The use of condensed tannins and nitrate to reduce enteric methane emission and enhance utilization of high forage diets in sheep

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DOCTOR OF PHILOSOPHY

Animal Production Management

by

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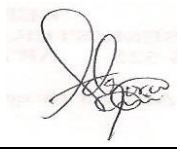
YUNIBESITHI YA PRETORIA

For every house is builded by someone; but he that built all things is God

Holy Bible (Heb 3: 4)

Declaration

I, **Festus Adeyemi Adejoro**, declare that the thesis, which I hereby submit for the degree of Doctor of Philosophy in Animal Nutrition and Management at the University of Pretoria, is my own work and has not been previously submitted by me or anybody for any degree at this or any other Institution.

Signature: 

Date: 5th February, 2019

Dedication

To:

Jehovah El-Shaddai, the all-sufficient God, to whom I owe all that I have been, and all that I would ever be.

To the memories of my grandmother, Mrs Felicia Ajayi Adebiyi; my uncle, Roland Akinola Adebiyi and my cousin, Adewale Adebiyi, who all departed the world in this course of this programme. May their souls rest in peace. Amen.

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When books are opened, we discover that we have wings.

— Helen Hayes

Preface

This thesis is based on the work conducted by me in the Department of Animal and Wildlife Sciences, University of Pretoria, South Africa under the supervision of Prof. Abubeker Hassen. The study has been divided into chapters, which are written as separate manuscripts published, submitted or ready for submission for publication in peer-reviewed academic journals.

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List of Abbreviations

µmol: micro-mole
a: immediate fermentable fraction
a: soluble fraction of fibre
ADF: acid detergent fibre
ADG: average daily gain
ADL: acid detergent lignin
ADL: Acid detergent lignin
AEC: Animal Ethics committee
ANOVA: analysis of variance
AOAC: Association of Official Analytical Chemists
ATE: Acacia tannin extract
b: slowly fermentable fraction
b: the insoluble, but slowly fermentable fraction of fibre
BUN: Blood urea nitrogen
$BW^{0.75}$: metabolic body weight
$BW^{0.75}$: metabolic bodyweight
c: rate of fermentation of b
Ca: calcium
CH_4: methane
CO_2: carbon dioxide
CP: crude protein
CT: condensed tannins
DM: dry matter
DMI: dry matter intake
DMI: dry matter intake
EDTA: Ethylenediaminetetraacetic acid
FCR: feed conversion ratio
g: gram
GC: Gas chromatography
Gg: giga gram
GHG: greenhouse gas
GLM: general linear model
GP: gas production
H_2: Hydrogen
H_2SO_4: sulphuric acid
Hb: Heamoglobin
IVDDM: *in vitro* digestible dry matter
IVOMD: *in vitro* organic matter digestibility
K: potassium
kg: kilogram
ME: metabolizable energy
Met-Hb: Methemoglobin

mg: milligram
MJ: mega-Joule
mL: milli-Liter
mm: milli-meter
Mmol: milli mole
MW: molecular weight
N: nitrogen
NDF: neutral detergent fibre
NDIN: neutral detergent insoluble nitrogen
NH_3-N: ammonia nitrogen
NPN: non-protein nitrogen
NRC: National Research Council
^oC: degrees celsius
OM: organic matter
pH: potency of Hydrogen
RCBD: randomised complete block design
S/O/W: Solid-in-oil-water
SAS: Statistical Analyses Software
SD: standard deviation
SEM: standard error of the mean
TMR: total mixed ration
TP: total phenols
TT: total tannins
TVFA: Total volatile fatty acid
VFA: volatile fatty acid

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By

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Degree: PhD in Animal Production Management

ABSTRACT

Methane emission in ruminant production is not only a global greenhouse gas, but also a loss of feed energy. Therefore, there is huge interest in identifying mitigation strategies that reduce ruminant derived methane, which guarantees sustainable ruminant production. Tannins have been reported to inhibit methanogenic activity, but may also limit feed intake and nutrient digestibility. While dietary nitrate has been noted as an efficient hydrogen sink in the rumen, its effectiveness may be dependent on the application method among others. The objectives of this study are: (i) to evaluate the effects of nitrate supplementation and nitrate treatment on the *in vitro* digestibility and methane production in Eragrostis hay; (ii) evaluate the effect of supplementing acacia tannin extract and nitrate on feed intake, nutrient digestibility, methane emission and health status of Merino lambs; (iii) prepare and evaluate an encapsulated acacia tannin extract formulation suitable for ruminants; (iv) evaluate the effect of encapsulated acacia tannin extract on feed intake, nutrient digestibility and enteric methane emission in Merino rams. A series of experiments (laboratory trials, and stall-feeding experiments) were conducted at the NUTRILAB and Hatfield Experimental Farm of the University of Pretoria, South Africa to evaluate the potential improvements in the use of nitrate and tannin extract using Sheep as model animal.

Treating *Eragrostis* hay with urea improved its *in vitro* organic matter digestibility better than treatment with nitrate. However, nitrate treatment significantly reduced enteric methane and improved digestibility compared with the control, this suggests that nitrate could be incorporated as a hydrolytic agent in treating poor-quality roughage feeds, with the aim of improving their utilization. The combination of urea or calcium nitrate with or without Acacia tannin extract as dietary supplements in a growth trial revealed that lambs receiving nitrate-

based TMR diets experienced superior growth performance compared to those on the urea-based TMR diets. Meanwhile, tannin inclusion did not improve growth or reduced methane emission from the lambs irrespective of the non-protein nitrogen source. A slight increase in haemoglobin, haematocrit and RBC count was associated with the use of calcium nitrate compared to urea. In this study, no clinical or subclinical signs of morbidity or tannin intoxication symptom was detected from the haematology and biochemical parameters evaluated in the Merino lambs.

The trial on the preparation and evaluation of an encapsulated Acacia tannin extract (ATE) showed that Gum Arabic-maltodextrin and native starch could only encapsulate the tannin extract at low inclusion levels while the *in vitro* release was not sustained. However, palm oil was found to be an effective wall material in encapsulating ATE using the double phase solid-in-oil-in-water encapsulation method where up to 80% w/w inclusion of tannin extract in the lipid wall material was achieved. This extract exhibited good morphological characteristics and high encapsulation efficiency even under high loading percentage. The lipid-encapsulated extract significantly reduced enteric methane production *in vitro*. Under *in vivo* evaluation with cannulated Merino rams, encapsulated Acacia tannin extract resulted in considerable reduction in methane per neutral detergent fibre intake, compared to the crude extract.

GENERAL INTRODUCTION

Livestock production and anthropogenic methane emissions

With a significant increase in human population which is expected to double from 2005 to 2050 (Steinfeld et al., 2006), the demand for increased production of meat and milk among other food sources are becoming increasingly high. Ruminants are capable of utilizing feeds with high fibre content, generally less nutritious and not easily digested by monogastrics to produce meat, milk and various fibre products. Thus, they occupy a significant ecological niche for meeting global demand for high quality and nutritious food (Bayat and Shingfield, 2012). However, ruminants have both local (nitrogen and phosphorus pollution) and global (greenhouse gas emission) footprints (Gerber et al., 2013; Pelletier et al., 2010). Greenhouse gas (GHG) emissions have risen worldwide since the beginning of industrialization as a result of human induced activities-anthropogenic factors (Vijaya Venkata Raman et al., 2012). Carbon dioxide (CO_2; 77%) is the major GHG, mainly from energy and land-use activities, followed by methane (CH_4; 14%) and nitrous oxide (N_2O; 8%) mainly from livestock production and waste management (Hristov et al., 2013; Montes et al., 2013a). Emissions from agriculture account for about 14-15% of global GHG, evenly shared between CH_4 and N_2O (45% each; rest CO_2) (Dong et al., 2006; Lassey et al., 1997). The largest agricultural source of GHG emissions are from soil management activities, mainly tillage and cropping practices (Staerfl et al., 2010) while the second largest source is livestock husbandry, where CH_4 is emitted during enteric fermentation in the rumen and through storage of manure (Steinfeld et al., 2006). One other important agricultural source of GHG (CH4) includes rice paddy fields (IPCC, 2006).

Compared to CO_2, the control of methane emission offers a greater prospect of reducing total anthropogenic greenhouse emission since the rate of increase in atmospheric methane concentration is higher than the rate at which net carbon dioxide is increasing (Moss, 1993; Takahashi, 2011). Furthermore, methane is estimated to have a global warming potential that is 21 times higher than that of CO_2 (Steinfeld et al., 2006), with a 12-year atmospheric lifetime, therefore making it more potent than CO_2. It is the second largest anthropogenic greenhouse gas, with carbon dioxide being the first (Forster et al. 2007; IPCC 2001). Also, methane is noted as capable of increasing stratospheric water vapour, which in turn increases the radiative force of the gas by approximately 70% (Lashof and Ahuja, 1990). Enteric methane from ruminant animals is the principal source of agricultural mmethane (Ellis et al., 2008; Hook et

al., 2010). The magnitude of nitrogen (N) excreted from animals and released as waste to the soil have been estimated to be 30 to 50% of global agricultural N_2O emissions (Grainger et al., 2009; Oenema et al., 2005). Apart from enteric fermentation, the manure, especially those stored in liquid form (slurry) constitute a significant source of methane and nitrous oxide emission (Külling et al., 2002). The amount of manure-derived methane is dependent on the duration of storage, moisture content, amount of volatile solids, storage temperature and proportion of anaerobically decomposed manure (Dong et al., 2006; Steinfeld et al., 2006).

Although global warming has been the main reason behind GHG research, there are other reasons why abating methane emission is very important in the livestock production system. Methane is a source of energy-loss to the animal (Waghorn et al., 2002) while animal manure is a source of nitrogen pollution of the environment (Eckard et al., 2010). The amount of methane released by ruminants fed a typical forages diet range from 7% to 17% of metabolizable energy (ME) intake depending on diet characteristics (Hristov et al., 2013; Robertson and Waghorn, 2002; Woodward et al., 2001). The methane emitted by a single dairy cow has been estimated to average 250g/day or 91 kg/year (Clark 2001; cited in: Waghorn et al. 2002). Methane contains 892.6 KJ mol^{-1} energy at 25°C and 1,013hPa, which is lost instead of contributing to the total supply of energy for metabolism in the ruminant animal (Takahashi, 2006). According to Leng (1991), methane emission in ruminant production systems in developing countries (where feed resources are usually of poor quality) may be higher because the diets are usually deficient in vital nutrients which results in slower growth and higher acetate to propionate production from high forage diets.

Therefore, it is important to consider enteric methane mitigation approaches in order to reduce footprints per unit of livestock product produced worldwide. The challenge of research is to develop mitigation technologies that are adaptable, show long-term effects and improve the efficiency of the energy and N-cycles in ruminant production for a more sustainable production system. A review of literature was conducted to understand the principles of enteric methane formation, identify knowledge gaps, and provide further innovative approaches to existing technologies.

CHAPTER ONE

Literature Review

1.1 Overview of rumen ecology

The digestive system of ruminants is characterized by a functional and anatomical adaptation that allows them to digest fibrous feeds and other complex carbohydrates otherwise indigestible by monogastrics. This advantage is conferred by the occurrence of a microbial fermentation process in the rumen prior to gastric and intestinal digestion (Niwinska, 2013). The rumen, which plays a significant role in the digestive system of ruminant animals has been described as an anaerobic fermentation chamber with a large diversity and dense microbial population living symbiotically with one another (Wolin et al., 1997). The microbial environment is noted for being a stable, but dynamic environment well established in performing the function of bio-conversion of feed into volatile fatty acids (Kamra, 2005). Fermentation of carbohydrate and protein results in intermediate products such as simple sugars and amino acids, which are utilized by the rumen microbes to produce microbial biomass, volatile fatty acids (VFA), ammonia nitrogen, carbon dioxide and methane. The primary VFAs produced by the rumen microbes include acetate, propionate and butyrate and to lesser extent the branched chain VFAs (valerate, isobutyrate, isovalerate) and lactic acid. These fatty acids are absorbed through the rumen walls, while microbial biomass and feed residues flowing to the abomasum provide a source of protein, vitamins and lipids to the animal (Niwinska, 2013). The rate and extent of this fermentation determines the availability of protein, vitamins and VFA supply to the animal and ultimately its productivity in terms of growth, meat and milk production.

The efficiency of the ruminant digestive system to utilize a wide variety of feed is highly dependent on its ability to sustain a highly diverse rumen microbial ecosystem (Niwinska, 2013). The rumen microbial environment comprise of bacteria species (10^{10}-10^{11} cells/ml, over 50 genera), anaerobic fungi (10^{3}-10^{5} zoospores/ml, five genera), ciliate protozoa (10^{4}-10^{6}/ml, 25 genera) (Kamra, 2005) and about 10^{9} methanogen cells/ml of rumen fluid (Cieslak et al., 2013). The synergistic and antagonistic relationship existing among the different classes of microbes is both diverse and highly complicated (Niwinska, 2013). The net result is responsible for the conversion of feed into various forms of fermentation byproducts utilizable by the animal. This mainly includes volatile fatty acids and ammonia nitrogen.

Bacterial species are the dominant group in the rumen. The major fibre degrading species (cellulolytic) in the rumen include *Ruminococcus albus*, *Ruminococcus flavefaciens* and

Fibrobacter succinogenes (Russell, 2002). Other bacteria species such as *Butyrivibro fibrisolvens*, and *Eubacterium cellulosolvens* have been noted to have extracellular cellulase, but have minor contribution to cellulolytic activity in the rumen (Shreck, 2013). These ruminal cellulolytic bacteria must be attached to feed particles as their cellulolytic enzymes are retained on the cell surface (Weimer, 1996). Another group of bacteria include the proteolytic species such as *Bacteroides amylophilus*, *Bacteroides fibrisolvens*, and *Streptococcus bovis*; lypolytic species such as *Anaerovibrio lipolytica* and amylolytic species such as *Bacteroides amylophilus*, and *Bacteroides ruminicola* (Conklin, 1995).

The role of rumen fungi have been identified to include enhancing the attachment of bacteria to feed particles (Akin and Borneman, 1990). Rumen anaerobic fungi actively colonize plant cell walls, accounting for up to 8-12% of the microbial biomass in the rumen (Rezaeian et al., 2004). Such fungi species include those belonging to *Neocallimastix*, *Caecomyces* and *Piromyces* genera (Nagpal et al., 2009). During the life cycle of the fungi, rhizoid development grows root-like structures that penetrate the feed particle and allow for greater space (Russell, 2002). These fungi species secrete high levels of enzymes that degrade fibre (cellulases, hemicellulases, xylanases, avicelases, glycosidases etc.) found to be associated with rhizomycelia (Lee et al., 2001; Williams and Withers, 1994). A parasitic relationship exist between some bacteria species and fungi species in the rumen. Nagpal et al. (2009) noted that chitin is one of the main structural component of the fungal cell wall. Therefore, the growth of these anaerobic fungi is likely to be inhibited by rumen chitinolytic bacteria such as *Clostridium species*. Co-culturing of an anaerobic fungi with chitinolytic *Clostridium tertium*, significantly reduced solubilization of crystalline cellulose, production of short-chain fatty acids and release of endoglucanase was observed *in vivo* (Hodrová et al., 1995). This suggests the role of chitinolytic bacteria in controlling fungal activities and therefore rumen fungi do not appear to attain their optimal fibre-degrading potential in the rumen due to inhibition by some bacteria. A similar association between rumen fungi with protozoa is also observable in the rumen ecosystem. Small sized fungal zoospores are likely to be prey for protozoa. Co-incubation of protozoa with anaerobic fungal species revealed that protozoa are able to ingest and digest fungi (Morgavi et al., 1994). Thus fungal growth and cellulolysis is negatively affected by rumen protozoa, possibly because of protozoal predation on zoospores (Nagpal et al., 2009). The protozoal population in the rumen are divided into two subclasses: flagellates and ciliates. Flagellates occur in young animals and the population continually decreases as the animal ages. Ciliate protozoa are known to engulf bacteria as their main nitrogen source thereby

influencing bacterial nitrogen recycling in the rumen (Williams and Coleman, 1997). This has a major negative impact on total N recycling because of its reduced bacterial outflow to the duodenum (Jouany, 1996; Lapierre and Lobley, 2001) due to the protozoa population outflow being slower than ruminal fluid dilution rate (Weller and Pilgrim, 1974).

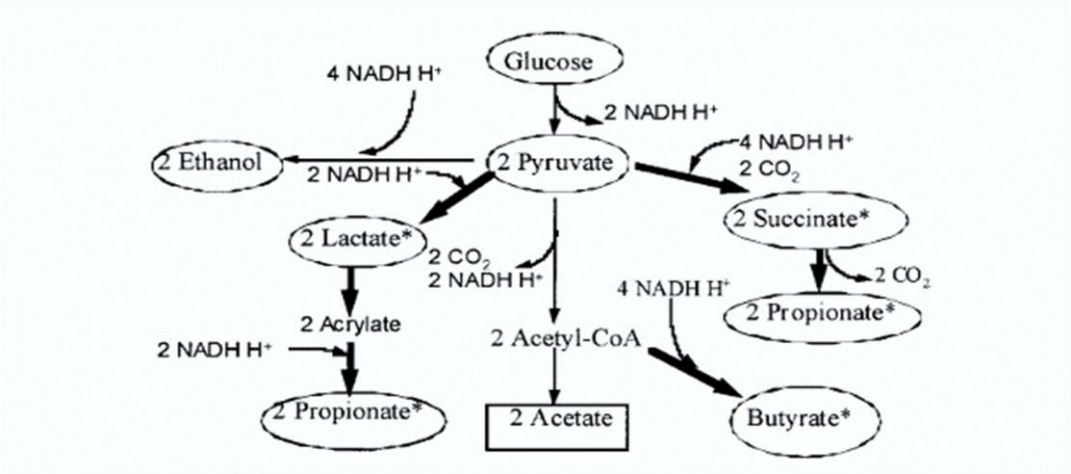
Ciliate protozoa also exhibit a symbiotic relationship with methane producing archaea in the rumen. The symbiotic relationship between ciliate protozoa and the rumen methanogenic archaea has been observed using microscopic observations (Vogels et al., 1980) and more recently by PCR-based techniques (Regensbogenova et al., 2004), revealing that methanogens have both ecto and endo-symbiotic relationship with ciliate protozoa (Patra and Saxena, 2010; Rahman, 2007). Methanogens not only attach to the cell surface of ciliates, but also exhibit endosymbiotic attachment by distributing themselves in the ciliate cell as reported by (Finlay et al., 1994) and Irbis and Ushida (2004). The major methanogenic species in the rumen include *Methanobrevibacter ruminatium*, *Methanomicrobium mobile* and *Methanobacterium formicicum* (Conklin, 1995).

1.2 Rumen carbohydrate fermentation and methane production

During rumen fermentation, anaerobic microorganisms (bacteria and fungi) ferment carbohydrates to produce volatile fatty acids (VFAs), energy and other waste product such as gases (methane and carbon dioxide) and heat. Three major volatile fatty acids are produced by rumen fermentation which include acetate, propionate and butyrate. The energy and the VFAs produced are used by the anaerobes for microbial growth (i.e., mainly to synthesize microbial biomass). Hydrogen is generated as one of the major intermediate products of fermentation by rumen bacteria and fungi, and it does not accumulate in the rumen because it is immediately used by other microbes present in the mixed microbial population (Moss et al., 2000). The collaboration between fermenting species and hydrogen-utilizing microbes (mainly, methanogens) is called “interspecies hydrogen transfer” (Miller, 1995). Methane is one of such metabolites that exchange hydrogen between hydrogen-producing microbes such as fibrolytic fungi and bacteria and hydrogen-consuming microbes such as methanogens (Kobayashi, 2010).This continuous production and removal of hydrogen facilitates continuous fibre degradation in the rumen (Ushida et al., 1997). Because of the interspecies hydrogen transfer relationship in the rumen, the population of fibrolytic bacteria are positively correlated to methanogens in the rumen of most ruminant species (Morgavi et al., 2010; Morvan et al., 1996). The H_2 and CO_2 simultaneously produced during microbial fermentation of feeds are

the main electron donor and acceptor respectively in the rumen ecosystem. These processes involve the cooperation of different groups of anaerobic microorganisms (Morgavi et al., 2010).

