylanase enzymes is shown in Table 5.7. The addition of cellulase and xylanase enzymes at more than 0.5 mg g^{-1} DM increased NDF disappearance for the *Eragrostis curvula* and maize stover. In the case of the TMR, cellulase increased NDF disappearance at an application rate higher than 0.5 mg g^{-1} DM, while similar increase in NDF disappearance was recorded for xylanase at an application rate greater than 0.25 mg g^{-1} DM. The NDF disappearance increased at a linear and quadratic function with increasing application levels of cellulase and xylanase enzymes.

Table5.5 Total and individual volatile fatty acid (mmol L^{-1}) production, acetate to propionate ratio (A: P), and ammonia–N (mg 100 ml^{-1}), in supernatant after 72 hr incubation of *Eragrostis curvula* hay, maize stover and TMR with cellulase enzyme

Feed and levels of enzymes use	Ammonia nitrogen and volatile fatty acids							
	NH_3-N	Acetic	Propionic	Iso butyric	Butyric	Valeric	TotalVFA	A : P ratio
Eragrostis curvula 0	11.7±0.04^{a}	23.0	7.29	0.73	2.63	1.08	34.74	3.155
0.5	11.6±0.01^{b}	23.05	7.33	0.74	2.68	1.08	34.87	3.147
1	11.0±0.01^{c}	33.42	10.13	1.03	3.80	1.36	49.74	3.299
2	10.9±0.05^{d}	34.09	10.30	1.01	3.71	1.44	50.56	3.311
3	10.8±0.01^{e}	37.20	10.40	1.33	5.16	1.16	55.25	3.576
4	10.74±0.05^{f}	38.49	10.55	1.43	5.25	1.47	57.20	3.647
Maize stover 0	10.8±0.05^{a}	36.8	13.86	1.00	4.59	1.45	57.7	2.654
0.5	10.2±0.05^{b}	38.64	13.98	1.08	5.00	1.61	60.31	2.764
1	9.5±0.03^{c}	47.25	18.68	1.51	6.84	1.98	76.26	2.529
2	9.2±0.05^{d}	52.7r6	15.09	1.60	7.19	1.60	78.24	3.497
3	9.0±0.01^{e}	55.63	14.18	1.52	6.16	1.62	79.10	3.923
4	9.0±0.01^{f}	50.01	19.38	1.62	6.91	2.02	79.95	2.580
TMR 0	19.4±0.03^{e}	42.08	26.50	1.47	4.28	2.81	77.14	1.588
0.5	19.2±0.01^{f}	54.53	19.38	2.19	9.68	3.12	88.90	2.814
1	23.3±0.03^{a}	54.67	19.91	2.20	9.21	3.05	89.04	2.746
2	21.2±0.02^{b}	57.64	23.98	1.72	7.58	2.03	92.96	2.404
3	20.6±0.02^{c}	58.98	25.19	1.83	8.08	1.20	95.28	2.342
4	20.3±0.01^{d}	58.63	21.10	2.37	10.44	3.35	95.89	2.779

Means with different letters (superscripts) within a column differ significantly at $p<0.05$ for each enzymes

Table 5.6 Total and individual volatile fatty acid (mmol L^{-1}) production, acetate to propionate ratio (A : P), and ammonia-N (mg 100 ml^{-1}), in supernatant after 72 hr incubation of *Eragrostis curvula* hay, maize stover and TMR with xylanase enzyme

Feed and levels of enzymes use		Ammonia nitrogen and volatile fatty acids							
		NH_3-N	Acetic	Propionic	Iso butyric	Butyric	Valeric	Total VFA	A : P ratio
Eragrostis curvula	0	11.7±0.05[a]	23.0	7.29	0.73	2.63	1.08	34.74	3.155
	0.5	11.7±0.01[b]	29.36	9.16	0.93	3.37	1.33	44.15	3.21
	1	11.3±0.05[c]	34.87	10.59	1.11	3.92	1.64	52.13	3.29
	2	11.0±0.01[d]	35.53	9.99	1.32	5.16	0.90	52.90	3.56
	3	10.9±0.05[e]	37.71	11.15	1.48	5.49	1.48	57.31	3.38
	4	10.9±0.05[e]	39.20	10.80	1.48	5.76	1.30	58.54	3.63
Maize stover	0	10.8±0.01[a]	36.8	13.86	1.00	4.59	1.45	57.7	2.654
	0.5	9.0±0.05[d]	50.26	12.01	1.58	6.01	0.72	70.57	4.19
	1	9.02±0.08[b]	43.12	17.85	1.44	6.69	1.96	71.06	2.42
	2	8.9±0.04[c]	44.10	17.48	1.34	6.57	1.93	71.42	2.52
	3	8.54±0.03[d]	51.46	11.84	1.47	5.90	1.21	71.87	4.35
	4	8.4±0.01[e]	52.83	11.55	1.62	6.07	0.90	72.97	4.57
TMR	0	19.4±0.02[e]	42.08	26.50	1.47	4.28	2.81	77.14	1.59
	0.5	19.2±0.02[f]	49.26	17.55	2.07	9.30	2.99	81.17	2.81
	1	20.8±0.01[a]	50.57	18.18	2.10	9.32	2.92	83.08	2.78
	2	20.3±0.01[c]	51.14	18.84	2.09	9.02	3.00	84.09	2.71
	3	20.5±0.01[b]	55.95	23.59	1.57	7.79	1.94	90.84	2.37
	4	20.3±0.01[d]	61.21	26.29	1.93	7.82	1.55	98.81	2.33

Means with different letters (superscripts) within a column differ significantly at $p<0.05$ for each enzymes

Table 5.7 Effects of cellulase and xylanase enzymes on the *in vitro* 48hr NDF disappearance (%, mean ±SE) of *Eragrostis curvula* hay, maize stover and TM at seven levels

Enzyme levels	*Eragrostis curvula*	Maize stover	TMR
Cellulase 0	33.7±0.88^{f}	39.4±0.27^{f}	59.5±0.27^{e}
0.5	35.2±0.35^{f}	40.0±0.91^{f}	60.1±0.46^{e}
1	39.7±1.32^{e}	40.9±0.20^{e}	62.7±0.86^{d}
2	44.6±0.91^{d}	44.6±0.79^{d}	65.1±0.44^{c}
3	47.2±0.45^{c}	49.0±0.09^{c}	68.1±0.03^{b}
4	48.9±0.91^{b}	53.8±0.44^{b}	75.4±0.06^{a}
5	51.0±0.29^{a}	56.1±1.06^{a}	80.2±1.02^{a}
Linear (P)	*<0.001*	*<0.001*	*<0.001*
Quadratic (P)	*<0.001*	*<0.001*	*<0.001*
Xylanase0	33.7±0.9^{d}	39.4±0.27^{d}	59.5±0.3^{f}
0.25	33.9±0.6^{e}	40.4±0.33^{d}	60.0±0.36^{f}
0.5	34.8±0.2^{e}	41.4±0.44^{d}	62.9±0.51^{e}
1	37.5±0.4^{d}	45.7±1.85^{c}	66.0±1.00^{d}
2	41.2±0.8^{c}	49.1±0.57^{b}	68.8±0.24^{c}
3	45.7±1.8^{b}	53.9±0.79^{a}	74.2±0.62^{b}
4	48.9±0.9^{a}	55.9±1.25^{a}	77.2±0.53^{a}
Linear (P)	*<0.001*	*<0.001*	*<0.001*
Quadratic (P)	*<0.001*	*<0.001*	*<0.001*

Means with different letters (superscripts) within a column differ significantly at p<0.05 for each enzymes

SE: standard error

5.4.6. Addition of fibrolytic enzymes on CH_4 production

Increased volumes of methane gas were recorded with increased levels of enzymes (Table 5.8). The addition of enzymes increased ($p<0.01$) the volume of CH_4 gas produced from incubated feeds. The volumes of CH_4 gas increased ($p<0.05$) with increasing levels of enzymes at linear and quadratic functions. The TMR produced relatively the lowest CH_4 volume during all incubation periods when compared with the two roughages tested. Methane production expressed in mass (g per INDDM) varied significantly ($p<0.05$) among the various levels of enzymes (Tables 5.9, 5.10 and 5.11). Significantly ($p<0.05$) lower values of these parameters were recorded for the lower levels, while higher values were recorded for higher levels of both enzymes. It was observed that CH_4 expressed in mass increased with increasing levels of enzymes both at linear and quadratic functions. A similar trend was observed for the ratio of CH_4 to digested NDF (CH_4: NDF digested), as well as ratio of CH_4 production to GP_{24} which showed a significant ($p<0.05$) increase with increasing levels of enzymes both for maize stover and the TMR. For *Eragrostis curvula* hay, however, significantly ($p<0.05$) lower ratio of CH_4 to total gas production was obtained at the highest levels of enzyme addition.

There was a significant negative correlation between methane production and CP (-0.96), ash (-0.63*), EE (-0.75*), NFC (-0.88*), NDFN (-0.94*), and ADFN (-0.77*) of the test feeds (Table 5.12). A significant positive correlation was noted between CH_4 production and NDF (0.93*), ADF (0.96*), ADL (0.99*), and cellulose (0.94*).

Table 5.8 Volume (ml g^{-1} DM) of methane produced due to addition of cellulase and xylanase enzymes to total mixed ration (TMR), maize stover (MS) and *Eragrostis curvula* hay (ECH) feeds at seven levels

Enzyme	Level	TMR				MS				ECH			
	(mg g^{-1} DM)	2	12	24	48	2	12	24	48	2	12	24	48
Cellulase	0	0.03^{d}	0.36^{g}	2.36^{g}	4.56^{g}	0.05^{g}	0.85^{f}	4.94^{g}	9.02^{g}	0.06^{e}	1.83^{d}	4.15^{g}	9.14^{g}
	0.5	0.03^{d}	0.49^{f}	2.64^{f}	5.32^{f}	0.063^{f}	1.04^{e}	5.26^{f}	9.45^{f}	0.06^{e}	1.75^{e}	4.39^{f}	9.53^{f}
	1	0.04^{c}	0.62^{e}	2.99^{e}	6.14^{e}	0.073^{e}	1.13^{d}	6.06^{e}	11.6^{e}	0.08^{d}	1.93^{c}	5.01^{e}	10.4^{e}
	2	0.04^{c}	0.76^{d}	3.26^{d}	6.87^{d}	0.077^{d}	1.49^{c}	7.01^{d}	14.1^{d}	0.12^{c}	2.10^{b}	5.54^{d}	11.2^{d}
	3	0.05^{b}	0.89^{c}	3.55^{c}	7.78^{c}	0.08^{c}	1.89^{b}	8.20^{c}	16.9^{c}	0.12^{c}	2.10^{b}	6.14^{c}	12.1^{c}
	4	0.05^{b}	1.02^{b}	3.88^{b}	8.85^{b}	0.082^{b}	2.16^{a}	9.26^{b}	19.3^{b}	0.13^{b}	2.22^{a}	6.76^{b}	12.9^{b}
	5	0.06^{a}	1.16^{a}	4.28^{a}	10.2^{a}	0.108^{a}	2.16^{a}	9.85^{a}	20.9^{a}	0.14^{a}	2.23^{a}	7.45^{a}	13.9a
	SEM	0.001	0.03	0.026	0.05	0.003	0.07	0.21	0.63	0.004	0.03	0.16	0.23
Xylanase	0	0.03^{c}	0.36^{bc}	2.36^{g}	4.56^{g}	0.05^{g}	0.85^{g}	4.94^{f}	9.02^{g}	0.06^{e}	1.83^{d}	4.15^{f}	9.14^{g}
	0.25	0.03^{c}	0.32^{e}	2.43^{f}	5.07^{f}	0.08^{e}	1.10^{f}	5.37^{e}	9.29^{f}	0.07^{d}	1.84^{d}	4.06^{g}	9.02^{f}
	0.5	0.03^{c}	0.34^{cd}	2.46^{e}	5.71^{e}	0.06^{f}	1.03^{e}	5.37^{e}	9.45^{e}	0.07^{d}	1.89^{c}	4.72^{e}	9.96^{e}
	1	0.04^{b}	0.35^{bcd}	2.57^{d}	6.44^{d}	0.17^{b}	1.12d	5.59^{d}	10.2^{d}	0.11^{c}	1.98^{b}	5.18^{d}	10.7^{d}
	2	0.04^{b}	0.36^{bc}	2.61^{c}	7.10^{c}	0.11^{d}	1.29^{c}	5.92^{c}	11.2^{c}	0.11^{c}	1.62^{f}	5.45^{c}	11.1^{c}
	3	0.05^{a}	0.37^{ab}	2.71^{b}	8.06^{b}	0.16^{c}	1.51^{b}	6.32^{b}	12.3^{b}	0.16^{b}	1.71^{e}	6.04^{b}	11.9^{b}
	4	0.05^{a}	0.39^{a}	2.78^{a}	8.90^{a}	0.28^{a}	2.12^{a}	7.25^{a}	15.8^{a}	0.21^{a}	2.23^{a}	7.19^{a}	13.6^{a}
	SEM	0.001	0.003	0.06	0.19	0.008	0.07	0.24	0.59	0.007	0.03	0.14	0.21

Means with different superscript (letters) across the column for each parameter are significantly different at $p<0.001$

SEM: standard error of the mean

Table 5.9 Effect of cellulase and xylanase addition on methane production expressed in mass, and ratio of methane to fermentation parameters from the TMR after 24 hours' incubation

Enzyme	Level (mg g^{-1} DM)	g kg^{-1} DDM	g kg^{-1} NDF	CH_4:ME	CH_4:gas prod	CH_4:NDF deg	CH_4:TVFA
Cellulase	0	1.69^{f}	2.84^{f}	0.068^{g}	0.053^{g}	0.83^{f}	0.0306
	0.5	1.89^{f}	3.14^{e}	0.076^{f}	0.058^{f}	0.84^{f}	0.0320
	1	2.14^{e}	3.42^{d}	0.085^{e}	0.062^{e}	0.88^{e}	0.0337
	2	2.33^{d}	3.58^{c}	0.092^{d}	0.066^{d}	0.91^{d}	0.0366
	3	2.54^{c}	3.73^{b}	0.100^{c}	0.070^{c}	0.95^{c}	0.0382
	4	2.78^{b}	3.68^{bc}	0.109^{b}	0.073^{b}	1.05^{b}	0.0407
	5	3.07^{a}	4.03^{a}	0.119^{a}	0.076^{a}	1.06^{a}	0.0447
	SEM	0.008	0.012	0.001	0.001	0.016	
Xylanase	0	1.69^{g}	2.84^{g}	0.068^{g}	0.053^{f}	0.83^{f}	0.0306
	0.25	1.74^{f}	2.92^{f}	0.070^{f}	0.054^{f}	0.83^{f}	0.0307
	0.5	1.91^{e}	3.19^{e}	0.077^{e}	0.058^{e}	0.84^{e}	0.0329
	1	2.08^{d}	3.30^{d}	0.083^{d}	0.060^{d}	0.88^{d}	0.0349
	2	2.22^{c}	3.37^{c}	0.088^{c}	0.063^{c}	0.92^{c}	0.0369
	3	2.52^{b}	3.66^{a}	0.099^{b}	0.068^{b}	0.96^{b}	0.0387
	4	2.66^{a}	3.54^{b}	0.104^{a}	0.070^{a}	1.05^{a}	0.0377
	SEM	0.005	0.013	0.001	0.001	0.011	

Means with different superscript (letters) across the column for each parameter are significantly different at $p<0.001$

SEM: standard error of the mean

Table 5.10 Effect of cellulase and xylanase addition on CH_4 production, CH_4 expressed in mass and ratio of CH_4 to fermentation parameters from the maize stover after 24 hours' incubation

Enzyme	Level (mg g^{-1} DM)	g kg^{-1} DMD	g kg^{-1} NDF	CH4:ME	CH_4:gas prod	CH_4:NDF deg	CH4:TVFA
Cellulase	0	3.54^{f}	8.97^{d}	0.83	0.58^{b}	0.55^{g}	0.086
	0.5	3.76^{f}	9.41^{d}	0.86	0.58^{b}	0.56^{f}	0.087
	1	4.34^{e}	10.6^{c}	0.95	0.61^{ab}	0.57^{e}	0.079
	2	5.02^{d}	11.2^{bc}	0.94	0.51^{c}	0.62^{d}	0.090
	3	5.87^{c}	11.9^{ab}	1.08	0.64^{a}	0.69^{c}	0.104
	4	6.63^{b}	12.3^{a}	1.19	0.63^{a}	0.75^{b}	0.116
	5	7.05^{a}	12.6^{a}	1.20	0.62^{ab}	0.78^{a}	0.118
	SEM	0.11	0.14	0.003	0.005	0.012	
Xylanase	0	3.54^{f}	8.97^{e}	0.83	0.58^{d}	0.55^{f}	0.086
	0.25	3.85^{e}	9.75^{a}	0.90	0.63^{b}	0.55f	0.082
	0.5	3.84^{e}	9.51^{c}	0.88	0.65^{a}	0.56^{e}	0.079
	1	4.01^{d}	9.67^{b}	0.89	0.61^{c}	0.58^{d}	0.083
	2	4.24^{c}	9.27^{d}	0.87	0.52^{e}	0.64^{c}	0.088
	3	4.53^{b}	9.22^{d}	0.86	0.48^{f}	0.69^{b}	0.099
	4	5.19^{a}	9.28^{d}	0.78	0.37^{g}	0.78^{a}	0.093
	SEM	0.07	0.016	0.007	0.005	0.011	