Fermentation microbes require a supply of NAD^+, which is converted to NADH and H_2, which are subsequently released as waste product. Both NADH and H_2 are used up by methanogenic archaea to reduce CO_2 to methane, and NAD^+ is regenerated for continuous fermentation (Figure 1.1). The success of microbial fermentation depends on the continual production of NAD^+ and removal of H_2 (Nolan et al., 2010). In terms of organic matter digestibility, methanogenesis ensures a fairly stable microbial fermentation process.



Source: Moss et al., (2000)

Figure 1.1. Metabolism of NADH H+ in the rumen

The CH_4 produced during carbohydrate fermentation is mainly eliminated via the nose and mouth of ruminants by belching and eructation (Murray et al., 1976), and thus the energy of the CH_4 is lost. Unless it is required to maintain body temperature, part of the heat energy is also lost by dissipation while the VFAs are the major product of microbial fermentation and are absorbed through the rumen wall into the portal circulation. As methane is lost to the atmosphere, the ruminant animal suffers a partial loss of utilizable feed energy (Johnson and

Ward, 1996). Methane production is therefore a by-product of anaerobic fermentation of feed in the rumen, mainly due to the metabolic activity of the methanogenic archaea population (Bayat and Shingfield, 2012).

1.3 Diet characteristics and methane production

Enteric methane emissions are dependent on the amount of feed consumed by the animal and the composition of the diet (Johnson and Johnson, 1995) with emission values increasing from concentrate diet, mixed concentrate-forage diet to forage diets (Mirzaei-Aghsaghali and Maheri-Sis, 2011). Structural carbohydrates such as cellulose and hemicellulose generally generate more methane than non-structural carbohydrates (Eckard et al., 2010). However, digestion of a unit of cellulose yields three times more methane than the same unit of hemicellulose. Roughages, such as grass hay, agro-industrial by-products and residues from crop production constitute the main feed resources for livestock feeding in larger parts of tropical and subtropical Africa. Preston and Leng (1987) have emphasized that with the global demand for grains and cereal crops, the most appropriate ways to improve feed resources for ruminants are through efficient utilization of these feed resources. They can be considered as a potential ingredient in the diet of producing animals if their digestibility can be improved (Cameron et al., 1991, 1990; Wanapat et al., 2009). During anaerobic fermentation, rumen microbes usually degrade the major fractions of carbohydrate and protein in feed to produce microbial biomass (microbial protein, B-vitamins etc.) and other useful by-product (mainly volatile fatty acids) and some waste product (mainly CH_4 and CO_2) (Singh et al., 2011). The availability of ammonia is often a primary deficiency when protein-deficient feedstuffs such as grass hay and crop residues are fed to ruminants, limiting rumen microbial biomass, digestibility and feed intake (Hao Phuc et al., 2009). The concentrations of these end-products depend largely on the chemical composition of the feed (mainly CP, NDF, ADF and ADL), digestibility and intake (Singh et al., 2011).

Roughages with a high content of easily fermentable carbohydrates such as sugars and starch and low amount of slowly digestible carbohydrate like ADF generally yield lower CH_4, a lower molar proportion of acetate and a higher molar proportion of propionate. Feed containing larger proportion of ADF and ADL produce higher methane, higher molar proportion of acetate and lower molar proportion of propionate (Dougherty, 1984; Zhang and Yang, 2012). In an *in vitro* study, CH_4 per unit of organic matter degraded was two times higher in tropical grass forage than legume forage (Widiawati and Thalib, 2007). The higher methane emission in these feed

resources is because of the high lignin-associated fibre fraction (indigestible polysaccharides) resulting in lower digestibility and a shift to more acetate than propionate. van Soest et al. (1991) characterized polysaccharides in the plant cell wall as belonging to two classes based on their biological associations and nutrient availability as (a) polysaccharides covalently bonded to core lignin-partially fermented (b) those that are not bonded-soluble and completely fermentable. The association of lignin to carbohydrates reduce its microbial degradation significantly (Jung and Deetz, 1993; Jung and Allen, 1995). Both concentration and composition of lignin appear to affect digestibility (Shreck, 2013). There exist a distinct relationship between feed organic matter digestibility and the proportion of concentrate or roughage fed and the pattern of ruminal fermentation. Bannink et al. (2008) reported that higher dietary soluble carbohydrates such as sugars and starches shifted rumen fermentation towards production of more propionate with a reduction in methane gas emitted. Therefore, ruminant systems based on low quality roughages, are expected to generate more methane gas per unit of the product produced than feedlot system.

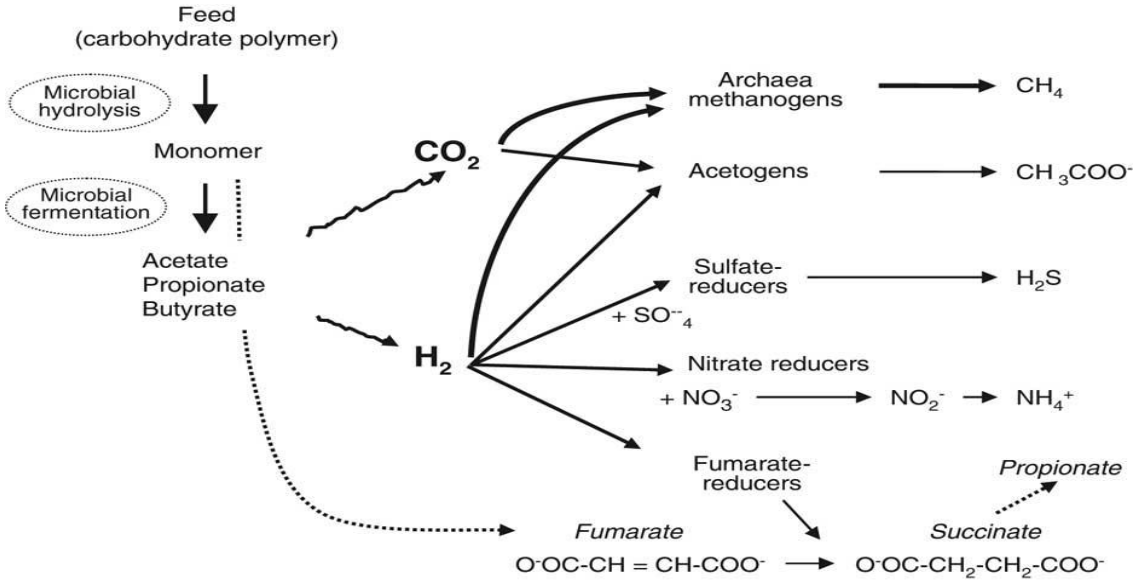
Supplementation of roughage based diet with non-protein nitrogen or high-protein feedstuffs have been noted to improve intake and digestibility of such feeds, although they may not always compensate for the presence of anti-nutritional factors, such as high ADL content and phenolic compounds (Chesson and Ørskov, 1984). Various types of treatment is possible to have a large increase in animal productivity with a relatively small increase in digestibility and feed intake (Reed and Goe, 1989). A successful treatment of feed must meet the following requirements: (1) improve enzymatic hydrolysis of feed, (2) avoid/minimize loss of soluble carbohydrate, (3) avoid formation of products that can inhibit rumen fermentation (4) improve palatability, and (5) is cost-effective (Sun and Cheng, 2002) as cited by Erickson et al. (2012). One of the effects of feed treatment is to cause a significant breakage in cell wall rigidity, to allow penetration by electrolytes and cellulolytic enzymes of rumen microbes (Weiske, 2005). Rumen microbes can subsequently colonize the vegetal matter more rapidly; decompose it more quickly and intensively because of the partial hydrolysis that had taken place during treatment, thus, resulting in better fermentation products.

Urea has been the NPN source of choice until recently. Renewed interest has been focused on nitrate due to its potential not only as a source of fermentable N, but because of its methane mitigation potential. Urea can be replaced with nitrate in low rumen degradable protein diets to provide a source of rumen ammonia while also reducing enteric methane production (Le

Huyen et al., 2010; Takahashi, 2011; Van Zijderveld et al., 2010). Feed treatment with NPN can improve the feed value of low quality roughage, particularly those with a high proportion of cell wall and a low level of crude protein (Bals et al., 2010). In the study by Le Huyen et al. (2010), rice straw and fresh grass mixtures treated with urea, sodium nitrate or ammonium nitrate as sources of fermentable N, showed no significant difference in feed intake and dry matter digestibility across treatments but significant reduction in methane occurring in the animals consuming nitrate. However, slightly higher ammonia concentration 11h after feeding was observed in animals consuming the nitrate diets, although the value was lower than the maximum threshold recommended by Perdok and Leng, (1989) for such class of animals. The *in vitro* trials by Le Thuy Binh Phuong et al. (2011) and Do et al. (2011) also reported similar results in methane reduction, although nitrate addition was by the supplementation method without further anaerobic storage. While nitrate has been proposed as an alternative source of microbial N in ruminant diets, crop residues have been known to vary in terms of efficiency of feed treatment (Van Soest et al., 1984). Therefore, research may be required to evaluate optimum utilization under particular dietary conditions.

1.4 Rumen hydrogen metabolism

The anaerobic fibrolytic microbes in the rumen produce CO_2, H_2, and VFAs (Mitsumori and Sun, 2008). Following continuous carbohydrate digestion, there is an accumulation of H_2, the removal of which allows the rumen microbes to function optimally, supporting the continuous degradation of feed dry matter (Hook et al., 2010; Sharp et al., 1998). The removal of hydrogen from the rumen environment, chiefly by methanogens, can therefore be termed a rate-determining step of carbohydrate fermentation. According to Wolin et al. (1997), methanogenesis is the major hydrogen consuming pathways among several other pathways already identified in the rumen as shown in Fig. 1.2.



Source: Morgavi et al., (2010)

Figure 1.2 Microbial fermentation and Hydrogen-consuming pathways recognized in the rumen

The production of methane contributes to the normal functioning of the rumen fermentation system by preventing the build-up of hydrogen to levels that might inhibit the enzymatic microbial activities involved in electron transfer reactions (NADH dehydrogenase) and ultimately impede rumen fermentation (Morgavi et al., 2010). Some of the methanogenic species that have been isolated from the rumen of ruminant livestock include *Methanobrevibacter ruminantium*, *Methanomicrobium mobile*, *Methanosarcina mazei*, *Methanobacterium formicicum and Methanosarcina barkeri* (Mitsumori et al., 2002). The same authors also showed that *Methanobrevibacter ruminantium*, *Methanobacterium formicicum* and *Methanomicrobium* utilise H_2/CO_2 and formate to produce methane while *Methanosarcina mazei* synthesizes methane from acetate, methanol and methylamines. *Methanosarcina barkeri* is noted to utilize H_2/CO_2, acetate, methanol and methylamines for methane synthesis (Jarvis et al., 2000). There is also the assumption that methyl coenzyme-M reductase (MCR) is common in all methanogenic species (Tatsuoka et al., 2004), because the final step of methane production by the methanogens is catalyzed by MCR (Ermler et al., 1997; Tatsuoka et al., 2004). Mitsumori and Sun (2008) noted the interaction between rumen ciliate protozoa and methanogens and observed that methanogenes attach themselves to protozoa cell surfaces, where they derive hydrogen from it for their metabolism (Vogels et al., 1980).

During the production of VFAs, which are the main products of rumen fermentation, there is also the production of reduced co-factors like NADH and NADPH that must be re-oxidized and re-used in the anaerobic system, failure of which further fermentation will be impeded because of hydrogen accumulation hindering further release of feed energy. The high concentration of electron acceptors such as CO_2, SO_4 and NO_3 in the rumen ecosystem helps in this recycling process by converting NADH, NADPH and FADH to NAD^+, $NADP^+$ and FAD+ respectively while CO_2, NO_3 and SO_4 are themselves reduced to methane, ammonia and hydrogen sulphide respectively (Kamra et al., 2012; McNeely, 2010; Thauer, 2010). These processes also involves the activities of special co-factors and co-enzymes, culminating in the production of ATP. The hydrogenotrophic pathaway is the main methanogenic pathway but at least four other pathways have been recognized in the rumen as shown as follows:

Hydrogenotrophic: $CO_2 + 4H_2$ ----------> $CH_4 + 2H_2O$

Methylotrophic: $CH_3OH + H_2$ ----------> $CH_4 + H_2O$

$4CH_3OH$----------> $3CH_4 + CO_2 + 2H_2O$

$CH_3NH_2 + H_2$ ----------> $CH_4 + NH_3$

Aceticlastic: CH_3COOH ----------> $CO_2 + CH_4$ (minor in the rumen)

In addition, recent research has identified newly recognized methanogenic species (e.g., rumen cluster C) that use a range of methyl donor compounds and CO_2 in methane formation (Poulsen et al., 2013), suggesting that there are other, yet to be identified, methanogenic pathways in the rumen (Hill et al., 2016).

Other significant hydrogen consuming pathways in the mixed rumen ecosystem include the fumarate reduction pathway, reductive acetogenesis, nitrate reduction, sulphate reduction and hydrogenation of unsaturated fatty acids as depicted in figure 1.1. Therefore, mitigation of enteric methane emission strategies are usually aimed at enhancing alternative hydrogen sinks to methane in the rumen, and/or inhibiting the activities of methanogenic bacteria that convert hydrogen to methane. The amount of hydrogen produced during feed fermentation is highly dependent on the diet and proportion of different types of rumen microbes prevalent in the rumen because the pathways for the final output (VFAs) differ in terms of hydrogen input/output. An understanding of how VFA output is controlled could enable a reasonable

approach to manipulate the rumen ecosystem to enhance the production of useful products while reducing the negative impacts such as methane emission (Ungerfeld and Kohn, 2008). The formation of propionic acid is a hydrogen-consuming pathway (equation 3) while the formation of acetate (equation 2) and butyrate (equation 4) are hydrogen releasing pathways (Mirzaei-Aghsaghali and Maheri-Sis, 2011).

Glucose ----> 2 Pyruvate + 4H (carbohydrate metabolism)	[1]
Pyruvate + H_2O ----> acetate + CO_2 + 2H	[2]
Pyruvate + 4H ----> propionate + H_2O	[3]
2 acetate + 4H ----> butyrate + $2H_2O$	[4]

It is noteworthy to realise that if rumen fermentation pattern shifts from acetate to more propionate in the total VFA production, net hydrogen and methane production will be reduced while an increase in acetate production will result in higher methane emission. Therefore, dietary strategies that increase propionate production at the expense of acetate production will result in a reduction in methane emission, thus establishing a well understood relationship between methane emission and the molar proportion of the various VFAs (Knapp et al., 2011). This provides opportunities to reduce enteric methane emissions by manipulating VFA output (Mirzaei-Aghsaghali and Maheri-Sis, 2011). To avoid the accumulation of hydrogen in the rumen system, methanogenesis is the predominant hydrogen-removing pathway, and providing a competitive alternative pathway offers a great prospect of reducing enteric methane emission in the ruminant animal without interfering with fibre fermentation.

1.5 Animal-derived methane mitigation strategies

The contribution of animal-derived methane emission to global GHG and its implication on production has elicited much research interest to halt the trend of global warming and ensure sustainable livestock production. Comprehensive reviews on enteric and manure methane mitigation technologies have been published in literature (Beauchemin et al., 2009, 2008; Cottle et al., 2011; Eckard et al., 2010; Ellis et al., 2008; Goel and Makkar, 2012; Hristov et al., 2013; Kebreab et al., 2006; Martin et al., 2010; Montes et al., 2013b). Enteric methane mitigation strategies can be broadly categorized into animal manipulation, diet manipulation

or rumen manipulation strategies (Eckard et al., 2010). Some of the rumen manipulation strategies highlighted in that review include biological control, the use of bacteriophages/bacteriocins, reductive acetogenesis, vaccination of animals against methanogens and chemical defaunation. Animal manipulation strategies already discussed in literature include breeding of animals for more efficient feed utilization and improvement in livestock systems. Diet manipulation strategies identified include improvement in forage quality, plant breeding or supplementation with dietary additives.

This review will briefly discuss the dietary additives capable of reducing enteric methane emission in ruminants. These additives can be classified as either those that can directly inhibit methanogens/methanogenic activity or indirectly by redirection of hydrogen away from methanogenesis via provision of a competitive pathway for hydrogen metabolism and modulation of rumen fermentation to favourable products other than methane or providing competitive organics for methanogenic bacteria within the rumen ecosystem (Hook et al., 2010). Research has shown potential for the direct inhibition of methanogens in the rumen through the administration of polyphenolic compounds/plant extracts (such as tannins, saponins, essential oils etc.). Methods aimed at indirect inhibition of methanogenesis/ redirection of hydrogen that has received wide scientific attention includes the use of ionophores (such as monensin), fatty acids/dietary lipids, organic acids (Cottle et al., 2011; Ungerfeld et al., 2005; Van Nevel and Demeyer, 1996), nitrate and sulphate supplementation (Brown et al., 2011; Gutierrez-Bañuelos et al., 2007; Leng, 2008). The application of bactereocins, ionophores, organic acids and sulphate are discussed briefly, while the application of nitrate and condensed tannins in rumen methane mitigation are reviewed in more detail with a view to identifying research gaps.

1.5.1 Bacteriocins and bacteriophages

This is a biological control method involving exogenous administration of a bacteriophage or bacteriocin to control rumen fermentation pattern. It has been found effective in inhibiting methanogens and redirection of hydrogen to other pathways such as the fumarate reduction pathway or reductive acetogenesis in the rumen (Ascensão, 2010). Bacteriophages are obligate microbial viruses that infect both bacteria and methanogenic archaea, attaching to them and undergoing lysis in their hosts during the lytic phase of their development (McAllister and Newbold, 2008) while bacteriocins are naturally occurring anti-bacterial agents often cultured from rumen bacteria populations. Other significant effects as described by Teather and Forster,

(1998) include prevention of animal metabolic disorders such as lactic acidosis and bloat. One of the earliest described bacteriocin, nicin, produced by *Lactococcus lactis*, exhibits a methane-mitigating response in a similar way to monensin supplementation *in vitro* without any negative effect on volatile fatty acid (VFA) production (Callaway et al., 1997). Nicin resulted in increased propionate production and selective anti-microbial activity against gram-positive rumen bacteria. Like bovicin, Nicin was reported to be as potent as monensin against methanogenesis and remain active even at low rumen pH (Kobayashi, 2010). Another bacteriocin called Bovicin, produced by *Streptococcus bovis* has also been reported as a possible methane-mitigating agent in the rumen. Supplementation with bovicin inhibited methane production by as much as 53% in a mixed rumen culture (Kobayashi, 2010).

1.5.2 Ionophores

The most popular ionphore is monensin, which is often added to the diet of feedlot animals to control bloat, improve feed efficiency and improve feed intake (Beauchemin and Mcginn, 2001). Research has shown monensin to reduce methane emission in beef cattle feedlot by between 7.5% and 25% in feedlot beef and dairy cattle, respectively (Gerber et al., 2013; Goodrich et al., 1984; Hristov et al., 2013) although its effectiveness is not persistent over a long term (Johnson and Johnson, 1995). There is a restriction on the use of antibiotics as a dietary supplement in animals across the European Union. Because monensin is a polyether ionophore antibiotic, its continued use in animal production systems remains controversial due to food safety concerns (Gerber et al., 2013).

1.5.3 Organic acids

Organic acids have shown some potential reduction in enteric methane emission in ruminant animals both *in vitro* and *in vivo* (Castillo et al., 2004; Newbold et al., 2005; Ungerfeld et al., 2003; Wood et al., 2009). Organic acids such as fumarate, malate and acrylate are propionate precursors in the succinate-propionate pathway and therefore can act as an alternative hydrogen sink in the rumen, thus restricting methanogenesis (Eckard et al., 2010). Fumaric acid, being the most commonly used carboxylic acid reduces H_2 or 2H to succinate, which is then converted to propionate, which benefits the animal (Kim et al., 2001). However, the relatively high amount of fumarate required to reduce one mole of methane makes it less promising. About four mole of fumaric acid is required to prevent the formation of one mole of methane(Gomez et al., 2005). The drop in rumen pH also makes it less favorable especially in intensive livestock systems (Castillo et al., 2004).

1.5.4 Sulphate

Sulphates act as electron receptors in the rumen ecosystem. Recent research with sheep (Van Zijderveld et al., 2010) has shown promising results with sulfate decreasing methane emission by up to 16%, and when both nitrate and sulfate were added, the effect on methane reduction was additive (Le Phuong et al., 2012; Van Zijderveld et al., 2010). However, the potential effect on animal health remains controversial especially due to the potentially high production of hydrogen sulfide in the rumen.

1.5.5 Dietary lipids/fatty acids

Dietary supplementation of animals with lipids/fatty acid within limits that do not affect rumen fermentation and animal performance, has shown to reduce CH_4 production (Beauchemin et al., 2008; Martin et al., 2010; Popova et al., 2011). The methane mitigation effect of dietary lipid has been noted to vary depending on the concentration, chain length and degree of unsaturation of fatty acids, and the interactions between diet composition and fatty acid (Eugène et al., 2008; Johnson and Johnson, 1995). Some *in vitro* studies reveal that medium-chain fatty acids such as lauric and myristic acids were more effective in reducing CH_4 production compared with the long chain or short chain fatty acids (Dohme et al., 2000; Dong et al., 1997). Depressed CH_4 production as a result of dietary lipid supplementation has also been attributed to a reduction in the total amount of organic matter fermented and established inhibitory effect of lipids on rumen microbial activity (Ivan et al., 2004; Johnson and Johnson, 1995). Eugène et al. (2008) also noted a significant reduction in methane emission due to dietary lipid supplementation as a result of a reduction in dry matter intake (DMI) of animals supplemented with lipid.

1.5.6 Dietary nitrate

1.5.6.1 Nitrate metabolism in rumen

The use of dietary nitrate is one enteric methane mitigation approach that has received significant research effort in the last few years. The exogenous inclusion of nitrate salts in the diet of animals can help provide alternative electron acceptors in the hydrogen pathway, thereby reducing the amount of methane that will be produced during feed fermentation (Leng, 2008). Methanogenesis is the main pathway for hydrogen consumption in the rumen, followed by propionate production (via the fumarate reduction pathway) while other less significant pathways identified include nitrate and nitrite reduction, reductive acetogenesis, sulfate reduction and hydrogenation of unsaturated fatty acid and use of nitroethane (Brown et al.,

2011; Gutierrez-Bañuelos et al., 2007; Kobayashi, 2010). Nitrate has been identified as an effective inhibitor of methanogenesis in all anaerobic fermentation systems such as the rumen ecosystem and anaerobic bio-digesters (Akunna et al., 1993; Allison et al., 1981; Hungate, 1966) based on its ability to compete favorably with methanogens for available hydrogen.

Nitrate reduction in anaerobic systems has been noted to occur via three distinct pathways, namely dissimilatory nitrate reduction to nitrogen gas (denitrification), dissimilatory nitrate/nitrite reduction to ammonia (DNRA) and assimilatory nitrate reduction to ammonia (ANRA) which is also called nitrite ammonification, but only the latter two have been reported in the rumen (Leng, 2008). The fact that ammonia is one of the end product of DNRA and ANRA means it will theoretically complement rumen fermentation and microbial protein synthesis by providing a non-protein nitrogen source to the fibrolytic bacteria. The DNRA usually occurs when redox potential values in the anaerobic system are low and involves the reductive conversion of nitrate/nitrite to ammonia (Knowles, 1982) as cited by Leng (2008) and often in the presence of sulphide ions (Brunet and Garcia-Gil, 1996) or high organic matter (Hungate, 1966) as cited by Akunna et al. (1993) and Leng, (2008). In ANRA, nitrate is enzymatically converted to nitrite via the NADH reduction pathway while the nitrite intermediate is further reduced to ammonia and ATP by assimilatory nitrite ammonification (Simon, 2002).

The thermodynamics of converting nitrate to NH_3 has been noted to be more energetically favourable than methane production, and therefore can effectively compete with methanogenesis in the rumen ecosystem if adequate nitrate concentration is maintained (Morgavi et al., 2010). Assimilatory nitrate reduction has important bioenergetics advantages in that it aids in conserving energy for microbial growth compared to energy loss in methane generation, thus microbial yield is higher than other sources of N such as urea or protein (Leng, 2008). Based on their Gibbs free energy change estimation, nitrate reduction to ammonium yields -598 kJ and reduction of carbon dioxide to methane yields -131 kJ (Allison and Reddy, 1984; Allison et al., 1981; Leng, 2008).With a more energetically efficient use of hydrogen in the rumen, microbial yield is theoretically expected to increase (Nolan et al., 2010).

The end product of nitrate reduction is ammonia, which for ruminants consuming low quality diets will provide an opportunity to overcome the limitation of such diets often low in soluble nitrogen, by providing an additional source of N for microbial growth. Therefore, carbon dioxide can be replaced by dietary nitrate as an alternative electron acceptor to generate another

reduced product (ammonia) which can be recycled in the rumen system (Leng, 2008). The inhibition of methanogenesis has also been confirmed by even small amounts of NO and N_2O which has been found to be toxic to some methanogenic species (Kluber and Conrad, 1998a, 1998b). The amount of hydrogen required to reduce 1 mole of nitrate and carbon-dioxide is summarized in the equation indicated below:

Accordingly, 1 mol of nitrate would trap 4 mols of hydrogen (8 electrons) to produce 1 mol of NH_3; 4 mol of H_2 is required to produce 1 mol of CH_4. Therefore, 1 mol nitrate will reduce CH_4 production by 1mol which is equivalent to 16g or 22.4 L (Leng, 2008).

1.5.6.2 Effect of nitrate on enteric methane production

The impact of dietary nitrate in reducing enteric methane has been studied in sheep (Nolan et al., 2010; Sar et al., 2004b; Van Zijderveld et al., 2010) and in dairy cattle (Hulshof et al., 2012; van Zijderveld et al., 2011; Van Zijderveld et al., 2011) with nitrate decreasing methane production by up to 50%. Supplementary nitrate does not only act as an alternative sink for hydrogen, it can also potentially serve as a source of nitrogen for microbial biomass production. This is particularly of immense benefit in protein deficient diets such as crop residues and poor quality roughage during the dry season. In the study by Sophal et al., (2013) methane production was reduced by 43% and N retention per unit of organic matter digested increased by 28% when dietary nitrate (5% of diet DM) replaced urea (1.4% of diet DM) in cattle. A similar result was reported by Van Zijderveld et al., (2011) in cattle with 32% methane reduction and Nolan et al., (2010) with a 25% methane reduction in sheep. In a related *in vitro* experiment comparing calcium nitrate to urea, there was no significant difference in total gas production after 48 hour, but the production of methane per unit of digested substrate was reduced by 32% (Inthapanya et al., 2011). A significant reduction in enteric methane emissions following dietary nitrate have also been recorded by Sophea and Preston, (2011) and Thanh et

al., (2011). The characteristics of the feed offered, type, specie and age of animal as well as methane measurement technique might have influenced the varying magnitude of methane reduction arising from dietary nitrate administration reported in literature. Nevertheless, they all reported a consistent reduction of methane.

1.5.6.3 Nitrate toxicity in ruminants

Previously, most of the earlier research on nitrate in ruminant nutrition had largely been downplayed due to its toxicity to animals (Leng, 2008). The accumulation of nitrite, formed as an intermediate in the breakdown of nitrate to ammonia, induce methemoglobinemia in animals (Lewis, 1951). This occurs when animals consume large doses of nitrate resulting in either acute or chronic methemoglobinemia (Allison and Reddy, 1984). Following or before ingestion of nitrate, nitrite is formed and can be partially absorbed from the digestive tract, into the portal circulation where it oxidizes the iron in haemoglobin (Hb) from the ferrous state (+2) to ferric state (+3) resulting in the formation of a dark coloured pigment, Methemoglobin (Met-Hb). Methemoglobin has a poor affinity for oxygen thus greatly reducing the oxygen carrying capacity of red blood cells (Blood et al., 1979; Crawford et al., 1966; Rogers, 1999).

When the Hb to Met-Hb conversion rate is about 20%, the colour of blood may be light or grey-brown, at 50% it may be brown-black and at 80%, it is usually dark brown to black. Animals may not show visible signs of nitrite poisoning, or may show mild or chronic signs. When conversion rate exceeds 50%, severe signs manifest. Death due to oxygen starvation occurs when Hb to Met-Hb conversion nears or exceeds 80%. Because serum nitrite levels correlate with the conversion rate of Hb to Met-Hb, when nitrite levels increases from 50 to 150 mg/L, conversion rate increases from 24 to 70% and at 250 mg/L nitrite /L blood, met-Hb conversion nears 100%. Nitrite levels in the blood can peak within 1-6 hours after intake of a high nitrate diet (Guerink et al., 1982). The minimum single lethal dose of nitrite in cattle and sheep is 67- 100 mg/kg LW translating to 40- 66 g NO_2^- for a 600 kg cow (Bartik and Piskac, 1981; Rogers, 1999). Therefore, at a feed DM intake of 2.5% BW, nitrate concentration of 0.9-2.2% in feed DM could be toxic for sheep if consumed rapidly. However, 0.9% NO_3^- in feed DM was safe in sheep when fed slowly but above 1.2% resulted in high mortality within 6- 10 weeks (Rogers, 1999).

Acute signs of nitrite toxicity include cyanosis, severe respiratory distress with rapid respiration and rapid weak pulse, weakness, coma and death. Sub-acute signs could include salivation, lacrimation, grinding of teeth, vomiting, abdominal colic and diarrhoea, ataxia, muscle tremor

convulsions, a rapid heart rate and breathing difficulty. Chronic signs include listlessness, depressed feed intake, milk yield and fertility (Bartik and Piskac, 1981; Blood et al., 1979; Rogers, 1999). Increased production of indicators of oxidative stress in blood and tissue of laboratory animal models have been observed due to high nitrate intake (Chow and Hong, 2002; Ogur et al., 2000; Vatassery et al., 2004). When reactive oxygen species (ROS) production exceeds the antioxidant defense mechanisms in the body of the animal, oxidative stress occurs (Cigerci et al., 2009; Diplock, 1994) and this may exert negative effects on meat quality. Cherdthong et al. (2014) noted that increased blood NH_3 concentrations which may be due to increased dietary nitrate alter hepatic metabolism by increasing ureagenesis and may also affect glucose metabolism in the liver and peripheral tissues (Huntington et al., 2006; Taylor-Edwards et al., 2009). A blood sample collection is usually done for screening purposes to monitor and evaluate the health and nutritional status of animals (Aengwanich et al., 2009; Gupta et al., 2007).

Another form of nitrate poisoning called chronic nitrate toxicity that can occur when rumen ammonia levels are elevated. Clinical signs of the disease are not observable, but there is an induction of sub-optimal performance and depressed immune status in animals (Avci et al., 2012). In chronic nitrate toxicity, the clinical signs of methemoglobinemia may not be observed, but it commonly induces reduction in animal performance and a greater susceptibility to infections resulting in financial loss to the producer. This usually occurring when nitrate concentrations reach 800-2000ppm in the diet. However, some authors have noted that sub-lethal doses have no obvious effect on production performance in animals (Rogers, 1999).

Methemoglobinemia occasionally occurs in grazing ruminants and is usually associated with a sudden increases in nitrate intake from lush pasture that is also high in crude protein or in plants that accumulate nitrate due to high nitrogen fertilization (Wright and Davison 1964; O'Donovan and Conway 1968; Lovett *et al.* 2004; cited by Leng 2008). In most cases of nitrate poisoning discussed in literature, the crude protein content of the feed is high and generally between 18 and 38%, resulting in consistently high rumen ammonia levels, often up to thrice above the recommend levels for optimal microbial efficiency of the animals (Preston and Leng, 1987). It is noteworthy that nitrate toxicity can occur as much as urea toxicity in ruminants.

The significance of gradual adaptation of animals to dietary nitrate in low crude protein diet strategy has been identified as the foundation for any successful utilization of nitrates as either a source of ammonia N or in CH_4 mitigation (Leng, 2008). It is noteworthy also that the

adaptability of the rumen microbes to nitrate may be lost within a short time after nitrate withdrawal from their diet (Alaboudi and Jones, 1985) and intake following re-introduction must be gradual and regulated to prevent poisoning (Hristov et al., 2013). The rumen degradable nitrogen level in the feed should also be considered when supplementing with nitrate in any livestock system. The ability of sulfate to reduce nitrite accumulation has been recognized as a significant approach to curtail nitrite toxicity. Supplementation of sulfur or cysteine together with nitrate may help ensure efficient conversion of nitrite to ammonia by enhancing the activities of nitrate reducing, sulfide-oxidizing bacteria (Hubert and Voordouw, 2007). Sulfate, itself being an electron acceptor will also compete with methanogens for hydrogen thus lowering methane production. This positive relationship has been documented in sheep (Van Zijderveld et al., 2010). Nolan et al. (2010) and Van Zijderveld et al. (2010) followed a gradual adaptation period of 18 and 28 days, and reported positive responses without any significant signs of nitrite toxicity in the animals. Some loss of nitrogen in the form of urinary N may be expected following a dietary intake of nitrate (Takahashi et al., 1998), but the effect on total urinary N loss has not been extensively evaluated (Hristov et al., 2013).

1.5.7 Phytochemicals (plant secondary compounds)

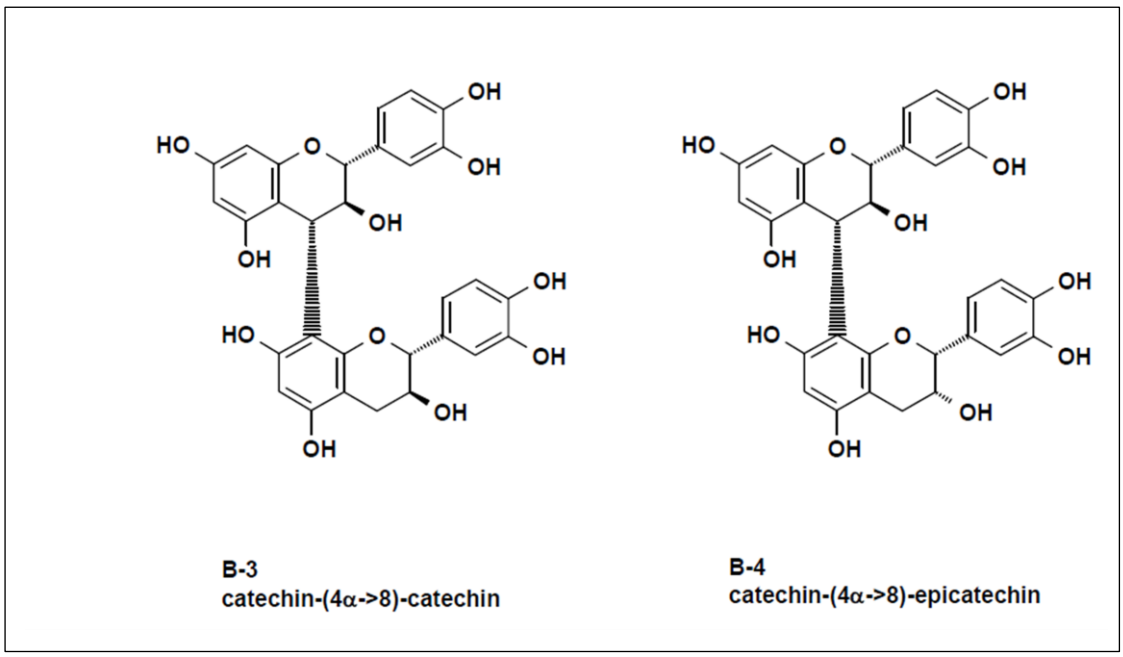
Plants produce a wide array of secondary compounds called phytochemicals to protect themselves against other plants, microbes, insects and herbivores (Wallace, 2004). These phytochemicals include terpenes, protein inhibitors, betalains, polyphenolic compounds such as flavonoids, saponins, essential oils (EO), tannins and organosulphur compounds. The antimicrobial properties of these polyphenolic compounds (mainly tannins, saponins and essential oils) have been reported in many biological systems and are able to selectively modulate rumen microbial populations (Cieslak et al., 2013; Patra and Saxena, 2009; Wallace, 2004) via their anti-microbial action and/or anti-protozoal action (Kamra et al., 2012; Patra and Yu, 2013). This results in a shift in rumen fermentation patterns, reduced rumen nitrogen digestion, reduced intestinal parasite infection, reduced incidences of nutritional disorders such as bloat or rumen acidosis and reduced methane emission (Kamra et al., 2006; Patra and Saxena, 2010; Rochfort et al., 2008; Wallace et al., 2002). It has been suggested that saponins decrease methanogen populations by reduction of methanogen-associated protozoa population (Patra and Saxena, 2010). A symbiotic association between ciliate protozoa and methanogens have also been noted (see also Finlay and Esteban 1994). Essential oils (EOs) are steam volatile extracts which also exhibit selective antimicrobial activity by interaction with the microbial cell membrane (Dorman and Deans, 2000; Kamra et al., 2012; Mcintosh et al., 2003). Essential

oils have selective anti-bacterial activity and have been found to be effective in inhibiting methane emission while enhancing propionate production *in vitro* (Macheboeuf et al., 2008). However, EOs vary widely in chemical properties and thus their antimicrobial activity (Lin et al., 2013). Tannins are water-soluble polyphenolic compounds with high molecular weight ranging from 500 to some thousand daltons (Beltran-Heredia and Beltran-Heredia, 2016). They are categorized into three types: hydrolysable tannin condensed tannins and ellagic tannins (Gould et al., 2009). Condensed tannins are the most widely distributed in plants. Condensed tannins are noted for high protein-binding properties, that aids the inhibition of methane production in the rumen. Condensed tannin is discussed in details in the next section.

1.5.8 Condensed tannins

1.5.8.1 Chemistry and occurrence of condensed tannin

Tannins are water-soluble, yellowish or brownish bitter-tasting polyphenolic compounds of high molecular weight, with strong affinity to form complexes with proteins and other macromolecules due to the presence of large numbers of phenolic hydroxyl groups. Chemically, they are polymeric flavonoids, a group of metabolites based on a heterocyclic ring system derived from phenylalanine and polyketide biosynthesis.



Source: Hagerman, (2002)

Figure 1.3 Chemical structure of condensed tannin sub units

They occur in many nutritionally important forage, shrub tree, legumes, fruits, cereals and grains (Patra and Saxena, 2010). They are usually classified as hydrolysable tannins (HT) and condensed tannins (also called proanthocyanidins) (Kamra et al., 2012). The hydrolysable tannins are not as widely distributed in plants as the condensed tannins. Tannins are widely known for their anti-nutritional properties in plants, which by nature serve as a defence mechanism against animals. There has been a lot of report in recent times on the beneficial effects of tannins in ruminant animals which include prevention of bloat, enrichment of conjugated linoleic acid, and mitigation of enteric methane production (Patra and Saxena, 2011). Different sources of CT differ in terms of the distribution of their monomeric units. For instance, *Lotus corniculatus* (birdsfoot trefoil) contain predominantly procyanidin-type CT made of catechin sub-units (67%) with an average molecular weight of 1900 (Foo et al., 1997) whereas *Lotus pedunculatus* (big trefoil) contain predominantly prodelphinidin-type CT, made of epigallocatechin subunits (64%) with an average molecular weight of 2200 (Foo et al., 1997). The lower molecular weight CTs have higher protein binding properties than the higher molecular weight CTs (Patra and Saxena, 2011). However, prodelphinidins-rich CT was more effective against abomasal nematodes than the procyanidin-rich CT (Brunet et al., 2008).