Means with different superscript (letters) across the column for each parameter are significantly different at $p<0.001$

SEM: standard error of the mean

Table 5.11 Effect of cellulase and xylanase addition on production of methane expressed in mass, and ratio of methane to fermentation parameters from *Eragrostis curvula* hay after 24 hours' incubation

Enzyme	Level (mg g^{-1} DM)	g kg^{-1} DMD	g kg^{-1} NDF	CH_4:ME	CH_4:gas prod	CH_4:NDF deg	CH_4:TVFA
Cellulase	0	2.97^{g}	8.98^{c}	0.70^{b}	0.29^{a}	0.46^{g}	0.117
	0.5	3.14^{f}	9.38^{d}	0.71^{b}	0.27^{b}	0.47^{f}	0.126
	1	3.51^{e}	9.96^{b}	0.74^{b}	0.24^{c}	0.49^{e}	0.125
	2	3.99^{d}	10.0^{b}	0.72^{b}	0.20^{e}	0.55^{d}	0.111
	3	4.50^{c}	10.1^{b}	0.89^{a}	0.28^{a}	0.63^{c}	0.121
	4	4.84^{b}	10.3^{b}	0.91^{a}	0.27^{b}	0.66^{b}	0.122
	5	5.33^{a}	10.9^{a}	0.88^{a}	0.23^{d}	0.68^{a}	0.130
	SEM	0.02	0.207	0.03	0.01	0.01	
Xylanase	0	2.91^{f}	8.78^{d}	0.68^{d}	0.28^{b}	0.46^{e}	0.117
	0.25	2.97^{f}	8.98^{d}	0.70^{d}	0.29^{b}	0.46^{e}	0.107
	0.5	3.38^{e}	10.3^{b}	0.79^{ab}	0.32^{a}	0.46^{e}	0.099
	1	3.71^{d}	10.7^{a}	0.80^{a}	0.28^{b}	0.49^{d}	0.103
	2	3.90^{c}	9.47^{c}	0.77^{bc}	0.24^{c}	0.58^{c}	0.105
	3	4.32^{b}	8.05^{e}	0.78^{ab}	0.22^{d}	0.75^{a}	0.123
	4	5.15^{a}	10.5^{ab}	0.74^{c}	0.17^{e}	0.68^{b}	0.117
	SEM	0.01	0.1	0.07	0.01	0.01	

Means with different superscript (letters) across the column for each parameter are significantly different at $p<0.001$; SEM, standard error of the mean

Table5.12 Pearson correlation between *in vitro* methane production and chemical constituents of test feeds

Major feed components	CH_4	Fibre components	CH_4	Nitrogen component	CH_4
Ash	*-0.63**	*NDF*	*0.93**	*CP*	*-0.96**
EE	*-0.76**	*ADF*	*0.96**	*NDFN*	*-0.94**
		ADL	*0.99**	*ADFN*	*-0.77**
		NFC	*-0.88**		
		Cellulose	*0.94*		

Significant at *p<0.001

5.5. Discussion

5.5.1. Enzyme activity

The effectiveness of enzymes was measured by their capacity to release sugar from the test substrate. The most common methods to measure cellulose activity from the test substrate were endo-glucanase and exo-glucanase. Endo-glucanase can randomly hydrolyse internal glycoside cellulose chains. Exo-glucanase, which is also called cellobio-hydrolase, was classified as exo-acting on the assumption that it cleaves β-1, 4-glycosidic bonds from cellobiose and some glucose molecules. In this study, the xylanase and endo-glucanase activities for the enzymes are relatively high when compared with other values that have been reported (Colombatto *et al.,* 2003; Enu & Beauchemin, 2007). However, it is difficult to extrapolate these results owing to differences in sources of enzymes and differences in analytical procedures. These enzymes showed optimum performance at a pH of 4.8, as recommended by the manufacturer. It appears that ruminants may not benefit optimally at the higher pH of the rumen unless applied at a higher level to compensate for the loss of efficacy. This indicates that the enzymes might benefit dairy cows and beef animals that depend on high concentrate feeds. In dairy and feedlot cattle, high-energy diets often result in a pH below 6.0 for much of the day, which is sub-optimal for efficient fibre fermentation; under such conditions fibre digestion is inhibited because of the depression of ruminal cellulolytic bacteria. Therefore, supplementation of these enzymes might positively influence fibre fermentation under such conditions. It is important to note that most of the enzymes were not produced with the intention of feeding ruminants.

5.5.2. *In vitro* gas production, feed fermentation and VFA production

In this study the enzyme treatment at different levels of application significantly (P<0.05) increased gas production for all the test feeds (Tables 5.3 and 5.4). Gas production is an indirect measure of feed fermentation, particularly the carbohydrate component (Menke *et al.,* 1979). The increment in gas production during fermentation shows the disappearance of the feeds in the rumen. This indicates an effect of the enzymes on the fermentation of incubated feeds. In contrast to this finding, Tricario (2001) and Colombatto *et al.* (2003), reported a short-term effect of enzymes on degradation of lucerne leaves incubated *in vitro,* with limited effects during fermentation. This continuous effect might be partly due to the pre-incubation effect that may form a stable enzyme-feed complex. The stable enzyme-feed complex increases the resistance of the enzymes to proteolysis and lengthens its residence during consecutive fermentation periods in TMR from lactating dairy cows (Kung *et al.,* 2000; Yang *et al.,* 2000).

Gas production increases at a quadratic rate with increasing levels of enzyme application. Higher gas production indicates higher feed fermentability. There is a significant and positive correlation between gas production and *in vitro* fermentation. Menke *et al.* (1979) reported similar findings on gas production from different feeds incubated under *in vitro* study.In agreement with this result, many authors noticed an increase in fibre fermentation of diets or feedstuffs with enzyme supplementation; Eun and Beauchemin (2007) from alfalfa hay under *in vitro*; Giraldo *et al.* (2008) from mixed grass hay: concentrate (70:30) fed to sheep and Pinos- Rodríguez *et al.* (2008) form different forage to concentrate ration fed to lambs.

The increased production of VFA and DM disappearance observed might have increased the flow of microbial-N and microbial colonization of the substrate, resulting in enhanced fibre fermentation. Similar to this finding, increased total VFA and acetate concentration was reported by Ranilla *et al.* (2008) from lucerne hay, barley straw, and isolated NDF cell walls with Fibrozyme. Pinos- Rodríguez *et al.* (2002) also reported an increased total VFA from lucerne and rye grass-based diet with Fibrozyme. Increased total VFA, acetate and propionate were also reported by Giraldo *et al.* (2008) from different proportions of forage in the diet and grass hay by addition of fibrolytic enzyme from *Trichoderma viride, Aspergillus niger* and *Trichoderma longibrachiatum.* Increased acetate, butyrate and methane production was also reported by Giraldo *et al.* (2007) from grass hay and diet with two different proportions

of concentrate treated with mixed fibrolytic enzymes from *Trichoderma longibrachiatum* and fumarate. On the other hand, a decreased acetate and increased proportion of propionate was reported by Krueger and Adesogan (2008) from Bahiagrass hay with the addition of cellulase and xylanase when combined with ferulic acid esterase.

The decrease in NH_3-N concentration for *Eragrostis curvula* and maize stover might be due to a low level of N compared with higher N in the TMR diet, which has substantially improved the fermentation of fibre. Enzymes resulted in subtle changes to the cell wall structure and facilitated microbial access to the cell contents; as a result the N located in these structures might have been exposed to microbial attach and resulted in better degradation of nitrogen (Colombatto *et al.,* 2003). The improvement of protein degradation by enzymes has also been reported by various authors: Colombatto *et al.* (2003) from lucerne hay under *in vitro*; Pinos-Rodriguez *et al.* (2002) from sheep fed on lucerne and rye grass and Pinos-Rodriguez *et al.* (2008) form different forage to concentrate ration fed to lambs.