1.5.8.2 Effect of condensed tannin on rumen microbes

The anti-microbial mechanism of condensed tannins is not fully understood (McSweeney et al., 2001b). However, the ability of tannins to complex cell wall and extra cellular enzyme secretions may likely be the mode of inhibiting nutrient transport to the cells therefore impeding cell growth. The effect of CTs on rumen fermentation show that CTs exhibit strong antibacterial activity on fibrolytic species often resulting in reduced digestibility of NDF and ADF. It has been observed that in animals fed tannin rich *Calliandra calothyrsus*, the population of rumen fibrolytic bacteria *Ruminococcus sp* and *Fibrobacter sp* were considerably reduced (McSweeney et al., 1999). Condensed tannin from *lespedeza curneata* have been found to inhibit pectinase and cellulase enzymes in rumen fluid (Bell et al., 1965; Smart Jr. et al., 1961) while endoglucanase activities of *Fibrobacter succinogens* were inhibited by CT from Lotus corniculatus (birdsfoot treefoil) at concentrations 100-400ug/ml *in vitro* (Bae et al., 1993). The growth of proteolytic bacteria *Butyrivibrio fibrisolvens*, *Ruminobacter amylophilus* and *S. bovis* were reduced by CT. However, certain strains of *Prevotella ruminicola* was found to be tolerant to CT from *Onobrychis viciifolia* (sainfoin) at concentrations less than 600ug/ml (Jones et al., 1994). Fungus species *Neocallimastix patriciarum* was unaffected at 100ug/ml CT from *L. corniculatus* (McAllister et al., 1994). Other studies show that fibre degrading fungi

species appear less sensitive to CT compared to fibre degrading bacteria (McSweeney et al., 1999). Tannin tolerant species are thought to secrete extracellular glycoproteins which bind to tannin or by production of extra cellular polysaccharides which shield the cell walls (Chiquette et al., 1988).

1.5.8.3 Effects of condensed tannins on methane production

Animals consuming condensed tannins either as tannin (CT)-containing forage (Puchala et al., 2005), or CT extract as an additive (Carulla et al., 2005) have been found to produce less CH_4 emission than control animals. Condensed tannins exhibit anti-methanogenic activity directly by inhibiting the growth of methanogens through the tanning action of their functional proteins (e.g., microbial enzymes) located in or at accessible sites on the methanogens (Field et al., 1989; Field and Lettinga, 1987). This results in bacteriostatic and bactericidal effects (Tavendale et al., 2005) or indirectly by the defaunating action on methanogen-associated protozoa population (Animut et al., 2008a, 2008b; Bhatta et al., 2009; Patra et al., 2006). Responses of methanogens to CT have been noted to be type and dose dependent.

Jayanegara et al. (2009) reported an inverse relationship between dietary tannin concentration and CH_4 production per unit of digestible organic matter (OM) in rumen fermentation. Tavendale et al., (2005) reported both bacteriostatic and bactericidal properties of *Lotus pedunculatus* (YLM-1 and DSM1093) on the common rumen methanogens *Methanobrevibacter ruminantium in vitro*. Waghorn et al. (2002) evaluated the potential of *Lotus pedunculatus* and reported a 16% reduction in methane production (in g CH_4 kg^{-1} dry matter intake) in lambs due to the presence of CTs in the forage. In a related study by Huang et al. (2010), condensed tannin extracts from Leucaena-hybrid Bahru (LLB) showed a significant reduction in *in vitro* methane production as the concentration of CT extract increased. In that study, both Linear and quadratic negative correlation were found between CT level and methane produced per unit of dry matter digested or methane to total gas ratio. Similarly, Carulla et al. (2005) observed a 13% reduction in CH_4 emissions when *Acacia mearnsii* CT extract was administered at 25g/kg feed DM to sheep.

Condensed tannin forage and condensed tannin extract used in literature have however shown wide variation in their anti-methanogenic effect, which according to Mané et al. (2007), is related to their average molecular weight and the degree of polymerization of the CTs. The chemical structure and molecular weight of CTs have been observed to play a significant role in their biological activity (Huang et al., 2011; Kraus et al., 2003; Naumann et al., 2014) and

condensed tannin-protein interaction (Soares et al., 2007). Some reports showed a very strong relationship between molecular weight of CT and their biological activity (Bate-Smith, 1973; Huang et al., 2011; Peleg et al., 1999) while some other studies have reported very weak or no correlation of CT molecular weight with biological activity (Harley D Naumann et al., 2013; Naumann et al., 2014; Tharayil et al., 2011). However, the effectiveness of a crude extract or tannin forage may depend on the range of molecular weight and/or the proportion of low or high molecular weight fraction in the unpurified extract.

1.5.8.4 Effect of condensed tannins on dry matter intake, nutrient digestion and animal performance

Tannins can complex with proteins, starch, cellulose and hemicellulose and reduce their digestibility (Degen et al., 1995). They can also impair the digestive process by complexing with digestive enzymes and endogenous proteins (Butler, 1992). The effect of CTs on protein metabolism in ruminant animals have shown potential beneficial effects. The reduction of protein degradation is largely due to the formation of tannin-protein complexes in the rumen and inhibition of proteolytic bacterial populations or inhibition of endogenous enzymes (Patra and Saxena, 2011). The quantity of protein (dietary and microbial protein) flowing from the rumen to the hindgut determines the productivity of animals (Patra and Saxena, 2011). According to Aerts et al. (1999), forage with moderate concentrations of 20–40g $CTkg^{-1}$ DM are capable of shifting the major site of protein digestion to the hind gut, resulting in increased bypass protein and increased sparing of good quality protein from microbial degradation. When ruminants are fed high protein diet there is a high proteolytic activity, leading to high ammonia nitrogen in the rumen, which is subsequently absorbed via the rumen wall and excreted via urine (Ulyatt and MacRae, 1975). Tannins help to reduce proteolytic activity in the rumen, thus ensuring a more efficient microbial synthesis (McNabb et al., 1996; Min et al., 2000; Molan et al., 2001; Patra and Saxena, 2011). Decreased excretion of urinary nitrogen is advantageous to the environment (Patra and Saxena, 2011; West et al., 1993).

Efficient microbial was observed in sheep by Bhatta et al. (2000) and Puchala et al. (2005) when they supplemented quebracho tannin at 10 and 20g/kg diet DM but not at 30g/kg diet DM (Al-Dobaib, 2009). However, McNeill et al. (1992) reported that urinary excretion of N was unchanged due to dietary tannin administration. The tannin-protein complex is usually broken in the abomasum (pH <3.5) and intestine (pH >7), thus making the proteins available for digestion in the lower gut. However, tannins differ in their binding ability and thus

dissociation ability (Patra and Saxena, 2011; Waghorn, 1990). A shift in dietary energy and N loss from urine to faeces with dietary CT administration was also reported. This signifies reduced N digestion by the animal, but also reduced volatile N loss to the environment because urine N is more volatile than feces (De Klein and Eckard, 2008). The main N-component in urine is urea, which is rapidly hydrolyzed to ammonia, then nitrified to nitrate (De Klein and Eckard, 2008) and subsequently results in nitrous oxide production (Whitehead, 1995). However, the main N component in faeces is organically bound nitrogen, which is not as readily denitrified to produce nitrous oxide (N_2O) as urea-N in urine (Grainger et al., 2007).

Reduced dry matter intake and nutrient digestibility particularly of crude protein, with increasing dietary tannin concentration has also been reported by Hristov et al. (2013) and Patra and Saxena (2010) due to its astringenic properties and tannin forming complexes with salivary proteins (Makkar, 2003a) and binding of dietary protein (Huang et al., 2010). Previous studies on the effects of CT on feed intake in ruminants have yielded inconsistent results. The first point of interaction of dietary tannins with the digestive system of animals is in the mouth during ingestion, mastication and salivation. Ruminant animals produces proline rich proteins as components of their saliva that readily complex dietary tannins. Proline rich proteins (PRP) are highest in deer, followed by goat, sheep and cattle respectively (Reed, 1995) although some researchers (Austin et al., 1989) argue that sheep and cattle do not have PRP. It has also been noted that long-term consumption of tannin-rich diets have been found to stimulate the development of the salivary glands to secrete more PRP in ruminants.

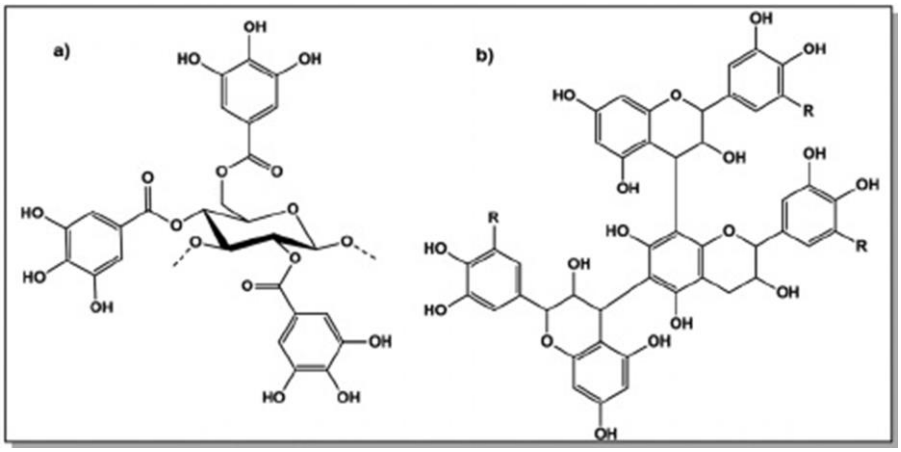
The type and concentration of CT present in a feed have been suggested to greatly influence feed intake, digestion and performance of animals (Patra et al., 2011; Waghorn, 2008). Supplementing CT extract in the diet of dairy cattle decreased intake of DM, OM, CP, NDF, and ADF, regardless of forage level (Dschaak et al., 2011). A decrease in dry matter intake as a result of dietary CT intake was reported in cows at 55 g CT/kg feed DM (McNabb et al., 1996) and 25 g CT/kg feed DM (Priolo et al., 2000); in sheep at 75-100 g CT/kg feed DM (Barry and McNabb, 1999). The depression of dry matter intake has been attributed to degraded palatability (Dschaak et al., 2011) or to a short-term effect of astringency (Landau et al., 2000a). In some other studies, CT did not affect dry matter intake. Quebracho tannin (*Schinopsis quebracho-colorado*) administered to beef steers at the rate of 20 g CT/kg feed DM showed no effect on intake and performance (Beauchemin et al., 2007). In sheep, 25 g CT/kg feed DM from carob pulp (*Ceratonia siliqua*) resulted in a decrease in growth rate of experimental lambs from 140g to less than 50g per day (Priolo et al., 2000) whereas the CT

from *Hedysarum coronarium* at 72 g CT/kg feed DM did not affect daily weight gain of lambs (Douglas et al., 1999). The depression of feed intake that is associated with condensed tannin has been found to be related to the predominance of *procyanidins as against prodelphinidins* in the extractable tannin fractions. This was observable within different accessions of *Calliandra calothyrsus* where greater feed intake and nutrient digestibility was recorded in the prodelphinidins-rich cultivar (San Ramon) compared to the procyanidins-rich cultivar (Patulul) (Lascano et al., 2003). Therefore the biological activity of CT forages or extracts in terms of methane reduction, protein-binding action etc. will largely depend on its astringency (i.e. protein binding property) and structure of the CT source (Grainger et al., 2009).

It is understood that most of the free CT in feed, are bound to protein and fibre fractions in the gastro intestinal tracts and are present in faeces, in the bound or un-extractable form. This was further confirmed by Degen et al., (1995) who reported significantly higher CT and protein in ADF and ADL fractions of faeces than the feed offered, thus creating a negative balance in ADF and ADL digestibility. The result of ADL and ADF digestibility in a high tannin diet therefor require cautious analysis because of the possibility of high tannin-protein and tannin-fibre complex formation in fecal materials (Makkar, 2003a). The pH of the rumen favours the protein-tannin complexation process. Post-rumen dissociation of protein-tannin complex, often depends on the nature of the tannin, its affinity to bind proteins, and the presence of other macro molecules (Makkar and Singh, 1995). Following the dissociation of tannin from feed-protein, there is also the tendency for tannin molecules to bind with endogenous proteins. Tannins help in nitrogen re-cycling to the rumen by lowering the rate of protein degradation in the rumen thereby resulting in lower concentration of rumen ammonia nitrogen and blood urea nitrogen (BUN). With lower BUN and increased stimulation of saliva production, there is increased recycling of nitrogen.

1.5.8.5 Tannin toxicity and the fate of tannins in ruminant digestion

Animal poisoning resulting from the consumption of high amount of tannins have been well documented. These have been largely attributed to presence of hydrolysable tannins (McSweeney et al., 2001b) as reported for Clidemia (Murdiati et al., 1991), Oak (Quercus) (Camp et al., 1967), and Terminalia (Doig et al., 1990).



Source: Lochab et al., (2014)

Figure 1.4. Chemical structure of (a) hydrolysable tannin (HT) and (b) condensed tannin (CT)

Hydrolysable tannins have a central core of polyol molecules (usually D-glucose) and hydroxyl groups esterified with phenolic groups such a gallic acid (gallotannin) and ellagic acid (ellagitannin) in their outer rings while condensed tannins are polymeric forms of flavonoid units (flavan-3-ol) and often linked by carbon-carbon units (Lochab et al., 2014). Hydrolysable tannins can be hydrolyzed by mild acids or mild bases and by microbial action of the rumen to yield carbohydrate and a phenolic acid. The deglycosylation of polyphenols, like flavonoids and hydrolysable tannins, ensure the release of their sugar moieties to rumen microbes. During rumen digestion, the ester linkages in hydrolysable tannins are broken down to produce gallic acid and ellagic acid, which are subsequently decarboxylated through pyrogallol as a major intermediate product to produce acetate and butyrate (Bausch and Carson, 1981). Some of the rumen microbes responsible for breakdown of HTs include *Selenomonas ruminantium* and *Streptococcus bovis*, *Eubacterium oxidoreducens*, *Coprococcus sp*, *Syntrophococcus sucromutans* (McSweeney et al., 2001b). During rumen fermentation (microbial metabolism) and in the lower GIT (gastric digestion), low molecular weight phenols such as resorcinol glucuronide and diphenyl lactone, pyrogallol, resorcinol, phloroglucinol and dephenyl lactone are produced from the hydrolysis of HTs and are absorbed into the portal circulation (Murdiati et al., 1992). Tannic acid and gallic acid, the simplest and major phenolic component of hydrolysable tannins, at doses exceeding 0.4 g/kg BW per day resulted in toxicity symptoms in sheep (Murdiati et al., 1992). High levels of phenols in the blood stream is a typical sign of tannin toxicity and was observed in steers fed 50 g of oak tannin (an hydrolysable tannin) for 14 days while the LD50 for tannin toxicity in rabbits is 6.9 g/kg body weight per day for 5 days

(Bausch and Carson, 1981). The LD50 value is the dose at which 50% of the target organisms will die under exposure to the toxic substance. Clinical signs of tannin toxicity include anorexia, depression, a clear watery nasal discharge that may be blood stained, distended rumen, constipation that may occur for 2- 10 days and may be followed by dark, mucoid and often bloody diarrhoea (Bausch and Carson, 1981). Pathophysiological signs include haemorrhagic lesions on the terminal intestine, necrosis of the liver, kidney damage and proximal tuberal necrosis and beyond the detoxifying capacity of the liver, death may occur. Therefore, while HT are easily hydrolysable by microbial action of the rumen, condensed tannins are not susceptible to hydrolysis (Degen et al., 1995; Makkar and Singh, 1995; McSweeney et al., 2001b).

It is understood that CTs do not directly constitute any physiological risk to vital organs such as the liver, spleen, or kidney under normal physiological conditions because they are indigestible and not absorbed into the blood stream, but excreted completely via feaces (Terrill et al., 1994). However, a very high concentration of CT leads to intestinal damage and therefore higher permeability of condensed tannin into the bloodstream, which may result in toxicity symptoms. Similarly, in the presence of saponins, there is the tendency for higher absorption of tannins (CT and HT) into the blood stream (Makkar, 2003a). Mucosa damage also result in decreased nutrient absorption through it and therefore, a loss of dietary nutrient uptake (Reed, 1995).

1.5.8.6 Prospect for the encapsulation of dietary tannin extract to mask its astringency and its potential for improved utilization in ruminant feeding

Addition of CTs decreased methanogenesis significantly compared to control diets in a number of studies in sheep and dairy cattle (Carulla et al., 2005; Grainger et al., 2009) while in other studies, methane production was not effectively reduced (Beauchemin et al., 2007; Min et al., 2006). This has been attributable to the possibility of low CT concentration used in some of these studies where CT was ineffective (Patra and Saxena, 2010). High dietary concentrations that have proved to be consistently effective, may significantly depress dry matter intake, nutrient digestibility and animal productivity (Aerts et al., 1999). Compromising voluntary dry matter intake will impose serious production performance challenge on the animals especially in low quality forage systems. Tannin consuming herbivore species have been observed to exhibit pre-ingestive and post-ingestive countermeasures to protect themselves against the negative digestive effects of high CT consumption (Foley and Moore, 2005; Shimada, 2006).

Pre-ingestive countermeasures include self-regulation of CT consumption while post-ingestive countermeasures include the production of tannin-binding proteins in the saliva and increased degradation of tannins by microorganisms in the GIT (Dearing et al., 2005; McArthur et al., 1991; Shimada, 2006). Tannin binding salivary proteins (TBSPs) have been reported to act as a first line of defence by ruminants against dietary tannins in the oral cavity during digestion and may slightly limit the amount of tannin that eventually becomes available in the rumen (McArthur et al., 1991; Yisehak et al., 2012). It is widely recognized that saliva production plays a functional role in the digestive process of ruminant animals. Browser species such as mule deer with more tannin in their natural diets secrete more tannin-binding salivary proteins (TBSPs) than intermediate feeders like goat and grazers such as cattle and sheep with lower tannin content in their natural diets (Clauss et al., 2005; Estell, 2010; Mole et al., 1990). Studies on the effects of increased consumption of high dietary CT on the induction of increased salivation and production of salivary proteins have been inconsistent in ruminants. While some reports have shown increasing production of TBSPs and increased saliva secretion, which may affect rumen outflow following tannin ingestion, other studies have revealed no such counter measures (Alonso-Díaz et al., 2010; Hanovice-Ziony et al., 2010; Makkar and Becker, 1998; Salem et al., 2013; Shimada, 2006; Yisehak et al., 2012). However, it is generally noted that increased salivation and changes in saliva properties may significantly alter digestibility in the ruminant animal. Encapsulation of condensed tannin extract has the potential of masking its effects on salivary proteins and saliva production in the oral cavity and thus will confine the effect of CT to the rumen and post-rumen environment, where it influences rumen fermentation and digestion in the post-rumen segment.

Encapsulation technology has been used successfully to shield and maximize the biological effects of additives such as essential oils, micro nutrients, drugs and other phytochemicals as dietary supplement in ruminant diet (Ribeiro et al., 2013). Encapsulation technology in various forms such as emulsion of oil by formaldehyde-treated proteins, saponification of lipids (Doreau and Chilliard, 1997) or more advanced forms such as packing of liquid or solid micronutrient or additives in tiny capsules has been used to deliver active ingredients to target location and concentration in the digestive system of animals. This technology of encapsulation has been used in modulating the release of urea in ruminant diets resulting in slow release urea with improved N utilization as well as enhanced blood metabolites in ruminants (Cherdthong et al., 2011). Various encapsulation techniques have been investigated for use in ruminant animals (Paula et al., 2011). In the study conducted by Lin et al. (2013), essential oil

compounds such as eugenol, carvacrol, citral, and cinnamaldehyde were absorbed in microporous starch and the complex was subsequently encapsulated with sodium alginate to provide a stable dietary additive to improve rumen digestibility and mitigate methane emission. In that report, addition of the encapsulated essential oil compounds (EOC) and monosodium fumarate at up to 1g/day did not significantly depress rumen pH or nutrient digestibility in the animals. However, dry matter intake was significantly increased in the supplemented animals compared to un-supplemented animals, showing that encapsulation can be an effective way to promote the use of such compounds in ruminant animals.

To achieve optimum interaction of tannin with methanogenic archaea, it is noteworthy that the rumen is a batch fermentation vessel rather than a steady state fermentation vessel (Emanuele et al., 2006), therefore resulting in fluctuating rumen ecosystem characterized by microbial biomass, pH, ammonia nitrogen concentration etc. (Russell, 2002). Wood et al. (2009), encapsulated fumaric acid (organic acid) with high-melting point coconut and palm oil and evaluated its effect on feed intake and methane emission. A higher response in terms of feed intake, weight gain and reduced methane emission in the animals fed encapsulated-fumaric acid (EFA) was observed, compared to those fed unencapsulated-fumaric acid (FA) or the control diet. In that study, methane emission was reduced by 76% and 62% in the EFA and FA supplemented animals respectively. These may have been due to the effect of encapsulation in modulating the negative consequence of pH reduction and acidosis often associated with dietary fumaric acid inclusion in ruminant diets. An encapsulation method that limit tannin interaction in the mouth or provides a controlled or sustained release of tannin extract in the rumen has the potential of improving the utilization of tannin in ruminants.

1.6 Conclusions

It appears that dietary nitrate and condensed tannins as dietary mitigation options have shown promising potential for adoption in much of small ruminants' production systems of Africa. Application method may be a significant determinant of the use of dietary nitrate with feedstuffs deficient in protein to improve performance of animals and mitigate methane emission. The amount of nitrogen available in the rumen, the amount of residual nitrate that is able to trap free hydrogen in the rumen and nitrate toxicity level may all be influenced by the application method. Limitations in terms of the use of tannin either as crude extract or as component of feed include its astringency, which limit dry matter intake, and its effect on

reducing nutrient digestibility. These conclusions have resulted in the development of the following research objective.

1.7 Objective of the study

1.7.1 General objective of the study

To identify effective means that enhances the utilization of nitrate and tannins as dietary mitigation options suitable for modulation of rumen fermentation and reducing greenhouse gas emissions in small ruminant production systems of South Africa.

1.7.2 Specific objectives of the study

1. Evaluate the effects of replacing urea with nitrate in two methods of application (as a feed supplement and in pre-treatment of grass hay) on digestibility, *In vitro* gas and methane production of *Eragrostis curvula* hay.

2. Evaluate the effect of Eragrostis hay treated with graded levels of urea and calcium nitrate on *In vitro* digestibility, gas production and methane emission.

3. Evaluate the effects of different sources of NPN with or without condensed tannin extract on dry matter intake, nutrient digestibility, growth performance and methane emission in growing South Africa Mutton Merino lambs.

4. Evaluate the effect of NPN sources, with or without condensed tannin inclusion on the haematology and serum biochemical parameters of growing Merino lambs.

5. Prepare and characterize an encapsulated-tannin extract for improved utilization of condensed tannins in ruminant production.

6. Evaluate the effect of the encapsulated-tannin extract on feed intake, nutrient digestibility and methane production in sheep.

1.8 Justification of the study

1. Sustainable ruminant production in much of the tropics will continue to depend on low-quality feed resources because of the increasing cost of grains, therefore, higher methane emissions.

2. Application method may be a significant factor in determining the effectiveness and physiological safety of the animals with regard to the use of dietary nitrate. The type of

nitrate salt (solubility), dose, mode of administration and the associated diet characteristics will determine the usefulness of nitrate supplementation, and therefore this has to be validated for different production systems.

3. Much of the research on nitrate supplementation or condensed tannin in roughage based feeding system in ruminants has been limited to *in vitro* studies. There is a need to further evaluate their effect using animal evaluation trials to validate the *in vitro* results.

4. The tropics and subtropics remains a rich source of tanniferous plant biodiversity, and therefore, harnessing their beneficial properties for livestock husbandry purposes is expected to continue to gain wider attention. For example, the recent interest in the modulation of rumen degradability of protein cakes using tannin extracts have made them become important commercial additive. Their potential antimethanogenic properties will therefore be important in sustainable production systems.

5. Encapsulation of tannin extract has the potential of providing better utilization of tannins in ruminant animal nutrition applications.

6. With adequate encapsulation, animals may be able to tolerate higher levels of tannin supplementation without experiencing the adverse effects of tannin on dry matter intake and nutrient digestibility.

7. If nitrate supplementation alone is capable of reducing enteric methane emission, one can expect a greater reduction of methane when nitrate and tannin are combined as additives in the diet of animals. In this regard, depression of total organic matter digestibility by tannin may be reduced with nitrate as the major rumen nitrogen source.

8. There is a possibility of a synergistic effect when two or more feed additives that can exhibit positive interaction on the ruminal ecosystem are combined appropriately with the aim of improving nutrient utilization and methane reduction.

CHAPTER TWO

In vitro methane production of Eragrostis hay treated with graded levels of urea or nitrate

Abstract

Urea treatment of protein deficient feeds serve as a source of ammonia nitrogen supply for rumen fermentation. This study was undertaken with the objective of determining the effect of treating *Eragrostis curvula* hay with varying levels of urea or nitrate on digestibility and *in vitro* fermentation. Grass hay was sprayed with urea solution at 0.5%, 1.0%, and 1.5% DM and calcium nitrate was used as a replacement of urea on an iso-nitrogenous basis. This was followed by 30 days anaerobic storage in airtight bottles with each treatment having three replicates. Following anaerobic treatment, hay samples were dried, milled and evaluated for their chemical composition, *in vitro* organic matter digestibility, and *in vitro* ruminal fermentation and methane production. Feed treatment with both urea and nitrate reduced ADF content of hay, while crude protein content was increased. *In vitro* organic matter digestibility of treated hay increased with inclusion levels, although urea recorded higher values than nitrate. Nitrate treatment significantly reduced *in vitro* methane by 14-33% while there was no significant methane reduction in the urea treated diets. Total volatile fatty acid, ammonia N and pH across treatments were statistically similar ($p > 0.05$). While urea treatment had the most improved digestibility, it did not confer additional benefits when compared to the nitrate treatment that provided an acceptable level of improvement in feed digestion and fermentation with the additional benefit of methane reduction. Nitrate can thus be incorporated into feed treatment to improve the nutritional value of poor quality hays.

Key words: ammoniation, digestibility, Eragrostis hay, nitrate, methane emission

2.1 Introduction

In much of the tropical and sub-tropical areas of Africa and Asia, small ruminants contribute to a significant proportion of the farmer's income, who exploit their ability to convert roughage feeds to edible meat or milk (Ben Salem and Smith, 2008). With changing climatic patterns, and decline in rangeland resources, there remains a shortfall in total feed resources available to these class of animals. They therefore often rely on hays, straws, and other crop residues to meet their dietary requirements, especially during the dry seasons (Ben Salem and Smith, 2008).

Various chemical treatment methods have been developed to improve the feeding value of these poor quality roughage feeds, such as alkali treatment, use of aqueous ammonia, or by urea treatment (ammoniation) under anaerobic conditions (Mapato et al., 2010; Wanapat et al., 2009). Urea treatment involves spraying with aqueous urea followed by anaerobic storage for up to 3 weeks prior to feeding. It not only improves ammonia nitrogen supply to the rumen but its ammoniation process helps to soften the fibre structures in feed for greater microbial attachment, thus leading to improved digestibility (Vadiveloo, 2003). Urea hydrolyses to ammonia during feed treatment incorporates NPN into the treated material and is also an effective preservative or fungicide (Oji et al., 2007). Non-protein nitrogen (NPN) in chemical treatment of feed is particularly valuable with feedstuffs high in fermentable carbohydrates but low in crude protein (Do et al., 2011). There is usually an increase in feed intake and nutrient digestibility in ruminants feeding on low quality roughages with supplemental proteins or non-protein nitrogen. This is because digestibility of these materials is dependent on adequate colonization of cellulolytic bacteria, which needs adequate supply of ammonia nitrogen for their own microbial synthesis (Mahr-un-Nisa et al., 2004).

Replacing urea with nitrate salts have recently received wide consideration because of its potential benefits in mitigating enteric methane production (Sophal et al., 2013). Nitrate is able to recycle hydrogen ions that are easily converted to methane by rumen archaea (Thanh et al., 2012). Nitrate is able to compete favourably with these methanogenic archaea for the available hydrogen ions in the rumen, thus capable of reducing methane by up to 80% (Leng, 2008). It is hypothesised that the method of nitrate application will determine its effectiveness, optimum dose and toxicity level in ruminant animals as earlier suggested by Zijderveld *et al.* (2010). Therefore, the focus of this study is to know the effect of Eragrostis hay treated with graded

levels of urea and calcium nitrate on *in vitro* digestibility, gas production and methane emission.

2.2 Materials and Methods

This study was conducted at University of Pretoria's Experimental Farm after the approval of the trial protocol by the Animal Ethics Committee of University of Pretoria (No. EC061-14).

Feed treatment and experimental design

Eragrostis curvula hay was collected from the University of Pretoria Experimental Farm's feedstock, chopped to about 5cm and used as the experimental diet. Feed treatment was carried out following the procedure described by Tesfayohannes *et al*. (2013). Approximately 3 kg of hay was mixed with urea or nitrate at iso-nitrogenous level in each batch. Both urea and calcium nitrate were feed-grade fertilizers obtained from Introlab Chemicals (Pty.) Ltd., Pretoria, South Africa. The experimental treatments were arranged to include two nitrogen sources (urea or nitrate) at three levels of inclusion (2.33g, 4.66g and 6.99g nitrogen kg^{-1} hay DM) in a 2 x 3 factorial plus a control treatment that contained no additive. Urea or calcium nitrate was dissolved into 1 litre of water whilst stirring until all the urea was dissolved. The entire solution was then sprayed onto about 3 kg of hay (on a DM basis) and hand mixed inside a wide plastic bowl. The final mix had a total moisture content of approximately 40%. Grass hay was filled into glass bottles which served as mini silos, compressed, and subsequently sealed anaerobically after sucking out the air to create a vacuum. This was repeated in three replicate glass bottles per treatment group. A control diet was mixed with a the same amount of distilled water but had no added additives. Each bottle was stored at room temperature (25°C) for 30 days. After the 30 day period, the glass bottles were opened, the upper 5cm discarded, and the contents emptied into a big plastic container, then hand mixed and sub-sampled as urea or nitrate treated diets. All experimental diets were freeze-dried and milled to pass through a 1 mm screen (Wiley mill) before analysis.

In vitro fermentation

Rumen buffer, macro-mineral and micro-mineral solutions were prepared as described by Goering and Van Soest (1970) with the modifications of Mould *et al.* (2005). The *in vitro* fermentation procedure of Menke *et al.* (1979) was followed. The prepared buffer solution was kept inside a water bath at 40°C and continuously purged with CO_2 until the solution turned colourless. Rumen fluid was collected from two rumen-cannulated merino wethers fed Lucerne hay *(Medicago sativa) ad libitum*. Detailed procedures have been previously described by

Hassen *et al.* (2015). Gas pressure was taken at 2, 4, 8, 12, 24, and 48 h after commencement of incubation, while gas samples were taken inside Hamilton syringes for the analysis of methane concentration. At the end of incubation, fermentation was terminated by removing the serum bottles and immersing them in ice to impede microbial activity. Rumen fluid pH was measured after incubation using a pH meter (Metler Toledo 230 pH meter) while supernatant was collected and stored at -20°C for ammonia-N (Broderick and Kang, 1980) and VFA analysis.

In vitro organic matter digestibility

The *in vitro* organic matter digestibility (IVOMD) of diets was determined using the two-phase digestion method of Tilley and Terry (1963) as modified by Engels and Van Der Merwe (1967). During the first stage, 200 mg of feed samples were incubated in four replicates of each diet with rumen liquor for 48 h at 39°C under anaerobic conditions. Blanks and a standard feed were included in each batch of incubation. This was followed by an acid-pepsin digestion phase for 48 hours. After digestion, the residual material was oven dried at 105°C for 18 hours, weighed, and subsequently ashed in a muffle furnace at 550°C for 3 hours. Three repeated batches/runs were carried out while replicates within each incubation run were pooled together as analytical replicates. The amount of *In vitro* organic matter digested was estimated from the weights of the starting material and residuals.

Methane production measurement

Gas samples from the *in vitro* incubations were taken using a Hamilton syringe on duplicate incubation bottles at 2, 12, 24 and 48 h incubation time (Gemeda and Hassen, 2015). Two blanks were included to correct the methane produced from the inoculum in each run, and two runs were executed. Methane concentration was analysed with gas chromatography (8610C BTU Gas Analyser GC System; SRI Instruments GmbH, Bad Honnef, Germany). The GC was pre-equipped with a solenoid column, packed with silica gel and a flame ionization detector (FID). Methane concentration values were related to the total gas production in order to estimate its concentration (Tavendale *et al.* 2005). Methane concentration was subsequently converted to energy and mass values (Santoso et al., 2007).

Chemical composition analysis

Urea and calcium nitrate treated hay samples were analysed for dry matter, ash and crude protein by the Leco/Dumas method according to AOAC, (2000) as indicated in ID 934.01, ID 942.05 and ID 968.06 respectively. Neutral detergent fibre (NDF), acid detergent fibre (ADF)

and acid detergent lignin (ADL) were determined according to Robertson and Van Soest (1981). The NDF content was determined inclusive of residual ash without the use of heat-stable amylase.

Calculations and statistical analysis

The concentrations of acetate, propionate, butyrate, valerate, and iso-butyrate were evaluated as molar proportions (mmol100 mol^{-1}) while total volatile fatty acid (TVFA) concentration was expressed in $mmolL^{-1}$ and ammonia-N concentration in mg dL^{-1}. Total gas and net methane was expressed in mL and in mass values per unit of IVOMD (g kg^{-1} IVOMD) and TVFA per unit methane ($mmolmmol^{-1}$). The ratio of non-glycogenic to glucogenic volatile fatty acid is expressed as acetate/propionate molar ratio. The chemical composition of samples were analysed as a one-way analysis of variance while *In vitro* data were analysed as a 2 X 3 factorial plus control, using the GLM procedure of SAS (Statistical Analysis System, version 9.3) using the model statement: $Yij = \mu + Bi + Tj + + eij$

Where, μ= overall mean, Bi = block effect (replicate), Tj = treatment effect, Yij= mean of individual observation and eij= residual error. Mean separation was done using Tukey's test and significance was declared at $p < 0.05$. Using single degree of freedom contrasts, comparison of means were done between i) control vs NPN-treated hay ii) urea treated vs nitrate treated hay iii) the linear effect of urea levels iv) the quadratic effect of urea levels v) the linear effect of nitrate levels vi) quadratic effect of nitrate levels.

2.3 Results

The chemical composition of untreated, urea treated and nitrate treated Eragrostis hay is shown in Table 2.1. The anaerobic treatment increased ($p < 0.05$) crude protein (CP) content of Eragrostis hay by between 8-25% in the urea diets and between 15-33% in the nitrate diets. However, nitrate was more effective in increasing the nitrogen content of the treated hay than urea at the same level of inclusion. There was a decrease in cell wall content (NDF and ADF) of treated hay as inclusion levels of urea increased ($p < 0.05$). However, there was no reduction ($p \geq 0.05$) in the NDF content of treated hay with increasing inclusion levels of nitrate but the ADF content decreased ($p < 0.05$). Urea or nitrate inclusion did not affect ($p \geq 0.05$) the ADL content of the diet across the treatments.

Table 2.1 Chemical Composition (Mean ± SE) of untreated, urea treated and nitrate treated Eragrostis hay

Parameters[1]	Untreated	Urea-treated hay[2]			Nitrate-treated hay[2]			*P*-value
		I	II	III	I	II	III	
DM (g kg^{-1} DM)	912.5±1.8	882.5±0.6	863.2±0.8	850.7±1.4	836.7±1.1	875.4±3.2	880.5±2.5	0.325
CP (g kg^{-1} DM)	53.8^{e}±0.16	58.3^{d}±0.38	62.6^{c}±0.15	67.0^{b}±0.11	61.9^{c}±0.15	65.0^{b}±0.14	71.7^{a}±0.26	0.002
Ash (g kg^{-1} DM)	30.4±0.4	29.7±0.1	29.5±0.3	30.0±0.2	29.6±0.3	28.8±0.4	29.4±0.6	0.152
NDF (g kg^{-1} DM)	746.6^{a}±3.2	732.8^{b}±1.7	712.5^{c}±1.4	709.8^{c}±2.8	736.3^{b}±1.1	734.0^{b}±2.0	731.0^{b}±1.5	0.0002
ADF (gkg^{-1} DM)	449.4^{a}±0.9	442.8^{b}±2.1	433.5^{c}±2.5	414.1^{d}±1.2	452.1^{a}±2.6	443.6^{b}±1.8	428.3^{c}±2.5	<0.0001
ADL (gkg^{-1} DM)	79.1±1.5	79.0±0.6	78.8±1.1	78.6±0.8	78.6±1.2	79.1±3.2	78.7±2.4	0.275

[1]DM, dry matter; CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre; ADL, acid detergent lignin. [2]I, II, III; 2.33, 4.66 and 7.0g N kg^{-1} hay & equivalent to 5, 10 and 15g urea and 13.6, 27.3 and 40.9g calcium nitrate kg^{-1} hay respectively. SE, standard error. Means in the same row with different superscripts are significantly different ($p < 0.05$).

The result of the *in vitro* gas production and digestibility of Eragrostis hay, untreated or treated with urea or calcium nitrate is shown in Table 2.2. Simple effects of nitrogen source showed that total gas production at 48 h was generally not affected by the average effects of nitrogen treatment when compared to the untreated hay, (105 vs 98.9 mL) but within the treated group, gas production was on average, higher ($p < 0.05$) in urea treated hay compared to nitrate treated hay (105 vs 84.5 mL). Increasing levels of inclusion did not affect gas production in both urea and nitrate treated hays ($p \geq 0.05$). Generally, urea treated hays showed higher methane production ($p < 0.05$) compared to nitrate treated hays although increasing inclusion levels of both urea and nitrate did not show any linear or quadratic response ($p \geq 0.05$). As high as 32% reduction in methane production was recorded in nitrate treated hay at 6.99 g nitrogen kg^{1}DM of calcium nitrate inclusion level compared to the untreated hay.

Both nitrogen sources improved *in vitro* organic matter digestibility (IVOMD) of treated Eragrostis hay compared to control. There was both linear and quadratic increase in IVOMD with increasing inclusion levels in both urea and nitrate treated hays. Values ranged from 455 g kg^{-1} in untreated hay to 502 g kg^{-1} in urea treated hay, with urea treated hays consistently resulting in better improvement in digestibility compared to nitrate treated hays across all three levels of inclusions (463 vs 458; 476 vs 460; 501 vs 485 g kg^{-1}) respectively. The ratio of methane to total gas produced showed that nitrate treated hay recorded lower methane ($p < 0.05$) as a proportion of the total gas when compared to the urea treated hays. Similarly, there was a reduction ($p < 0.05$) in the ratio of methane produced per unit of organic matter digested in the nitrate treated hay compared to urea treated hay. However, methane produced per unit of organic matter digested did not produce any linear or quadratic response across the inclusion levels in the nitrate treatment. In contrast, there was a linear increase in methane per unit of organic matter digested with increasing levels of urea inclusion. The ratio of TVFA to methane showed that nitrate treated hays recorded higher TVFA per unit of methane when compared with urea treated hays, ($p < 0.07$) however, there was no linear or quadratic response with increasing inclusion levels.