This study showed that cellulase and xylanase enzymes could improve fibre fermentation and the rate of fermentation of these feeds. However, the mechanism of this improvement is not clearly known. The improvement in the attachment of micro-organisms to the plant cell wall (Nsereko *et al.,* 2000; Wang *et al.,* 2001), an alteration in the cell wall structure due to the enzyme effects (Giraldo *et al.,* 2008), coupled with the increased colonization, which would have shortened the lag time, could all be possible reasons for the observed improvement. When enzymes act on the structures of plant cell walls, the microbes will easily access to the potentially fermentable cell wall (Sutton *et al.,* 2003; Elwakeel *et al.,* 2007). In addition, the 24 hr pre-incubation of feed sample with enzymes in this study might have enhanced the attachment of enzymes to the cell wall component and improved fermentation of the feeds. The positive effect of pre-feeding treatment was elaborated by many researchers due to the enzyme-substrate pre-incubation interaction period (Elwakeel *et al.,* 2007; Krueger & Adesogan, 2008; Alvarez *et al.,* 2009).

5.5.3. Change in CH_4 production associated with level of enzyme application

The observed linear increase in volume of methane production with increasing level of enzymes application might be partly explained by an observed increase in fermentation of OM associated with a higher VFA production, but shifted more towards acetate production.

The increase in acetate or acetate: propionate formation with an increase in the level of enzyme application resulted in the formation of more H_2, which could be utilized by methanogens to produce methane. The shift in the pattern of VFA seems to be influenced by the type of diet and enzyme preparations (Wang *et al.,* 2001; Giraldo *et al.,* 2008). The finding in this study agrees with a number of researchers who found an increased VFA production: Arriola *et al.* (2011) from lactating cows fed low to high concentrate diets; Giraldo *et al.* (2007) from mixed grass hay: concentrate (70:30) *in vitro*; and Gado *et al.* (2009) from dairy cows fed on TMR (70% forage). The increased VFA was associated with increase in methane production due to fibrolytic enzymes and their mixtures and was reported by Geraldo *et al.* (2007). Chung *et al.* (2012) also reported that increasing the dosage of enzyme supplementation linearly increased enteric CH_4 production of dairy cows fed a mixed ration at low, medium and high enzyme application levels when compared with a control. On the other hand, McGinn *et al.* (2004) found no effect of the enzyme on fibre fermentation and methane production in steers fed a barley-based silage diet, whereas, Beauchemin *et al.* (1999) and Yang *et al.* (1999) reported no effect of fibrolytic enzymes on rumen fermentation. In contrast to this finding, a decrease in acetate: propionate ratio in the rumen fluid was reported by Arriola *et al.* (2011) in lactating cows fed low to high concentrate diets. The reported variations by researchers might be due to types of microbial sources for enzymes and their preparations, types of substrates evaluated and their methods of application.

Generally, a higher volume of methane was produced relatively from hay and maize stover substrate compared with TMR (comparison not indicated). This might be due to the associated high levels of cell wall components (ADF, NDF, hemicellulose and cellulose) and lower CP and ME in the roughage substrate. In this study, methane production was positively correlated with the fibre component, while it was negatively correlated with N and EE parameters (Table 5.12). This is mainly because fermentability of feed to its end products is determined primarily by digestibility, which depends mainly on its composition (McDonald *et al.,* 2011). For example, VFA concentration and their relative proportion that mainly influence methane production are affected by the nature of carbohydrate (Getachew *et al.,* 2008). According to Eun *et al.* (2004), the amount of fermentable carbohydrate and levels of fibre in the diet are major drivers of methane production in the rumen. However, the variations in amount of methane production with enzyme application might be influenced by the types and sources of enzymes, diet under consideration, pH considered, and rumen

microbial population (Wang *et al,* 2002; Giraldo *et al.,* 2008, Jalilvand *et al.,* 2008; Krueger *et al.,* 2008). This is because the feeds fermented with enzymes determine the amount and proportion of VFAs and level of methane production. It is therefore important to consider the ratio of methane to OM and NDF fermented or digested to compare the effects of different enzymes and their levels of application in reducing or increasing methane production.

The increase in methane production with enzyme addition might also be related to changes in rumen microbial populations, as the addition of exogenous fibrolytic enzymes may cause a shift in the type of VFA production, especially an increase in the acetate proportion. Unfortunately, the rumen microbial population change was not assessed in this study , but other studies have shown an increase or a shift in the methanogen population (Zhou *et al.,* 2011) and increased number of cellulolytic bacteria (Wang *et al.,* 2001; Giraldo *et al.,* 2007; Giraldo *et al.,* 2008) for ruminants supplemented with exogenous fibrolytic enzymes. Although supplementation of exogenous fibrolytic enzymes are reported to cause a shift in the molar proportion of VFA, the shifts in pattern of VFA seemed to be influenced by the type of diet and enzyme preparations (Wang *et al.,* 2001; Giraldo *et al.,* 2008) suggesting the need to include additive diets that may play a complementary role by serving as a hydrogen sink.

5.6. Conclusion

The enzymes activity profile in this study indicated that high activity was recorded at pH of 4.8, while it declined as pH increases for both enzymes. Enzyme product such as EFE should better perform under conditions closer to that of the rumen environment to be effectively used as a potential product for ruminants. Thus, to get similar response at rumen pH condition of ruminant animals' higher application rate than the manufacturer recommendation should be applied. For example, cellulase and xylanase used in this study were recommended at 0.5 mg g^{-1} DM and 0.25 mg g^{-1} DM of feed, respectively. However, at this application level their effect was not significant compared with the control sample. This is because in most cases EFE are not produced with the intention of feeding ruminants. The *in vitro* gas production in this study indicated that cellulase and xylanase enzymes have a marked effect on total gas production and rate of gas production of tested feeds. Moreover, the increase in application of levels of cellulase and xylanase increased gas production with linear and quadratic functions.

The gas production results were also supported by the observed increase *in vitro* DM and NDF disappearance of tested feeds with increased volatile fatty acid production. The increased production of VFA and DM disappearance observed might have increased the flow of microbial-N and microbial colonization of the substrate, resulting in enhanced fibre fermentation. Although their cumulative effect increases with increasing level of application, their efficiencies decrease above 1 mg enzyme g^{-1} DM application level. In addition the associated cost related to higher enzyme application rate needs be considered to justify the return. Thus taking into consideration the efficiency of application different rates studied, the pre-treatment of these low-quality forages with cellulase and xylanase at 1 mg g^{-1} DM resulted in better *in vitro* ruminal fermentation and disappearance of NDF. This study also found out that the addition of fibrolytic enzyme increased methane production. The increase of methane production was due mainly to a shift in VFA profiles, which favoured acetate. Since the increased fermentation of these feed resulted in increased H^+ due to acetate shifted fermentation it will be very important to consider other hydrogen sinks or additives that have some complementary role to directly capture H^+ produced so that addition of enzymes could be very efficient, and one may achieve better fermentation effect with reduced methane emission. It is important to verify whether the recommendedrate of 1 mg of enzyme per g of these feeds would provide similar responses under *in vivo* trials.

CHAPTER 6

Rumen fermentation and methane production of Merino rams supplemented with a fibrolytic enzyme mixture and nitrate

6.1 Abstract

This study investigated the effect of exogenous fibrolytic enzymes and nitrate as feed additives on rumen fermentation and methane production of Merino rams. A 4 by 4 latin square design was used, which consisted of a control diet total mixed ration (TMR), TMR plus fibrolytic enzyme supplement, TMR plus a nitrate supplement and TMR plus a fibrolytic enzyme and a nitrate supplement. The fibrolytic enzyme contained an equal amount of cellulase and xylanase, while calcium nitrate was used as a source of nitrate. Each experimental period lasted for 30 days, with 19 days of adaptation, 7 days of data collection on intake, digestibility and rumen fermentation in metabolic crates and 4 days of methane production measurement in an open-circuit respiration chamber. There was a significant interaction effect between enzyme and nitrate additives in terms of feed intake and apparent digestibility of nutrients, ruminal NH_3-N concentration, nitrogen retention and daily methane production of rams. No significant interaction effect was noted between enzyme and nitrate additives in terms of ruminal pH and total volatile fatty acid (TVFA) concentration. Simultaneous addition of nitrate and enzyme increased feed intake, digestibility of nutrients, NH_3-N concentration and nitrogen balance, but reduced ruminal pH, nitrogen loss through faeces and urine and daily methane production. However, enzyme alone reduced methane production per unit of dry matter intake and organic matter digested. Supplementation of enzyme with nitrate reduced methane emission compared withcontrol or enzyme alone diets. If one opts for improving fibre fermentation and methane reduction simultaneously, the combination of these two additives can be an option. However, the amount of nitrate mixed with enzyme needs further study.