Table 2.2 *In vitro* gas production, digestibility and fermentation efficiency in *Eragrostis curvula* hay ammoniated with different levels of urea and nitrate

Parameter[1]	Control	Urea[2]			Nitrate[2]			SEM	Contrast *P*-Values[3]					
		I	II	III	I	II	III		C vs N	U vs N	U_L	U_Q	N_L	N_Q
48H Gas (mL)	98.9	106	103	106	88.7	93.8	82.4	3.63	0.78	0.02	0.21	0.94	0.61	0.78
48H Methane (mL)	23.2	26.3	27.6	26.6	19.7	18.7	15.8	1.37	0.79	0.002	0.07	0.96	0.40	0.48
IVOMD (g kg^{-1} DM)	455.1	463.0	475.8	501.9	457.6	459.8	486.1	3.64	<.0.001	0.001	<.0.001	0.001	0.003	0.001
Methane/Total Gas	0.24	0.25	0.26	0.25	0.22	0.20	0.18	0.01	0.59	0.001	0.01	0.82	0.45	0.29
Methane/IVOMD (g kg^{-1} IVOMD)	91.0	101.8	103.9	94.7	76.6	72.7	54.5	2.02	0.57	0.003	0.04	0.87	0.79	0.38
TVFA/Methane	6.85	5.82	6.70	7.29	8.82	8.93	9.34	0.03	0.56	0.07	0.16	0.87	0.76	0.62

[1]IVOMD, *in vitro* organic matter digestibility; TVFA, total volatile fatty acid.[2]I, II, III; 2.33g, 4.66g and 6.99g N kg^{-1} hay DM respectively & equivalent to 5 g, 10 g, & 15 g urea kg^{-1} hay & 13.6 g, 27.3 g, & 40.9 g calcium nitrate kg^{-1} hay. [3]Contrast analysis across treatments: C vs. N, control vs. the average of NPN-treated diets; U vs. N, average of urea-treated diet vs. average of nitrate-treated diets; U_L, linear effect of urea levels; U_Q, quadratic effect of urea levels; N_L, linear effect of nitrate levels; N_Q, quadratic effect of nitrate levels.

In vitro fermentation characteristics of urea and nitrate treated Eragrostis hay is shown in Table 2.3. Total VFA production was not influenced by nitrogen source or levels of inclusion across the diets. Nitrate treated hays recorded higher acetate concentration compared to urea treated hay ($p < 0.05$). Increasing inclusion levels showed a linear response in the nitrate treated hays. Butyrate, and valerate concentrations did not show any differences between the nitrogen sources ($p \geq 0.05$) while propionate and iso-butyrate concentrations were lower in nitrate treated hays compared to urea treated hays ($p < 0.05$). This reduction in propionate also led to an increase in the ratio of non-glucogenic to glucogenic volatile fatty acids as shown by the A/P molar ratio where the average effect of nitrate treated hays showed higher A/P molar ratio compared to the average effect of urea treated hays ($p < 0.05$). There was no difference in rumen ammonia-nitrogen concentrations between urea and nitrate diets ($p \geq 0.05$) while the pH of the rumen fluid, after 48 h incubation was also not affected by the nitrogen source or levels of inclusion.

Table 2.3 *In vitro* fermentation parameters of *Eragrostis curvula* hay as influenced by ammoniation with different levels of urea and nitrate

Parameter[1]	Control	Urea[2]			Nitrate[2]			SEM	[3]Contrast*P*-values					
		I	II	III	I	II	III		C vs N	U vs N	U_L	U_Q	N_L	N_Q
TVFA (mmolL^{-1})	157	132	166	173	162	173	153	12.0	0.87	0.97	0.98	0.69	0.67	0.85
Acetate (mmol 100ml^{-1})	64.6	60.2	64.1	62.0	61.9	64.5	68.7	2.89	0.35	0.003	0.41	0.50	0.04	0.25
Propionate (mmol100mol^{-1})	24.9	25.9	25.0	25.4	24.3	24.1	21.1	1.90	0.37	0.002	0.001	0.04	0.75	0.61
Butyrate (mmol100mol^{-1})	5.93	7.88	6.64	8.09	8.65	7.11	7.37	0.50	0.003	0.64	0.18	0.01	0.02	0.58
Isobutyrate (mmol100mol^{-1})	2.49	3.28	2.01	2.25	2.30	1.93	1.33	0.33	0.11	0.001	<.0.001	0.03	0.09	0.28
Valerate (mmol100mol^{-1})	2.12	2.81	2.31	2.23	2.80	2.37	2.36	0.24	0.08	0.69	0.73	0.07	0.84	0.05
A/P Molar ratio	2.62	2.32	2.57	2.44	2.55	2.67	3.25	0.14	0.66	0.01	0.12	0.25	0.60	0.04
NH_3-N (mg dL^{-1})	11.9	12.2	13.5	15.8	12.7	14.7	14.8	2.1	0.84	0.54	0.55	0.97	0.63	0.55
pH	6.78	6.77	6.78	6.78	6.87	6.87	6.87	0.02	0.57	0.12	0.18	0.85	0.96	0.95

[1]TVFA, total volatile fatty acid; A/P Molar ratio, acetate to propionate molar ratio; NH_3-N, ammonia nitrogen. [2]I, II, III; 2.33 g, 4.66 g and 6.99 g N kg^{-1} hay DM respectively & equivalent to 5 g, 10 g, & 15 g urea kg^{-1} hay & 13.6 g, 27.3 g, & 40.9 g calcium nitrate kg^{-1} hay.[3]Contrast analysis across treatments: C vs. N, control vs. the average of NPN-treated diets; U vs. N, average of urea-treated diet vs. average of nitrate-treated diets; U_L, linear effect of urea levels; U_Q, quadratic effect of urea levels; N_L, linear effect of nitrate levels; N_Q, quadratic effect of nitrate levels.

2.4 Discussion

Treating Eragrostis hay with urea or calcium nitrate up to 1.5% of DM, increased its nitrogen content. A similar trend of increased nitrogen content after anaerobic treatment of roughage feeds has been observed in the urea treatment of hays and crop residues as reported in literature (Fadel Elseed et al., 2003; Oji et al., 2007). The solubilization of NPN (urea or calcium nitrate) during anaerobic treatment was more pronounced in the urea treated diet as judged by the lower residual nitrogen content of urea treated hay compared to the nitrate treated hay which recorded higher residual nitrogen content. For the urea treatment, the breakdown of urea can be related to the optimal conditions that favour the activity of the urease enzyme which enables the hydrolysis of urea to ammonia. According to Oji *et al.* (2007), moisture content of treated forage above 375g kg^{-1} favours the urea hydrolysis to ammonia. The dissimilatory reduction of calcium nitrate to ammonia has been noted as the major pathway for nitrate breakdown under anaerobic conditions often in the presence of high organic matter concentrations. However, high ammonia accumulation has also been noted to impede the further breakdown of nitrate under such conditions (Simon, 2002; Leng 2008). The higher IVOMD of Eragrostis hay in urea treated hay compared with nitrate treated hay may also be an indication that the feed treatment process favours the hydrolytic reduction of urea more than the nitrate reduction process. The ability of aqueous ammonia to penetrate and soften the cell wall structure of roughage feeds for improved digestibility is an indication of an effective ammoniation treatment.

Generally, treatment with urea or nitrate decreased the cell wall content of Eragrostis hay and this was consistent with previous research. Oji *et al.* (2007) observed a trend of reduction in NDF and ADF contents of maize stalks, maize cobs and maize husks following urea and aqueous ammonia treatment, and this can be related to increased digestibility. Tesfayohannes *et al.* (2013) also noted that improved IVOMD following feed treatment is attributable to a reduction in NDF and hemicellulose content of poor quality feeds and increase in the degradable portion of ADF. Solubilization of fibre fractions due to linkage disintegration following treatment has been reported by Mason *et al.* (1988) while Zorrilla-Rios and Owens (1985) noted increased fragility of wheat straw following ammoniation. The process of ammoniation like many other hydrolytic treatment methods, helps to improve feed digestibility by disruption of the cell wall structure by ammonia and increased swelling resulting in a higher affinity for microbial attachment (Fahey et al., 1993). Treatment of poor quality roughage diets with urea or aqueous ammonia have been found very effective in improving their digestibility (Adejoro and Hassen, 2017; Uza et al., 2005). However, several factors have been found to influence the

feed treatment process, such as type of feedstuff, and other treatment conditions (Mason et al., 1988).

From the result of this study, it appears a considerable amount of nitrate was hydrolysed during feed storage to account for the differences in fibre composition and *in vitro* digestibility of treated hay, with a small residual nitrate remaining to potentially act as hydrogen scavenger during rumen fermentation *in vitro*. The activities of the residual nitrate in the treated hay generally played a role in mitigating methane production when compared to the average effects of urea treated hay, but the levels of inclusion of nitrate was not significant. In contrast, a linear response was observed in terms of increased methane production associated with the inclusion level of urea in the urea treated hay. This was similar to previous experiments involving nitrate supplementation, where nitrate resulted in methane reduction, both *in vitro* and *in vivo* (Hulshof et al., 2012; Nolan et al., 2010; Sophal et al., 2013; Thanh et al., 2012).

Values of ammonia nitrogen concentration did not show any differences across treatment groups. They were lower than the minimum of 15-20 mg dL^{-1}proposed by Preston and Leng (1987) for effective feed intake and fibre digestion in roughage based diets. The pH ranges observed in this study were however within the normal range of 5.5 to 7.0 for optimum rumen function (Krause and Oetzel, 2006).With considerable hydrolysis of both urea and nitrate during feed treatment, higher inclusion levels of urea or nitrate may thus be required in treating Eragrostis hay to provide allowance for residual nitrate or urea to meet the ammonia-nitrogen requirements of the animals. Inclusions levels of up to 20 g kg^{-1} DM have been reported for urea treatment of rice straws, (Fadel Elseed et al., 2003) but at the same time, considerations must be given to the total amount of nitrogen consumed by each animal per day to avoid excessively high ammonia nitrogen accumulation in the rumen. The reduction in the molar proportion of propionate in the nitrate treated hay is consistent with the findings of Nolan *et al.* (2010) for nitrate supplementation because nitrate tends to compete with propionate synthesis (Van Zijderveld *et al.* 2011). This also explains the higher A/P molar ratio recorded in the nitrate treated hays. The ability of nitrate to recycle hydrogen ions into ammonia, thus competing for reducing equivalents away from propionate synthesis have been noted as capable of limiting animal productivity, but the results of other *in vivo* trials have not shown any significant reduction in productivity of animals in terms of energy in milk, energy retention or nitrogen retention (Van Zijderveld *et al.* 2011; Sophal *et al.* 2013).

2.5 Conclusion

The treatment of Eragrostis hay with graded levels of calcium nitrate showed on average a significantly higher methane suppression than urea treated hay, with both urea and nitrate increasing *in vitro* digestibility compared to the untreated hay. However, the improvement in *in vitro* digestibility was somewhat smaller in the nitrate treated hay than with the urea treated hay. The additional benefit observed in terms of reduction in methane production implies the potential of incorporating nitrate into feed ammoniation as a possible climate smart agricultural practice. Considering the increasing improvement in digestibility and gas production across the graded levels, higher inclusion levels of urea and calcium nitrate in treating Eragrostis hay is required while a response surface analysis will help to determine the optimum level of NPN treatment.

CHAPTER THREE

Effect of supplementing or treating *Eragrostis curvula* hay with urea or nitrate on its digestibility and *in vitro* fermentation

Abstract

The potential of dietary nitrate to reduce enteric methane, apart from being a source of rumen-degradable nitrogen, has stimulated further research into its use. However, its suitability in feed treatment requires further investigation. The objective of this study was to determine the effects of urea or nitrate and two methods of non-protein nitrogen (NPN) application (anaerobic pre-treatment versus direct supplementation) to *Eragrostis curvula* hay, on its *in vitro* ruminal fermentation. An iso-nitrogenous level of NPN (7 g nitrogen/kg feed dry matter) from either urea or calcium nitrate was used to pre-treat hay by subjecting it to 30 days' anaerobic storage in airtight bottles, or by direct supplementation, each diet having three replicate bottles. Hay samples were dried, milled, and evaluated for *in vitro* organic matter digestibility (IVOMD), and *in vitro* gas and methane production, while rumen fluid was analysed for pH, ammonia nitrogen and volatile fatty acids. Compared with the untreated hay (control), NPN under both methods of application generally did not increase 48-hour gas production, but increased the IVOMD of *E. curvula* hay. However, pre-treatment of *E. curvula* hay improved IVOMD more than supplementation. Urea inclusion enhanced digestibility more than nitrate and the urea pre-treatment was more effective than supplementation. In contrast, nitrate inclusion significantly reduced methane production compared with urea and the control, and supplementation of nitrate was more effective in reducing methane than pre-treatment with nitrate. Pre-treatment with nitrate increased digestibility and reduced enteric methane emission similar to supplementation, indicating the potential of using nitrate as a hydrolytic agent in feed treatment.

Keywords: digestibility, *Eragrostis curvula*, feed treatment, nitrate, methane emission

3.1 Introduction

Roughages such as grass hay, agro-industrial by-products, and crop residues have remained the main feed resources for ruminant feeding in larger parts of tropical and subtropical Africa (Preston and Leng, 1987). The roughages could become significant ingredients, even in the diet of high-producing animals, if their digestibility could be improved (Wanapat et al., 2009). The availability of rumen ammonia is often a primary deficit when protein-deficient feedstuffs such as grass hays are fed to ruminants, limiting microbial biomass production, digestibility and feed intake (Hao Phuc et al., 2009). Enteric methane emissions are dependent on the amount of feed consumed by the animal and the composition of the diet, with lower emission values from concentrate rather than roughage-based diets (Mirzaei-Aghsaghali and Maheri-Sis, 2011). Methane per unit of organic matter degraded could be up to two times higher in tropical forages than in legumes (Widiawati and Thalib, 2007). In the past, many studies have been conducted globally on improving the feeding value of roughages (Shreck, 2013; Wanapat et al., 2009). One such method involves treating feedstuff with aqueous ammonia or urea through the process of ammoniation (Diaz et al., 2013; Mapato et al., 2010; Moreira Filho et al., 2013). Urea has been used as a hydrolytic agent that helps to disrupt the cell wall structure, and break the ester bonds between lignin, cellulose and hemicellulose, thus improving the digestibility of feedstuffs (Vadiveloo, 2003). Urea is also converted to ammonia in the rumen, thus contributing to the synthesis of microbial protein (Mapato et al., 2010; Schroeder and Titgemeyer, 2008; Shreck, 2013). Under anaerobic systems, such as in feed treatment, nitrate can be converted to ammonia via dissimilatory nitrate/nitrite reduction and assimilatory nitrate reduction to ammonia (ANRA) which is also called nitrite ammonification. This is particularly favoured under low redox potential values, high organic matter and in the presence of sulphide ions (Leng, 2008). Under ammoniation treatment, the presence of urease enzyme enables the hydrolysis of urea to ammonia. According to Oji *et al.* (2007), a range of optimum moisture concentrations favour urea hydrolysis to ammonia.

Urea was a popular non-protein nitrogen (NPN) source of choice in the feeding system of ruminants. More recently, however, there has been renewed interest in the use of nitrate because of its potential benefits as a source of degradable N, and for methane mitigation (Li et al., 2012). Urea could be replaced with nitrate in low rumen degradable protein diets to provide a source of rumen ammonia, while reducing enteric methane production (Le Huyen et al., 2010; Takahashi, 2011; Van Zijderveld et al., 2010). Under anaerobic conditions, nitrate could undergo conversion to ammonia (Leng, 2008). The major limitation to the use of nitrate and other NPN sources in ruminant feeding is their potential to induce methemoglobinemia as a

result of elevated ammonia-N and nitrite in the rumen (Lee and Beauchemin, 2014; Newbold et al., 2014; Van Zijderveld et al., 2010). However, the risk of toxicity could be reduced through gradually adjusting the animals to a high nitrate diet with regulated dosing (Lee and Beauchemin, 2014; Newbold et al., 2014; Nolan et al., 2010). Earlier studies have shown that the method of nitrate application has significantly influenced its utilization and toxic dose in animals (Van Zijderveld et al., 2010). However, the suitability of nitrate in the treatment of poor-quality roughages to improve their utilization and reduce the enteric methane has not been evaluated extensively in South Africa. *Eragrostis curvula* hay is a popular feed resource in South Africa, with mean crude protein content of usually less than 60g/kg DM, which may require supplementation or treatment with NPN (Tesfayohannes et al., 2013). Therefore, it is hypothesised that nitrate instead of urea, and treatment rather than supplementation will improve the *in vitro* organic matter digestibility and *in vitro* ruminal fermentation as well as reduce the enteric methane emission of *Eragrostis curvula* hay. This study, therefore, aims to compare the effects of replacing urea with nitrate in two methods of application (as a feed supplement and in pre-treatment of grass hay) on digestibility, *in vitro* gas and methane production of *Eragrostis curvula* hay.

3.2 Materials and Methods

This study was conducted at the University of Pretoria's Experimental Farm after the approval of the trial protocol by the Animal Ethics Committee of University of Pretoria (No. EC061-14). *Eragrostis curvula* hay was pre-treated or directly supplemented with NPN from two sources (urea and nitrate). The amount of urea or nitrate was set at iso-nitrogenous levels to provide 7 g nitrogen/kg hay DM as the supplemented group. For the second or treated group, hays were mixed and subsequently kept under anaerobic conditions for 30 days prior to sampling. An *in vitro* digestibility and gas production study was conducted to evaluate the response to NPN (urea or nitrate) supplementation or treatment. Urea and calcium nitrate are feed-grade fertilizers, which were obtained from Introlab Chemicals (Pty) Ltd, Pretoria, South Africa.

Eragrostis curvula hay was collected from the University of Pretoria Experimental farm's feedstock, chopped to about 5 cm, and used as experimental diets. Feed treatment was carried out according to the procedure described by Tesfayohannes et al., (2013). A weighted quantity of urea/calcium nitrate was dissolved into 4 litres of water whilst stirring until all the urea was dissolved. The 4-litre solution was then sprayed onto 10 kg of hay (on a DM basis), with the final mix having a moisture content of approximately 40%. Grass hay was thoroughly hand mixed with additives inside a big plastic container and subsequently sampled for the *in vitro* evaluation of urea or nitrate-supplemented diets. For the second treatment, grass hay was pre-

mixed (as for the supplemented group) with urea or nitrate solutions at the same iso-nitrogenous (7.0 g N/kg DM) and moisture level, filled into glass bottles which served as mini silos, compressed, and air was sucked out and subsequently sealed in three replicate glass bottles per treatment group. The Control treatment contained a similar amount of water but no additive was added prior to sampling. Each bottle was stored at room temperature (25°C) for 30 days. After the 30 day period, the glass bottles were opened, the upper 5 cm was discarded, and the contents were emptied into a big container, then hand-mixed and sampled for *in vitro* evaluation as urea or nitrate-treated diets. All experimental diets were freeze-dried and ground to pass through a 1-mm screen (Wiley mill) before analysis.

Rumen buffer, macro-mineral and micro-mineral solutions for the experiment were prepared as described by Goering and van Soest, (1970) with the modifications of (Mould et al., 2005). The prepared buffer solution was kept in a water bath at 40°C and continuously purged with CO_2 until the solution turned colourless. Rumen fluid was collected from two rumen-cannulated merino *wethers* fed lucerne hay *(Medicago sativa) ad libitum*, strained through four layers of cheesecloth into a pre-warmed thermos flask, and transported quickly to the laboratory. Rumen fluid was blended in the laboratory, mixed with the buffer-mineral solution in the ratio of rumen fluid to buffer solution (3:5), and added to each serum bottle, which already contained approximately 400 mg of each diet, under a continuous stream of CO_2. Each bottle was sealed with a rubber stopper and crimp seal cap. For each incubation run, four blanks, containing only rumen fluid, were used to correct for the effect of rumen fluid. In each incubation run, each test diet had four replicates per run, and three runs were conducted. All bottles were placed in the incubator at 39°C and 120 rpm. A semi-automated gas pressure system was used to measure gas pressure according to (Theodorou et al., 1994). The system consisted of a digital data tracker (Tracker 220 series indicators; Omega Engineering Inc., Laval, QC, Canada), fitted with a pressure transducer (PX4200-015GI, Omega Engineering Inc.) and a needle tip. Gas pressure was taken at 2, 12, 24 and 48 hours after commencement of incubation, and gas samples were taken to analyse the methane concentration. At the end of the incubations, *in vitro* ruminal fermentation was terminated by removing the serum bottles from the incubator, and immersing them in an ice bath to impede further microbial activity. Rumen fluid pH was measured after 48 hours' incubation using a pH meter (XLS 15 Accumet® pH meter, Fisher Scientific, Singapore). Supernatant (5 mL) from 48 h incubation bottles was collected into vials containing 1 mL 0.5M H_2SO_4 (for ammonia-N analysis) and 1 mL 25% orthophosphoric acid (for volatile fatty acid (VFA) analysis). All vials were frozen at -20°C until analysis, as described by Broderick and Kang, (1980) for NH_3-N, and Webb, (1994) for VFAs.

The *in vitro* organic matter digestibility (IVOMD) of diets was determined with the two-phase digestion method of Tilley and Terry (1963), as modified by Engels and Van Der Merwe, (1967). In the first stage, 200 mg of feed samples were incubated in four replicates for each diet with rumen liquor for 48 hours at 39°C under anaerobic conditions. Blanks and a standard feed were included in each batch of incubation and three incubation runs were carried out. This was followed by an acid-pepsin digestion phase for 48 hours. After digestion, the residual material was oven-dried at 105°C for 18 hours, weighed and subsequently ashed in a muffle furnace at 550°C for three hours. The IVOMD was estimated from the weights of the starting material and residuals.

Gas samples from the *In vitro* incubations were taken with a Hamilton syringe at 2, 12, 24 and 48 hours' incubation (Gemeda and Hassen, 2015). Methane concentration was analysed with gas chromatography (8610C BTU Gas Analyzer GC System, SRI Instruments Europe GmbH, Bad Honnef, Germany), pre-equipped with a flame ionization detector. Two blanks were included to correct the methane produced from the inoculum in each run, and two runs were executed. Methane concentration values were related to the total gas production to estimate its concentration (Tavendale et al., 2005), and subsequently converted to energy and mass values (Santoso et al., 2007).

The concentrations of acetate, propionate, butyrate, valerate, and isobutyrate were evaluated as molar proportions (mol/100 mol), while total volatile fatty acid (TVFA) was expressed as mmol/L, and ammonia-N concentration was expressed in mg/dL. Total gas and net methane were expressed in mL/g fermented dry matter and as mass values in relation to IVOMD (g/kg IVOMD) and TVFA. The ratio of non-glucogenic to glucogenic VFAs was calculated and expressed as molar ratio of acetate to propionate (A/P molar ratio).

The *in vitro* ruminal gas production experiments were examined in three experimental runs with four replicates (bottles) per treatment combination. In each run, individual bottles served as analytical replicates and were averaged prior to statistical analysis, while each run served as a statistical replicate. Gas volume produced was plotted against incubation time, and the Ørskov and McDonald, (1979) non-linear equation $Y = a + b\ (1 - e^{-ct}\ )$ was used to estimate gas production characteristics and to calculate effective gas production (EGP). Data were analysed using the GLM procedure of SAS 9.3 (2010). The model used is as follows:

Yij = μ+ Bi+ Tj + + eij

Where, μ= overall mean, Bi = block effect (replicate), Tj = treatment effect, Yij= mean of individual observation and eij= residual error. Single degree of freedom orthogonal contrasts were used to test i) control versus the average of NPN-included diets; ii) average of urea-included diet versus average of nitrate-included diet; iii) in urea subgroup, supplemented versus treated; and iv) in nitrate subgroup, supplemented versus treated on all parameters. Mean separation was done using Tukey's test and significance was compared at $P < 0.05$ and $P < 0.001$ as applicable.

3.3 Results

The chemical composition of the *E. curvula* hay substrate used in this study, had the following nutrient content: CP (48.9 g/ kg DM), neutral detergent fibre (NDF) (746 g/kg DM), acid detergent fibre (ADF) (447 g/kg DM), acid detergent lignin (ADL) (79 g/kg DM), neutral detergent insoluble nitrogen (NDIN) (21.5 g/kg), and ash (30.4 g/kg).

In vitro digestibility and gas production of *E. curvula* hay supplemented or treated with urea or nitrate are summarized in Table 3.1. Regardless of the source, generally NPN inclusion did not affect total gas production after 48 hours' fermentation compared with the control diet ($p \geq 0.05$) (Figure 3.1). From the two NPN sources, urea generally resulted in a higher 48 hour gas production ($p < 0.05$) than nitrate, while the application method did not affect 48-hour gas production in either the urea or the nitrate diet. Generally, NPN inclusion significantly reduced methane production compared with the control diet ($p < 0.05$) (Figure 3.2). However, of the two NPN sources, nitrate showed a significant ($p < 0.001$) reduction in methane production (by 57.5%) compared with urea, which showed a reduction of 12.5%. The application method (supplemented vs treated) generally did not affect methane production ($p \geq 0.05$). Although application method did not significantly affect the methane to total gas ratio in either the urea or the nitrate inclusions, there was a significant decrease in methane to total gas ratio with nitrate inclusion compared with urea inclusion, and with the control (untreated) diet ($p < 0.001$). NPN inclusion (urea or nitrate) generally improved ($p < 0.05$) the IVOMD of *E. curvula* hay, regardless of application method. While the untreated diet recorded 462 g /kg, with the inclusion of urea at 7 g N/kg hay, IVOMD increased to 493 g/kg DM and 502 g/kg DM in urea-supplemented and urea-treated hay, respectively. Similarly, compared with the control (462 g/kg IVOMD), nitrate-supplemented and nitrate-treated diets recorded 482 g/kg and 486 g/kg DM IVOMD, respectively. Urea resulted in significantly higher digestibility compared with nitrate in both the supplementation and the feed treatment. The volume of methane produced per unit of organic matter digested (expressed as a mass value) showed that NPN addition significantly ($p < 0.05$) reduced methane (g/kg IVOMD) compared with the control diet, with

the average of nitrate-included diets also resulting in significantly lower ($p < 0.05$) methane per unit of organic matter digested compared with urea-included diets. Application method did not result in significant differences in terms of methane per unit of organic matter digested in urea, but the nitrate-supplemented diet recorded lower methane per unit of organic matter digested compared with the nitrate-treated diet (37.9 g/kg versus 58.3 g/kg). Neither NPN source nor application method had any significant effect on TVFA, but the overall effect of NPN inclusion significantly improved TVFA per methane ratio when compared to the control group. However, of the two NPN sources, the nitrate inclusions showed significantly increased TVFA per methane production compared with the urea inclusions. The ratio of total gas production to IVOMD was not significantly different across the NPN sources and application methods.

Table 3.1 Comparison between direct supplementation or pre-treatment of *Eragrostis curvula* hay on 48 hour *in vitro* gas production and organic matter digestibility as influenced by urea or calcium nitrate

Parameter[1]	Control	Urea[2]		Nitrate[2]		SEM[3]	Contrasts[4]			
		Supplemented	Treated	Supplemented	Treated		C vs. A_N	U vs. N	U_S vs. U_T	N_S vs. N_T
Gas (mL/g DM)	99.7	101.8	105.7	83.8	81.1	3.83	NS	*	NS	NS
Methane (mL/g DM)	29.8	27.9	26.6	10.2	15.4	2.33	*	**	NS	NS
IVOMD (g/kg DM)	462	493	502	482	486	3.63	**	**	**	*
Methane/Total Gas	0.30	0.29	0.25	0.12	0.18	0.02	**	**	NS	NS
Gas/IVOMD (mL/kg)	0.22	0.21	0.21	0.17	0.17	0.01	NS	NS	NS	NS
Methane/IVOMD (g/kg)	115.4	101.4	94.7	37.9	58.3	7.73	*	*	NS	*
TVFA (mmol/L)	167	168	173	154	153	13.2	NS	NS	NS	NS
TVFA/Methane (mmol/mmol)	5.67	6.24	7.29	15.1	9.34	1.26	*	*	NS	NS

[1]IVOMD, *in vitro* organic matter digestibility; TVFA, total volatile fatty acid. [2]7.0g N /kg hay; equivalent to 15g urea and 40.9g calcium nitrate/ kg hay respectively; Supplemented, application of NPN; Treated, application of NPN was followed by 30 day anaerobic storage. [3]SEM, Standard error of mean; [4]Contrast analysis across treatments: C vs. A_N, control vs. the average of NPN-included diets; U vs. N, average of urea inclusions vs. average of nitrate inclusions; U_S vs. U_T, urea-supplemented diet vs. urea-treated diet; N_S vs. N_T, nitrate-supplemented diet vs. nitrate-treated diet; NS, not significant; *, $p < 0.05$; **, $p < 0.001$.

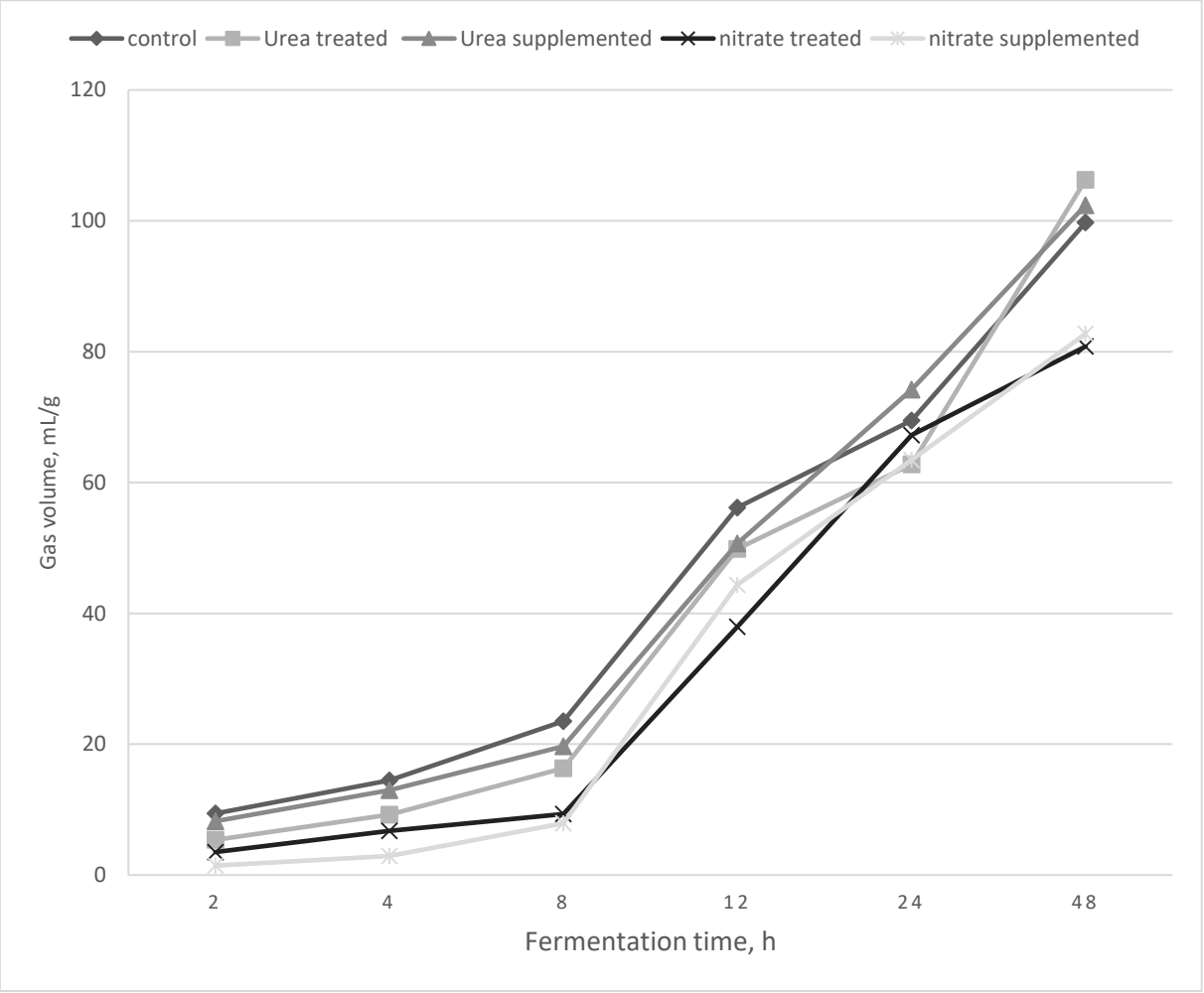


Figure 3.1 Gas production pattern of *Eragrostis curvula* hay showing the effects of NPN source and application methods

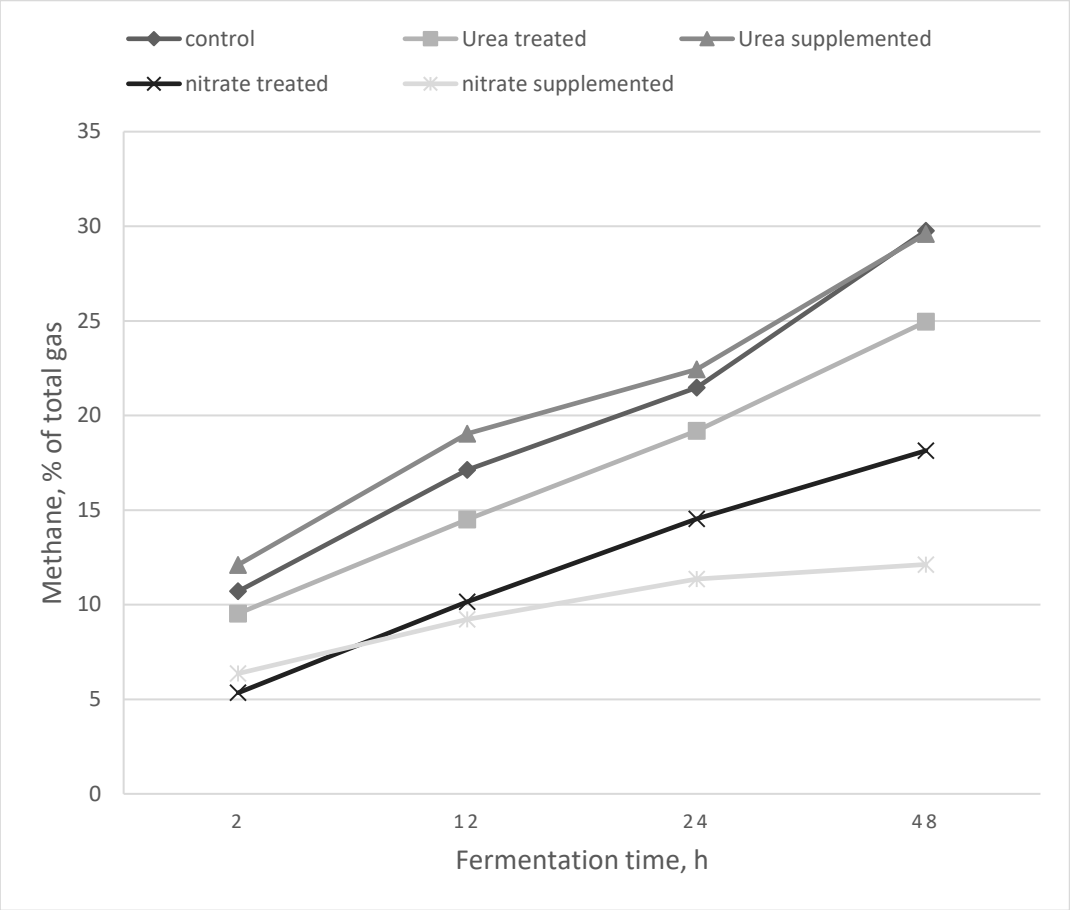


Figure 3.2 Fermentation time on methane concentration of total gas produced as influenced by NPN source and application method

The influence of NPN source and application method on gas production characteristics, which relates to the slowly fermentable fractions (b), rate of gas production (c), and EGP of *E. curvula* hay is presented in Table 3.2. Generally, NPN source did not influence the slowly fermentable fraction compared with the untreated hay. However, urea inclusion resulted in a higher b-value and EGP than nitrate-included diets. In particular, the urea treatment resulted in a higher ($p < 0.05$) b-value than the urea-supplemented diet. Neither NPN source nor application method had an effect ($p \geq 0.05$) on the rate of gas production. The pattern of cumulative gas production over the 48-hour period is shown in Figure 3.1. Cumulative gas production increased slowly from 2 to 8 hours. However, as the time of incubation increased, gas production increased more rapidly until 24 hours. Total gas production in urea-supplemented and urea-treated diets tended to increase more rapidly from 12 to 48 hours. The concentration of methane as a percentage of total gas produced across the *in vitro* incubation time is shown in Figure 3.2. Generally, methane concentration increased as incubation time increased ($R^2 \approx 0.99$), and regardless of the method of application, nitrate consistently reduced methane production over the 48-hour incubation period.

Table 3.2 Effects of non-protein nitrogen source and application method on *in vitro* gas production characteristics of *Eragrostis curvula* hay

Parameters[1]	Control	Urea[2]		Nitrate[2]		SEM[3]	Contrast[4]			
		Supplemented	Treated	Supplemented	Treated		C vs. A_N	U vs. N	U_S vs. U_T	N_S vs. N_T
b (mL/g)	114.7	125.8	144.6	107.5	108.4	4.74	NS	*	*	NS
c (h^{-1})	0.05	0.04	0.04	0.05	0.05	0.003	NS	NS	NS	NS
EGP (mL/g)	109.6	120.8	135.8	94.2	95.7	5.50	NS	*	NS	NS

[1]b, gas production from slowly fermentable fraction; c, rate of fermentation; EGP, effective gas production; [2]7.0g N /kg hay; equivalent to 15g urea and 40.9g calcium nitrate/ kg hay respectively; Supplemented, application of NPN; Treated, application of NPN was followed by 30 day anaerobic storage.[3]SEM, Standard error of mean. [4]Contrast analysis across treatments: C vs. A_N, control vs. the average of NPN-included diets; U vs. N, average of urea inclusions vs. average of nitrate inclusions; U_S vs. U_T, urea-supplemented diet vs. urea-treated diet; N_S vs. N_T, nitrate-supplemented diet vs. nitrate-treated diet; NS, not significant; *, $p < 0.05$; **, $p < 0.001$.

In vitro ruminal fermentation characteristics of *E. curvula* hay supplemented or treated with or without an NPN source are shown in Table 3.3. Neither rumen fluid pH nor rumen ammonia-nitrogen concentration was affected by NPN source or method of application ($p \geq 0.05$). Inclusion of NPN generally increased acetate production compared with the control. Of the two NPN sources, nitrate resulted in a higher acetate concentration than urea, regardless of the method of application. However, propionate concentration was significantly reduced ($P < 0.001$) in nitrate diets compared with urea diets. Regardless of the method of application, the acetate to propionate ratio was influenced by the addition of nitrogen source, with nitrate diets resulting in significantly higher acetate to propionate molar ratios than urea diets. The nitrate-treated diet had higher ($p < 0.001$) butyrate and valerate concentration than the nitrate-supplemented diet. Other ruminal fermentation parameters did not show significant ($p \geq 0.05$) differences as a result of NPN source or application method.