Key words: digestibility, fermentation, methane, nitrate, N-retention

6.2. Introduction

Tropical and sub-tropical forages are generally characterised by high proportion of poorly digested structural carbohydrates. In addition, they often have a low protein content that further limits digestibility (McDonald *et al.,* 2011). The imbalance of energy and nitrogen supply affects rumen fermentation and increases enteric methane (CH_4) production (McDonald *et al.,* 2011). Several studies indicated that the incubation of exogenous enzymes with fibrous feeds resulted in an increased degradation of cell wall components (Beauchemin *al.,* 2003; Morgavi *et al.,* 2000). The addition of cellulase and xylanase to high concentrate diet improved the fermentation of OM and fibre and increased total volatile fatty acid production of dairy cows (Arriola *et al.,* 2011). These authors also found that among volatile fatty acids, the proportion of acetate was particularly increased. Acetate production liberates hydrogen that is readily available for CH_4 production (Demeyer 1991, Janssen *et al*., 2010). Thus, the addition of exogenous enzymes could improve the digestibility of forages in the tropics. However, they could also increase enteric methane emissions.

Under acetate-dominated rumen fermentation, reducing the concentration of hydrogen in the rumen is an efficient way of decreasing CH_4 production (Gerber *et al.,* 2013). In this regard, the use of fumarate, nitrate, sulphate, and nitro-ethane as electron (H^+) acceptors in the hydrogen pathway, is a potential CH_4 mitigation strategy (Leng, 2008). Nitrates in particular are attractive in tropical and sub-tropical conditions where forages have low nitrogen content and negligible levels of nitrate (Gerber *et al.,* 2013). In these systems, supplementary nitrate can act as a source of non-protein nitrogen for the synthesis of microbial protein and, at the same time, provide an alternative sink for hydrogen. Nitrate is an effective inhibitor of methanogenesis in the rumen (Van Zijderveld *et al*., 2010). This is partly due to a toxic effect of nitrate and nitrite on methanogens (Iwamoto *et al*., 2002; Van Zijderveld *et al*., 2010) and also to the conversion of nitrate to NH_3 which traps up to eight electrons in the process and out-competes methanogens for electrons (Leng, 2008; Leng & Preston, 2010). In the process of nitrate reduction, energy is conserved for microbial use instead of being lost as CH_4 (Leng, 2008). It has been reported that nitrate reduced enteric CH_4 production by up to 50% in sheep (Nolan *et al.,* 2010; Van Zijderveld *et al.,* 2010) and in cattle (Van Zijderveld *et al.,* 2011; Hulshof *et al.,* 2012).

Combining exogenous enzymes with nitrate in supplements could promote forage digestibility and CH_4 reduction. Under tropical and sub-tropical conditions, there is limited information about the use of nitrate supplementation or combination of enzymes with nitrate to improve fibre fermentation without increasing CH_4 production per unit of digested organic matter. This study investigated the effect of supplementation of exogenous enzymes and nitrate additive fed to Merino rams on rumen fermentation, feed digestibility and enteric methane production.

6.3. Materials and methods

6.3.1. Experimental location and ethic approval

This study was conducted at University of Pretoria's Experimental Farm between July and October 2014 after the approval of the trial protocol by the Animal Ethics Committee of University of Pretoria (No. EC086-12).

6.3.2. Experimental diets

Total mixed rations (TMR) were formulated as indicated in Table 6.1. The experimental rations were formulated to meet the maintenance requirement of 60 kg adult ram according to the recommendations of the National Research Council (2001). The diets were formulated to be iso-nitrogenous and iso-energetic. To facilitate intake and to limit selection, all feed were uniformly milled after mixing all ingredients.

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Table 6.1 Ingredient composition (g kg^{-1} DM) of the experimental diets

Feed ingredients	Experimental treatments (g kg^{-1} DM)			
	Control	Enzyme	Nitrate	Enzyme+ nitrate
Lucerne hay	200	200	200	200
Eragrostiscurvula hay	500	500	500	500
Hominy chop	150	150	139	139
Wheat bran	50	50	20	20
Maize meal	50	50	55	55
Urea	10	10	1.4	1.4
Molasses	30	30	50	50
Calcium nitrate	0	0	35	35
Salt	5	5	0	0
Lime stone	5	5	0	0
Enzyme (cellulose & xylanase)	0	1	0	1

DM: dry matter

A 2 by 2 factorial design experiment was set up, which consisted of a control (TMR without enzyme or nitrate), TMR plus enzyme (1 g enzyme per kg DM of TMR), TMR plus nitrate (inclusion of 3.5 % nitrate in a TMR), and TMR plus enzyme and nitrate (inclusion of 3.5% calcium nitrate and 1 g enzyme per kg DM of TMR). The enzyme used was made up of a mixture of cellulase and xylanase at a ratio of 1:1. The amount of enzyme, nitrate and enzyme-nitrate mixture needed in the daily ration was first completely dissolved in water. Thereafter, the solutions were mixed to the diets and incubated for 24 hr before feeding to the rams.

6.3.3. Experimental design, procedures and description of chamber

Four Merino rams with an initial bodyweight (BW) of 60 (±3) kg were allocated to the four dietary treatments in a 4 x 4 Latin square design. Each experimental period lasted for 30 days, with 19 days of adaptation, 7 days of data collection on intake, digestibility and rumen fermentation in metabolic crates and 4 days of methane production measurement in open-circuit respiration chambers. The detailed methods of this section were described in Chapter 4 section 3.3.

6.3.4 Laboratory analyses

Feeds offered and refusals and faeces were dried at 55°C for 48 hr and ground to pass a 1-mm screen for chemical analyses. The details of samples collections andanalysis for DM, total ash, EE, NDF, ADF, ADL and CPwere done with similar methods as detailed in Chapter 2, section 3.2. Samples of rumen fluid were thawed and centrifuged; the supernatants were immediately analysed for ammonia N (McDonald et al., 1960) and VFA (Ottenstein & Bartley, 1971).

6.3.5 Methane measuring

Methane measures were performed as described in Chapter 4 section3.5.

6.3.6. Calculations, statistical analysis and model

A 4 by 4 latin square design was used in this experiment. Data were analysed with the generalized linear model (GLM) procedures of SAS (version 9.1.3; 2009; SAS, Cary, NC, USA) with diet, animal and experimental periods as sources of variation. Source of variation owing to the dietary treatment was further partitioned into effect due to enzyme and nitrate and interaction between enzyme and nitrate (t_i; $t_e + t_n + t_e x\ t_n$). The following model was used:

$Yijk = \mu + t_i + a_j + p_k + e_{ijk}$, where

t_i is the effect of i^{th} treatment (diets)

a_j is the effect of j^{th}animal

p_k is the effect of k^{th}period

e_{ijk} is the associated random error.

All multiple comparisons among means were done with Tukey's test.

6.4. Results

The chemical compositions of the diets used in the experiment are shown in Table 6.2. The formulated diets did not differ significantly ($p \geq 0.05$) in terms of organic matter composition (OM), crude protein (CP) and fibre (NDF and ADF) concentrations.

Table 6.2 Chemical composition (g kg^{-1} DM) of the experimental diets

Composition	Experimental treatments			
	T_1	T_2	T_3	T_4
OM	748	748	754	754
CP	113	113	112	112
EE	37.7	37.7	36.2	36.2
NDF	433	433	430	430
ADF	279	279	275	275
ADL	46.0	46.0	45.4	45.4
Hemi-cellulose	154	154	145	145
Cellulose	231	231	227	227

DM: dry matter; OM: organic matter; EE: ether extract; CP: crude protein; ADF: acid detergent fibre; NDF: neutral detergent fibre; ADL: acid detergent lignin

The intake and digestibility of OM, CP, NDF and ADF by Merino rams fed on diets containing enzyme and/or nitrate additives are summarized in Table 6.3. There was an interaction effect between enzyme and nitrate additives for feed intake and digestibility of OM, CP, NDF and ADF ($p<0.05$). In the absence of nitrate, the addition of fibrolytic enzymes to the diets significantly ($p<0.05$) increased their daily DM and OM intake, but the observed improvement in intake due to enzyme addition was relatively reduced when nitrate was simultaneously added to the enzymes. A similar trend was noted for daily feed intake expressed per unit of metabolic bodyweight. Regardless of the nitrate additive the digestibility of OM, NDF and ADF significantly ($p<0.05$) increased with addition of enzymes. Similarly, regardless of enzyme additive, nitrate had increased the digestibility of OM, NDF and ADF. In the absence of enzymes, the addition of nitrate had no effect on crude protein digestibility.

Table 6.3 Intake and digestibility (mean ±SE) of diets fed to Merino rams with the inclusion of enzymes, nitrate and an enzyme-nitrate mixture

	Experimental treatments				Contrast		
	Control	Enzyme	Nitrate	Nitrate + Enzyme	Enzyme	Nitrate	Nitrate * Enzyme
BW (kg)	63.1	66.7	65.6	67.8	*	ns	**
DM intake (g day^{-1})	1743	2200	1751	2125	*	ns	**
OM intake (g day^{-1})	1441	1816	1443	1740	**	ns	**
Intake per $BW^{0.75}$ (g kg^{-1} day^{-1})							
DM	78.2	94.4	80.4	90.2	**	*	**
OM	64.6	78.0	66.9	74.0	**	*	**
Digestibility (g kg^{-1} intake)							
OM	600	738	650	750	**	*	**
CP	490	510	490	514	**	ns	**
NDF	480	498	486	495	**	*	**
ADF	391	402	395	409	**	*	**

*significantly different ($p< 0.05$); ** significantly different ($p< 0.01$); ns, non-significant
BW: bodyweight; $BW^{0.75}$: metabolic bodyweight

The effects of the addition of enzymes nitrate and their combination to the diets of rams on ruminal pH, NH_3-N and total and individual VFA are presented in Table 6.4. No interaction effect was noted between enzymes and nitrate in terms of their effects on ruminal pH, NH_3-N, total VFA, and propionic acid. The addition of enzymes reduced ($p<0.05$) ruminal pH whereas nitrate had no effect ($p\geq0.05$).

Table 6.4 Ruminal pH, ammonia-N (mg 100 ml^{-1}), total and individual VFA concentrations (mmol L^{-1}) of Merino rams fed diets treated with enzymes, nitrateandanenzyme-nitrate mixture

	Experimental treatments				Contrast		
	Control	Enzyme	Nitrate	Nitrate + Enzyme	Enzyme	Nitrate	Nitrate * Enzyme
Rumen pH	6.63	6.51	6.61	6.49	**	ns	**
Rumen NH_3-N	6.00	6.15	6.12	6.20	*	*	**
Total VFA	96.0	108.5	103.3	120.4	**	*	**
Acetic acid	50.3	62.1	60.8	71.5	**	**	**
Propionic acid	30.6	31.1	30.9	32.5	ns	ns	*
Isobutyric acid	4.35	4.43	4.28	4.65	*	*	*
Butyric acid	4.89	4.55	4.29	4.59	**	**	*
Valeric acid	5.86	6.33	3.1	7.15	*	**	**
A : P ratio	1.64	2.00	1.98	2.20	*	*	*

*significantly different ($p< 0.05$); ** significantly different ($p< 0.01$); ns: non-significant

Supplementation of enzyme or nitrate additives alone increased ($p<0.05$) total VFA and propionic acid production. However, an interaction effect was observed between enzyme and nitrate additives for ruminal pH, NH_3-N concentration and proportions of acetic acid, isobutyric acid, butyric acid, valeric acid and A : P ratio. Addition of enzymes and nitrate increased rumen NH_3-N ($p<0.05$), but the highest response for enzyme additives was revealed in the absence of nitrate and vice versa. An increase in the concentration of acetic acid and A : P ratio was recorded due to enzyme as well as nitrate additive ($p<0.05$). However, the highest increase in acetate production associated with enzyme application was revealed in the presence of nitrate. Similarly, the highest response associated with nitrate was revealed in the presence of enzyme supplementation.

The nitrogen balance of rams fed on nitrate, enzymes and their mixture is indicated in Table 6.5. Enzyme and nitrate treatments increased N intake of rams ($p<0.05$). There was also a positive interaction between enzyme and nitrate additives in terms of daily nitrogen intake of rams ($p<0.05$).

Table 6.5 Nitrogen balance in Merino rams fed a total mixed ration supplemented with enzymes, nitrate and an enzyme-nitrate mixture

	Experimental treatments				Contrast		
	Control	Enzyme	Nitrate	Nitrate + Enzyme	Enzyme	Nitrate	Nitrate * Enzyme
Intake	31.5	39.7	32.8	38.4	**	*	*
Faeces	10.2	9.9	9.8	9.6	**	*	**
Urine	10.3	10.1	10.1	9.6	ns	ns	**
Balance	11.0	19.6	12.9	19.1	**	**	**

*significantly different ($p< 0.05$); ** significantly different ($p< 0.01$); ns: non-significant

Similarly, a significant ($p<0.05$) interaction was observed between enzyme and nitrate additives in terms of daily N loss through faeces and urine, and N balance. The addition of enzymes or nitrate significantly ($p<0.05$) increased the daily nitrogen balance of rams, but the highest value was obtained for enzyme additives in the absence of nitrate. Concomitantly, both enzyme and nitrate significantly ($p<0.01$) reduced daily nitrogen loss through faeces and urine, but the lowest loss was recorded for enzyme additives in the presence of nitrate.

The enteric CH_4 production of rams fed on diets treated with different additives is shown in Table 6.6. A significant ($p<0.05$) interaction effect between enzymes and nitrate additives was observed in terms of daily CH_4 production, daily energy loss through CH_4, CH_4 production per metabolic BW ($BW^{0.75}$), CH_4 production per DM intake, CH_4 production per OM digested, and CH_4 produced per digested NDF. Daily CH_4 production and energy loss through CH_4 were significantly ($P<0.05$) reduced when rams were fed on diets treated with a nitrate additive. In contrast, enzyme application increased the daily CH_4 production and daily energy loss as CH_4 in rams. In the absence of enzymes, nitrate was more effective in reducing daily CH_4 production, while a relatively higher amount of daily CH_4 was produced due to enzyme additives in the presence of nitrate. This means in the absence of nitrate, enzymes significantly ($p<0.05$) increased daily CH_4 production of the rams. On the contrary, rams that received a diet treated with nitrate, in the absence of enzyme, emitted the lowest amount of CH_4 and lost the least amount of energy. Daily CH_4 emission was reduced due to nitrate additive by 24 and 44% for diets treated with and without enzymes, respectively. Likewise, the daily energy loss of sheep as CH_4 was reduced by 27.9 and 45.8 % due to inclusion of enzyme for nitrate supplemented and non–supplemented group, respectively. No interaction effect was observed between enzyme and nitrate in terms of CH_4 produced per total VFA.

Table 6.6 Methane productions of Merino rams fed total mixed ration treated with the inclusion of enzyme, nitrate and enzyme-nitrate mixture

	Experimental treatments				Contrast		
	Control	Enzyme	Nitrate	Nitrate + Enzyme	Enzyme	Nitrate	Nitrate * Enzyme
l head^{-1} day^{-1}	24.1	27.2	13.5	18.3	**	**	**
l kg^{-1} BW$^{0.75}$	1.08	1.16	0.58	0.78	*	**	*
kJ /kg^{-1} BW$^{0.75}$	43.0	46.1	23.3	31.0	**	**	**
l kg^{-1} DM intake	13.8	12.4	7.70	8.66	*	**	**
l kg^{-1} OM digested	16.7	15.0	9.30	10.5	**	**	**
l kg^{-1}NDF digested	50.2	54.4	27.7	37.2	**	**	**
l total VFA^{-1}	0.25	0.25	0.13	0.15	ns	**	**

*significantly different ($p< 0.05$); ** significantly different ($p< 0.01$); ns: non-significant
BW: bodyweight; BW$^{0.75}$: metabolic bodyweight

Nitrate generally reduced ($p<0.05$) CH_4 produced per unit total VFA production, but enzyme application increased CH_4 produced per unit total VFA.

6.5. Discussion

6.5.1. Effect of enzyme addition

Addition of fibrolytic enzymes in diets of sheep improved feed intake and digestibility of OM and fibre. The greater feed intake might be the result of a higher rate of passage of forages that were rendered more easily fermentable by the enzyme treatment. According to Elwakeel *et al.* (2007), pre-incubation of feed for 24 hr with fibrolytic enzymes might alter fibre structure. This improves microbial access to the cell wall due to enhanced attachment of microbes to the cell wall component (Giraldo *et al.,* 2008). As a result, more energy and N were available to rumen microbes owing to increased digestion of carbohydrates and crude protein by enzyme addition (Elwakeel *et al.,* 2007; Giraldo *et al.,* 2008). In turn, this is expected to increase the flow of microbial-N (Kung *et al.,* 2000). Similar to our results, improved intake and digestibility were reported in lambs fed a diet supplemented with a preparation containing mainly xylanase activity (Cruywagen & Van Zyl, 2004, 2008; Pinos-Rodriguez *et al.,* 2002, 2008).

In this study, the addition of enzymes increased the production of enteric methane. This is certainly due to the increased fermentation and digestion of enzyme-treated rations that resulted in an increased VFA and acetate concentration. The synthesis of acetate increases the H^+ pool in the rumen that in turn is used by hydrogenotrophic methanogens to produce methane. This finding agrees with those of a number of researchers who found an increase in VFA production from dairy cows fed low to high concentrate diets (Arriola *et al.,* 2011), and dairy cows fed on TMR (70% forage) (Gado *et al.,*(2009), due to the addition of enzymes, which in turn were associated with an increase in methane production. According to Chung *et al.* (2012), increasing the dosage of enzyme supplementation linearly increased enteric CH_4 production of dairy cows compared with the control. In contrast, McGinn *et al.* (2004) found no effect of the enzyme on fibre fermentation and methane production in steers fed a diet based on barley silage. Beauchemin *et al.* (1999) and Yang *et al.* (1999) also noted no effect of fibrolytic enzymes on rumen fermentation. In contrast to this finding, which showed an increase in the A : P ratio, Arriola *et al.* (2011) found a decrease in acetate to propionate ratio in the rumen fluid during inclusion of fibrolytic enzymes in feeds of lactating dairy cows. However, the shift in the pattern of VFA seems to be influenced by the type of diet and types of enzymes (Giraldo *et al.,* 2008).

6.5.2. Effect of nitrate addition

Addition of nitrate alone had no effect on intake or CP digestibility, but improved OM and fibre digestibility and N retention. The improvement in fibre digestibility and N retention might be associated with the supply of NPN from nitrate to the rumen microbes (Leng, 2008), which might have enhanced microbial growth and microbial protein synthesis and improved N balance and fibre fermentation. Gradual introduction of nitrate in the diet might enable a large number of microbial species capable of fermenting fibre to adapt to nitrate and eventually reduce nitrate via nitrite to NH_3-N, as noted by Cheng and Phillippe (1988). Leng (2008) also reported that in well-adapted animals fibrolytic microbes increase, facilitating fibre utilization. Similar to our findings, increases in fibre digestibility were reported when nitrate was supplemented to low CP feeds in goats fed a forage-based diet (Trinh Phuc Hao *et al.,* 2009), goats fed sugar cane (Ngoc Huyen Le Thi, 2010), and cattle fed on rice straw and cottonseed meal (Nguyen Ngoc Anh *et al.,* 2010). Similar to our findings on intake, Van Zijderveld *et al.* (2010) reported no effect in feed intake due to nitrate supplementation for lambs fed silage-based diets. The modest increase in OM digestibility observed in this study

was associated with an increase in the concentration of total VFA, confirming the findings by Nolan *et al.* (2010) who reported the same in sheep fed chaffed oaten hay, but with no effect on DM intake.

The decrease in methane production due to supplementation of nitrate might be because of the high-affinity of nitrate to accept H^+ during reduction of nitrate to ammonia-N. This process is energetically more favourable than the process of utilizing CO_2 for the formation of methane (Van Zijderveld *et al.,* 2010, Nolan *et al.,* 2010). A reduction in methane production as a result of inclusion of nitrate in diets of sheep was observed in other studies (Van Zijderveld *et al.,* 2010, Nolan *et al.,* 2010; Hulshof *et al.,* 2012). However, the results obtained in this study were comparatively lower than other findings, which might be due to the difference in nitrate and the methods of inclusion.

6.5.3. Interaction between enzyme and nitrate

In this study, mixing enzymes with nitrate resulted in an increase in feed digestion and methane production. In the presence of nitrate, intake and rumen VFA moderately increased due to added enzyme, but methane production increased. However, in the presence of enzymes the expected decrease in methane production due to added nitrate was relatively moderate (30%) compared with nitrate alone (44%). The observed reduction in the efficacy of nitrate for the enzyme-treated diet might be due to the lower amount of dissolved nitrate that perhaps was not sufficient to sink all H^+ that arise from the acetate-dominated fermentation, caused by the use of both additives. Thus, the un-trapped H^+ might have been utilized by methanogens to produce methane. This was evident with comparatively high amount acetate formed by the enzyme-nitrate mixture ration. This suggested that the amount of nitrate used was not be enough, as suggested by Diego *et al.* (2010), to effectively utilize nitrate in improving feed utilization and methane reduction. This needs further study regarding the levels of nitrate and enzyme addition.

6.6. Conclusion

The addition of exogenous enzymes improved feed intake, digestibility and nitrogen retention in adult rams fed at maintenance levels. Although the enzyme treatment improved inake and digestibility of feed, methane production was increased. If the same effect is observed in fast-growing or highly productive dairy animals, the amount of methane produced per unit of animal product might be lowered. This is because of increased feed utilization efficiency and subsequently the overall GHG released into the atmosphere might be lower for enzyme-supplemented animals. Combining enzymes with nitrate produced effect by further increasing the levels of intake, digestibility and associated methane production. The relative effect of enzyme supplementation, however, was moderate in terms of relative improvement in intake and digestibility, while the relative methane production is very high. If one opts for improving fibre fermentation and methane reduction simultaneously, the combination of additives can be a good option. However, this could be more appropriate for livestock production that depends on feeds that have lower nitrogen and high fibre content. Nitrate alone has slightly improved intake, as well as digestibility of OM and NDF, while the reduction in methane was consistently high. In the presence of enzyme, however, nitrate reduced slightly the digestibility of NDF and intake of OM, but increased the digestibility of OM. The effectiveness of nitrate is moderate when used together with enzyme additives, partly due to acetate-oriented fermentation associated with supplemented enzyme or nitrate additives. However, the level of nitrate combined with enzyme that can maximize net benefit interms of improving productivity and GHG reduction needs to be investigated.

CHAPTER 7

Conclusion, recommendations and critical evaluation

7.1. Conclusion and recommendations

In the tropical and sub-tropical ruminant production systems of Africa, low-quality feed associated with low digestibility and high enteric methane production is a major challenge for ruminant production. In such systems, supplementing ruminants with concentrate or good-quality legumes could improve rumen fermentation and reduce energy losses through methane. However, such practices are not usually applicable as ruminants receive small quantities of concentrates due to high costs and direct competition with human and other monogastric animals. Under such conditions, livestock productivity can be heightened through improving the fermentation of low-quality feeds and reducing losses of energy through enteric methane production. In this regard, characterization of available feed resources in terms of their chemical and methane production is essential to identify nutritious plant types or varieties with low methane production potential. Such screening work is useful in formulating a ration for ruminants based on age, physiological status and breed.

The study, which was conducted with sixteen perennial grasses from the Kalahari area of South Africa, showed that ruminal fermentation and methane production in perennial grasses varied between species. The study confirmed that most palatable perennial grasses produced relatively lower CH_4, which might indicate that better-quality grasses could reduce methane and might be used as a mitigation option. The negative correlation between methane production and nitrogen content in the grasses might indicate that selecting species for higher N content could help to mitigate methane production in tropical grassland. Thus, it is also possible to consider improving grasses through breeding and selection. By doing so, it is possible to replace existing low-quality species gradually with selected species. In extensive grassland, oversowing could also be an option. The management of grazing land is another important issue to be considered. If the grasses are grazed at an early stage when N concentration is high and fibre is relatively low, there is more chance of reducing methane produced. However, if the grazing land is used when the grasses are more fibrous, as in most

tropical and subtropical regions, it is important to supplement with energy and protein rich feeds or legumes. In addition, the application of fertilizer could also be used to improve the N content of the grasses if moisture is not the limiting factor. However, owing to the diversity of the system, more research would be needed in the Kalahari area to produce more firm and conclusive information.

The study with tannin-rich browses showed that some of the species contained a high amount of CP and low to moderate NDF concentration. These browses could possibly be used as a supplementary diet during drought, or can be included in a ration forruminants to increase the protein content and reduce methane production. From the studies, tanniferous browses *Morus alba* and *Melia azedarach* were the most fermentable and digestible, with low ADF, ADL, and phenolic compounds. They are promising for use alone or mixed with diets to supplement for nitrogen or used as additives. However, for browse species with substantial amounts of N and high tannin content, more studies should be done to determine the optimum inclusion level that could result in methane reduction without reducing intake, OM and fibre fermentation. Using this approach it could be possible to improve the N content, OM and fibre fermentation of low-quality roughages. In this regard, it is important to consider the level of inclusion in a diet. In this study, replacing lucerne hay with *Monechma genistifolium, Acacia nilotica* and *Grewia flava* in the diets of Merino rams decreased methane emission without negative effects on feed intake, organic matter fermentation and the production of total volatile fatty acids. The use of *Monechma genistifolium* at30% inclusion rate in total mixed rations was found to reduce methane emission without reducing feed intake and digestibility and with better N balance. Thus, this could be used as a suitable level of inclusion in sheep rations. However, the inclusion of *Acacia nilotica* and *Grewia flava* at 30% resulted in reduction of N balance, CP degradation and fibre fermentation. This implies lower levels have to be tested for better fibre fermentation and methane reduction in the diets of sheep.

Crop residues and hays are the dominant feed types in most farming systems in tropical and sub-tropical Africa. They contain huge amounts of cellulose and hemi-cellulose, which are the most abundant fibrous carbohydrates on the planet. They are not fully utilized due to their associated low fermentation. In addition, they produce more enteric methane during fermentation in the rumen. The *in vitro* study conducted with exogenous enzymes at different

doses by *Eragrostis curvula* hay, maize stover and a total mixed ration showed substantial improvement of organic matter and fibre fermentation. The increase in the dose for cellulase and xylanase enzymes linearly increased OM and fibre fermentations and consequently increased VFA, NH_3-N and CH_4 from *Eragrostis curvula* hay, maize stover and a total mixed ration. Thus, it would be possible to use fibre-fermenting enzymes to improve the utilization of fibrous feeds in tropical and sub-tropical African livestock production systems. Although it was found that the addition of fibrolytic enzymes increased feed fermentation and digestibility of these feeds, there was an associated increase in methane production. Considering the efficiency of feed fermentation, the net return per unit of additional enzyme level and associated enteric methane production, the use of 1 mg g^{-1} DM of each enzyme could be an optimum level to improve fibrous feed utilization in low-quality roughages. However, this study involved only cellulase and xylanase enzymes produced from certain strains of microbes and considered only three feed substrates, namely hay, crop residue and total mixed ration out of a range of feed resources in the tropical and sub-tropical livestock production systems.

The supplementation of enzymes is not expected to decrease methane production, since the increased fermentation resulted in increased H^+ due to acetate shifted VFA production. In this regard, the result obtained *in vitro* study was confirmed during feeding of enzyme-incubated diets for Merino rams. Therefore, it would be important to consider other hydrogen sinks or additives that have some complementary role to directly capture H^+ produced, so that the addition of enzymes could be efficient and reduce energy loss through methane. On the contrary, rams that received nitrate in their diets showed a reduced daily methane production without a change on intake and fibre fermentation. When enzymes were mixed with nitrate, methane was reduced with substantial improvement of OM and fibre fermentation. Thus, it is possible to use the enzyme-nitrate mixture, especially in tropical feeds that often are lowin nitrogen content. Proper adaptation of animals to nitrate to overcome nitrate toxicity is important. However, the amounts of nitrate and enzyme, if mixed together, should be further studied for each feed type and species of ruminants as different animal have different threshold levels to nitrate toxicity. By generating such information, it is possible to improve digestibility of fibrous feeds, while reducing enteric methane production.

7.2. Critical evaluation

It is important to consider some important issues that werenot included in this study, but would have improved the findings and conclusion of this work.

- Perennial grasses need more than one season to complete their life cycle and the erratic nature of rainfall in the area indicated the importance of a more seasonal approach of the study. In addition, a grazing system is composed of different kinds of annuals, legumes, forbs, trees and shrubs; thus, these feed resources should be studied for their fermentation and methane production. Based on such information and intake data it could be possible to develop a model for estimating methane emissions from the Kalahari Desert by different classes of grazing ruminant animals. Such information could indicate the way forward for methane mitigation strategies or improving the rangeland for lower methane emission.
- The results of fermentation of nineteen tanniferous browses are documented in this study. For these with high tannin levels, their sole supplementation can reduce intake, fibre fermentation and ultimately their efficiency of utilization as ruminant feed. The best option for better utilization of high tannin-containing browses might be their extraction and inclusion in ruminant diets, and this needs more research.
- During study of species of plants for their tannin effects, the leaf samples from different plants of the same species in the same location should be made as a replicate. Moreover, the samples should be collected from different locations and comparison for variation within species across location has to be made. This can indicate if there are variations of tannin within species across different locations that can be attributed to climatic, edaphic and other factors.
- Tannic acid was used as a standard during quantification of 19 browse plants. However, tannins vary in structure, even within the same species. This implies that using tannic acid as a standard for all browses in this study might not resulted in the true potential quantity of tannins. Therefore, it is often suggested that each tannin source needs a separate standard with similar molecular structure and the best method to quantify might be the molecular method if it can be applied. This needs further investigation.
- To obtain the clear effect of tannin on feed fermentation and methane production under *in vitro* study the donor animals should be fed tanning-containing diets. If

rumen donor animals feed on tannin-free feeds, rumen microbes might be adversely affected and this might distort the true effect of tannins during the introduction of tannin-containing samples under *in vitro* fermentation study.

- The inclusion level of the tannin binding agent polyethylene glycol (PEG) with feed substrate needs further research. It should be included with feed samples based on tannin concentration in the incubated feeds samples. In some of the browses in this study, the inclusion of ration of 1:1 might not be sufficient to completely bindavailable tannin, especially for high tannin-containing browses. This is because in some cases tannins might form a complex matrix, making it difficult to assess the true effect of tannins in these browses when comparing incubations with and without PEG addition.
- The study of levels of inclusion of selected tannin-containing browses to roughage feeds or total mixed ration needs further research,because the level of inclusion to TMR or roughages feeds that give better supplementation value and reduce methane without compromising feed fermentation had to be identified under *in vitro* study before proceeding to *in vivo* studies.For example, the inclusion of tannins containing browses at 30% in the ration of rams did not result in better performance for all browses. Although methane was reduced with *Acacia nilotica* and *Grewia flava*, fibre and CP digestibility was reduced. This indicated that lower levels of *Acacia nilotica* and *Grewia flava* might lead to better utilization efficiency by reducing methane. Therefore, it should be mixed with roughage feeds or included in total mixed diets at appropriate levels. Such levels of inclusion should be studied under *in vitro* incubations and the best level selected and further validated by *in vivo*studies. This might be better achieved if *in vitro* inclusion-level study was conducted.
- The sustainability of tannins as a methane-mitigating additive depends on theirlong-term effect so such studies need further investigation.
- Enzyme assay is better known by quantifying their protein density. It is often suggested that screening enzymes based on protein density and enzyme activities can better indicate their potential in digesting feed. However, only two enzymes were included in this study and protein density was not performed.
- The supplementation of nitrate seems promising, as proven by this study; however, only one level of inclusion was tested. It may be important to study the level of inclusion of nitrate, especially in relation to the level of CP content of feeds.

- Tannins, enzymes, nitrates and enzyme-nitrate mixtures showed variable effect on ruminal fermentation and enteric methane production. In this work, the primary targets were the changes of rumen fermentation and associated methane production, while trials were not conducted on their effect on methanogenic archaea, protozoa, bacteria, fungi and protozoa, populations. On the other hand, recent molecular-based analyses of the rumen microbial community structure provided evidence that changes in rumen fermentation are not always accompanied by the changes in the target rumen microbial populations and vice versa. Thus, knowledge of methanogens and other microbes present in the rumen and their dynamism because of different supplementation strategies of host animals could enable a more targeted manipulation of the rumen system. In this study, there is no such information on the association of rumen fermentation of different dietary treatments and change in methanogenic archaea population. Therefore, such information needs further investigation.
- In describing the result of gas production, the model was not the one that best fits gas production curves. There are other models that could fit better to the curves for this result.

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