Table 3.3 Comparison between supplemented or treated *Eragrostis curvula* hay on ruminal fermentation characteristics at 48 h of *in vitro* incubation as influenced by urea or calcium nitrate inclusions

Parameter[1]	Control	Urea[2]		Nitrate[2]		SEM[3]	Contrast[4]			
		Supplemented	Treated	Supplemented	Treated		C vs. A_N	U vs. N	U_S vs. U_T	N_S vs. N_T
pH	6.77	6.82	6.78	6.85	6.88	0.02	NS	NS	NS	NS
NH_3-N (mg/dL)	13.5	21.1	15.8	15.1	14.8	1.7	NS	NS	NS	NS
Acetate (mol/100ml)	63.0	62.8	62.0	69.6	68.7	0.88	*	**	NS	NS
Propionate (mol/100mol)	25.9	25.0	25.4	22.4	21.1	0.54	*	**	NS	NS
Butyrate (mol/100mol)	6.11	7.46	8.09	5.51	7.37	0.28	*	*	NS	*
Isobutyrate (mol/100mol)	2.71	2.37	2.25	0.73	0.50	0.25	**	**	NS	NS
Valerate (mol/100mol)	2.25	2.32	2.23	1.69	2.35	0.08	NS	NS	NS	*
A/P molar ratio	2.44	2.51	2.44	3.11	3.25	0.09	*	**	NS	NS

[1]NH_3-N, ammonia nitrogen; A/P, molar ratio of acetate to propionate. [2]7.0g N /kg hay; equivalent to 15g urea and 40.9g calcium nitrate/ kg hay respectively; Supplemented, application of NPN; Treated, application of NPN was followed by 30 day anaerobic storage. [3]SEM, Standard error of mean; [4]Contrast analysis across treatments: C vs. A_N, control vs. the average of NPN-included diets; U vs. N, average of urea inclusions vs. average of nitrate inclusions; U_S vs. U_T, urea-supplemented diet vs. urea-treated diet; N_S vs. N_T, nitrate-supplemented diet vs. nitrate-treated diet; NS, not significant; *, $p < 0.05$; **, $p < 0.001$.

3.4 Discussion

The result shows that the feed is very low in crude protein, but high in NDF concentration. The very low crude protein content of the hay shows that the addition of an NPN source could improve its feeding value for ruminant animals. As expected, regardless of source, the addition of NPN resulted in higher *in vitro* digestibility of hay. The significant increase in IVOMD with NPN supplementation and NPN treatment confirms the earlier research hypothesis that very low quality feedstuff responds more readily to NPN inclusion (Mapato et al., 2010). Though total gas production was not significantly affected by the NPN application method, the treated diets resulted in a relatively higher IVOMD than the supplemented diets. The significant increase in digestibility after the urea treatment compared with urea supplementation agrees with previous studies (Uza et al., 2005; Verma et al., 2006). Mgheni et al. (1993) compared the effect of urea supplementation versus urea treatment and found a significant increase in *in vitro* digestibility, DM intake and growth performance of goats fed urea-treated rice straw, compared with urea-supplemented rice straw. Similarly, Tesfayohannes et al., (2013) reported significant improvement in the digestibility of *E. curvula* hay, wheat straw, barley straw, ryegrass, maize stover and oat hay following urea treatment (ammoniation) for 35 days, but noted variations in the responses, depending on the type of feedstuff. Poor quality feeds were reported to show a greater improvement in digestibility compared to good quality feeds, as observed earlier by Goto et al., (1991). The improvement in feed digestibility of the treated feed over the control diet has been attributed to a significant reduction in NDF and the hemicellulose content of poor-quality feeds. This is as a result of the solubilization of fibre fractions resulting from linkage disintegration (Mason et al., 1988; Tesfayohannes et al., 2013; Zorrilla-Rios et al., 1985), disruption of cell wall structure by ammonia and increased swelling of fibre structure, and higher affinity for microbial attachment (Fahey et al., 1993).

Under anaerobic systems, nitrate is able to undergo various biochemical pathways, but the dissimilatory reduction pathway is favoured under very low redox potential, and often in the presence of high organic matter concentrations (Leng, 2008; Simon, 2002). A partial conversion of nitrate to ammonia may have occurred during the nitrate treatment process. This ammonia is partly responsible for the improvements in hay digestibility observed in this study, while the residual nitrate in the treated hay played an additional role in inhibiting methane production *in vitro*. The release of ammonia and the success of hydrolytic treatment of feedstuffs depends on several factors such as biomass quality and conditions of storage, for example, amount of moisture, duration of treatment and temperature (Shreck, 2013). Not much has been reported in literature about feed treatment with nitrate, therefore the reaction kinetics

is not clearly understood. However, the results of this study show that a considerable breakdown of nitrate to ammonia may have occurred during the feed treatment to account for the significant differences in fermentation and digestibility parameters observed between the nitrate-supplemented and the nitrate-treated diets.

According to Inthapanya et al. (2011), increase in methane content of gas with a lengthened times of incubation and a more rapid gas production pattern after eight hours are probably because of the order of substrate degradation in the *in vitro* rumen microbial environment. Soluble carbohydrates and NPN are readily utilized initially, while structural carbohydrates such as NDF and ADF which generate more methane are degraded later, often requiring a considerable amount of energy utilization (Inthapanya et al., 2011). The ADF content of a feed may significantly affect its methane production pattern, particularly at the later stages of fermentation (Bannink and Smits, 2010). In the nitrate-supplemented and nitrate-treated diet, the amount of residual nitrate remaining across the incubation times may primarily influence the magnitude of methane reduction. Total methane production was significantly higher in nitrate-treated hay compared with the nitrate-supplemented hay, which indicated that some nitrate was hydrolysed to ammonia during storage, while a lower amount of residual nitrate resulted in a relatively higher value of methane output in nitrate-treated hay compared with nitrate-supplemented hay (15 versus 10 mL/g). The trend in methane concentration across time intervals was similar to that reported by Inthapanya et al., (2011). The significant reduction of methane in both nitrate inclusion methods is an indication that incorporating calcium nitrate via feed supplementation or during feed treatment could help to improve the utilization of poor-quality feed and might improve the efficiency of gross energy utilization. Enteric methane accounts for 7–17% loss of the metabolizable energy of feed in ruminant diets during the digestion process (Hristov et al., 2013; Robertson and Waghorn, 2002). Thus, methane reduction can be regarded as a means of conserving the dietary energy of feedstuff. The significant decrease in methane production with dietary nitrate reported in this study was in agreement with earlier findings by various researchers (Hulshof et al., 2012; Nolan et al., 2010; Sophal et al., 2013). According to Gemeda and Hassen, (2015), the feeding value of a feed is determined by its fermentation and digestibility characteristics. Although total gas production showed a significant reduction in nitrate diets compared with urea diets, it appears that the observed reduction is largely owing to the methane component of the total gas, as shown by the methane to total gas ratios. The ratios of methane to IVOMD and TVFA to methane indicate that nitrate diets provide a more efficient digestive process than the urea or control diets. The impact of the nitrate inclusion on rumen methanogenesis was more pronounced than their effect

on substrate degradation, as shown by the high TVFA/methane and low methane/IVOMD ratios of nitrate-treated and nitrate-supplemented diets compared with urea inclusions and the control diet. Ammonia nitrogen values in this study did not show significant differences across treatment groups. This may be an indication of comparable uptake of ammonia from the ammonia nitrogen pool for microbial biomass production or that the rate of NPN inclusions used in this study did not lead to the accumulation of ammonia nitrogen.

The reduction in the molar proportion of propionate is consistent with previous research findings. In this study, there was a significant reduction in propionate concentration in both nitrate-supplemented and nitrate-treated diets. This agrees with previous studies (Nolan et al., 2010; van Zijderveld et al., 2011). Nitrate has been noted to have a higher affinity for hydrogen ions than carbon dioxide, hence inhibiting the pathways that generate propionate and methane (Ungerfeld and Kohn, 2008). This explains the higher values of A/P molar ratios observed in this study.

While this study confirms the reports of earlier findings on the improvement in digestibility of roughages after urea treatment as shown in gas volume and IVOMD values, the result shows that increased digestibility of hay was followed with a concomitant increase in methane production. This study also shows that replacing urea with nitrate in the treatment of poor-quality roughage resulted in a relatively lower digestibility improvement compared with urea, yet the level of improvement observed for nitrate was better than for the control diet. Moreover, a significant reduction in methane production could be achieved with nitrate treatment to justify its use, because it leads to a more efficient digestion process, as shown by methane/total gas ratio, and TVFA/methane ratio. An appreciable improvement in digestibility, coupled with a considerable reduction in methane production, indicates that calcium nitrate could be incorporated alone or with urea in the feed treatment of poor-quality roughages. However, the cost: benefit ratio needs to be acceptable for wider application.

3.5 Conclusion

The results of this experiment suggest that treating *Eragrostis curvula* hay with urea improved its utilization better than treatment with nitrate. However, with a reduction of enteric methane production and the associated improvement in digestibility compared with the control, nitrate could be incorporated as a hydrolytic agent in treating poor-quality roughage feeds, with the aim of improving their utilization.

CHAPTER FOUR

Effect of non-protein nitrogen source and inclusion of condensed tannin on nutrient digestibility, growth performance and methane production in sheep

Abstract

The effect of urea or calcium nitrate as non-protein nitrogen (NPN) sources in a total mixed ration (TMR) with or without the inclusion of *Acacia mearnsii* tannin extract was evaluated for its effect on dry matter intake, nutrient digestibility, growth performance and methane emission of South African Mutton Merino lambs. Forty weaner lambs with an average body weight of 34.7±4kg were blocked into different groups after stratifying the lambs according to their body weight and sex. Individual lambs within a block were randomly allocated to four different groups that received randomly, one of the following dietary treatments: urea; nitrate; urea + tannin (tannin, 42 g/kg feed); nitrate+ tannin (tannin, 42 g/kg feed). Two animals were kept in each pen and the pens were replicated five times per treatment. The lambs were fed a TMR diet with roughage: concentrate ratio of 43:57. Generally, the dry matter and other nutrient intake of lambs were not significantly influenced by NPN source or the inclusion of tannin extract in the TMR diet with the exception of the CP intake, which was higher ($p<0.05$) in animals that received urea-based diets compared to those that received nitrate-based diets. In this study, the inclusion of tannin reduced dry matter intake and digestibility of DM, CP, NDF and ADF of the TMR diets but did not significantly affect the methane emission of lambs ($P>0.05$). Generally, animals on nitrate-based diets had lower total volatile fatty acid production compared to those on urea-based diets. The inclusion of tannin increased the propionate and reduced the acetate proportion, with a significant reduction in acetate to propionate ratio when used in urea-based TMR diet but not in the nitrated-based TMR diet. Although tannin inclusion increased faecal-N excretion, the urinary-N excretion and N-retention was not influenced by nitrogen sources or inclusion of tannin extract in the TMR diet. A higher average daily gain and lower methane emission were recorded for lambs fed nitrate-based diets compared to those fed urea-based diets. In contrast, the inclusion of tannin reduced the final body weight and average daily gain of the lambs. Feed conversion ratio was not affected by NPN source but was significantly reduced with the inclusion of tannin in the TMR diet. This study demonstrated that nitrate could be used safely as a source of non-protein nitrogen with co-benefit of reducing enteric methane emission and improving the average daily gain of South Africa Merino sheep. In contrast, the inclusion of acacia tannin extract reduced dry matter intake, nutrient digestibility, and average daily gain of sheep but increased the proportion of faecal nitrogen excretion.

Keywords: Growth performance, Methane emission, Nitrate, Non-protein nitrogen, Urea.

4.1 Introduction

The traditional view of tannins as an undesirable plant secondary compound has shifted in recent times. The beneficial properties of tannins have continued to be explored for a variety of uses in ruminant animal nutrition, and commercial tannin extracts have been proposed as additives for ruminant animals (Fernández et al., 2012; H D Naumann et al., 2013). One such beneficial effect of tannins is enteric methane reduction (Carulla et al., 2005; Hassen et al., 2016). Several studies have established a dose-response relationship between condensed tannins from different sources and mitigation of methanogenesis in the rumen (Animut et al., 2008a). Therefore, moderate to high tannin diets may be used depending on the source as a sustainable approach to mitigating methane emission in ruminants. High concentration of tannin, however, may result in reduced voluntary feed intake and decreased degradation of crude protein in the rumen leading to increased bypass protein (Eckard et al., 2010; Grainger et al., 2009; Silanikove et al., 1994). Nevertheless, higher dietary bypass-protein has been associated with higher immune response and savings in metabolic cost associated with detoxification of ammonia nitrogen (Parker et al., 1995). Dietary tannins have also been associated with increased resistance to intestinal parasites in sheep and goats (Min et al., 2004; Niezen et al., 2002).

On the other hand, where crude protein is limiting, tannins can reduce rumen protein degradation and therefore ammonia nitrogen availability for microbial biomass production. This drawback can be overcome by strategic supplementation of non-protein nitrogen (NPN). Urea has been the NPN of choice to supply ammonia nitrogen in ruminant feed, and although it requires careful addition to avoiding over-consumption by animals, it can cause ammonia toxicity (Adejoro and Hassen, 2017; Newbold et al., 2014). Urea is widely used in both smallholder and commercial farming systems. The hydrogen ions' scavenging properties of nitrate salts have presented them as an alternative to urea because they are not only able to meet the ammonia nitrogen needs of rumen microbes, but they also serve as an alternative hydrogen sink in proteolytic pathways, thus competing with methanogens. This results in considerable reduction in methane production associated with rumen fermentation (Adejoro and Hassen, 2017; Nolan et al., 2010; Van Zijderveld et al., 2011).

Although there have been a number of experiments that have shown the effects of tannins, and of nitrate in reducing methane emission in ruminants, there are few studies that have evaluated the long-term effect of tannin or tannin extracts in sheep. The possible interaction effect between tannins and the different NPN sources have not been assessed in ruminants to the best

of our knowledge. Concurrent use of tannin with nitrate will help to overcome the limitation of soluble nitrogen source in the rumen for optimal rumen fermentation while providing double benefit of reducing the enteric methane emission of ruminants. It is hypothesised that with reduced rumen protein digestion as a result of dietary tannin inclusion, the choice of NPN available to supply rumen ammonia nitrogen required by microbes, might have a significant effect on nitrogen metabolism, rumen fermentation, animal performance and enteric methane production. The objectives of this study were to assess the effect of different sources of NPN with or without condensed tannin extract on dry matter intake, nutrient digestibility, growth performance and methane emission in growing lambs.

4.2 Materials and Methods

Animal management protocols were carried out in accordance with the University of Pretoria animal ethics committee (AEC) guidelines as stipulated in the approval number EC061-14. The experiment was conducted at the small stock unit of University of Pretoria experimental farm (Pretoria, South Africa).

Animal, experimental design and adaptation

The experiment was designed as a randomised complete block design. Twenty-four ram-lambs were blocked into three groups while sixteen ewe-lambs were blocked into two groups based on body weight at the start of the experiment, making a total of forty animals in five blocks for the trial. Two animals from each block were then randomly allocated to each pen. Five pens, one from each block was therefore allocated to each dietary treatment. The treatments were urea-based TMR (Treatment 1); urea-based TMR + tannin (treatment 2), nitrate-based TMR (treatment 3) and nitrate-based TMR + tannin (treatment). Acacia tannin extract was included at 42 g/kg feed DM, and this was an equivalent to 1.7 g/kg (leucocyanidin equivalent) CT inclusion. The extract was obtained from UCL Company (Pty) Ltd. Dalton, South Africa, and has a molecular weight that ranges from 500 to 3000, with an average of 1250. Both TMR were formulated to be isocaloric and isonitrogenous. Experimental animals were housed in open pens in a group of two animals per pen and a total of five pens per treatment were used. A gradual introduction to diet was done over an initial 21 day period as recommended by Nolan et al. (2010). During the adaptation, animals were allocated 30%, 60% and 100% of the experimental diets in three 7 d periods, to replace the commercial pellets previously consumed by the animals. This was to allow sufficient time for the rumen environment to adapt to the new diet while stimulating the shift in microbial population and environment.

Diets and feeding

After the initial 21 day adaptation period, animals were maintained on their respective experimental diets for a continuous period of 60 d. Diets were offered *ad libitum* in two portions daily and fresh water was available at all times. Experimental diets (Table 4.1) consisted of roughage to concentrate ratio: 43:57 %. The concentrate ration was manufactured based on author's specification by AFGRI Pty (Ltd), South Africa while the roughage and tannin inclusions were added on the experimental farm and the final TMR re-mixed on weekly basis using a vertical mixer. During the growth trial, the average feed consumption for two animals within a pen was monitored daily while individual body weight of animals was measured weekly.

Nutrient digestibility, nitrogen balance and Rumen Fluid sampling

At the end of the growth trial, 20 male animals comprising five animals per treatment, were placed inside individual metabolic cages and fitted with faecal bags for total faeces and urine collection. Because the animals had been on the respective diets during the growth study, animals were adjusted to the cages for only 7 days, followed by 5 days of complete collection of faeces, feed refusal and urine output. Feed offered and feed refusal was monitored daily and recorded before the next ration was allocated and faecal output was weighed daily. Samples of feed offered, refusals and faeces were collected and frozen at -20 °C. Urine was collected inside plastic bottles containing 50 mL sulphuric acid (10%, v/v) to prevent N-volatilization. At the end of the collection period, samples of each animal's diet, refusals and faeces were pooled across days and sub-sampled for analysis. A portion was weighed, and oven dried at 95°C for 18 hours for dry matter determination, while a second portion was dried at 55°C for 48 hours, ground to pass a 1 mm sieve, and stored for chemical analysis.

Rumen Fluid sampling

Ruminal fluid was collected from each animal immediately after slaughtering at the abattoir. Once the animals were slaughtered, the entire rumen content was emptied into a plastic bucket and mixed thoroughly. Thereafter, samples were strained through 4 layers of cheesecloth into empty bottles (for pH and microbial analysis) and 100mL rumen fluid to 20mL of 25% orthophosphoric acid (for analysis of VFA), and 90mL rumen fluid to 15mL of 0.5M sulphuric acid (for ammonia-N analysis). All samples were thereafter transported to Nutrilab, The University of Pretoria in cooler boxes until frozen at -20°C.

Methane emission measurement

The open circuit respiratory chamber for methane measurement is similar to the description by Storm et al., (2012). The chambers are made of steel frames and transparent, UV-resistant PVC sheets covering every side and with a small opening in front to allow ambient air into each chamber. With speed-adjustable fans, air is withdrawn from each chamber in one direction and vented to the outside through a pipe connection. Connected to this pipe at a uniform junction is the hot wire anemometer that measures continuously the speed of air flowing out of each chamber as well as a tubing for air sampling. Each chamber was pre-calibrated with methane gas of known concentration and a recovery percentage was determined both at the beginning and at the end of each cycle. This recovery percentage was used as a correction factor to adjust the total volume of methane produced by each animal when they passed through each chamber. During each cycle of methane measurement, the sheep was adapted to the chamber for 1 day before methane output measurement in the four subsequent days. In every cycle, four animals coming from the same block were included, i.e. one from each treatment group was placed in each chamber and every day, the sheep was rotated to a different chamber until all the animals had passed through the 4 different chambers. Animals were fed once daily and water was provided *ad lib*. The chambers were cleaned and thereafter kept closed for about 22.5 h daily during which airflow speed was recorded automatically by the hot wire anemometers fitted with automatic data loggers. Samples of air flowing out from each chamber and the ambient air in front of each chamber over the 22.5 h were collected in deflatable Teflon balloons, using an 8-channel peristaltic pump (Masterflex 77292-50 L/S, Cole-Palmer Instr., USA). From each balloon, 5 different samples of gas collected were analysed using an SRI GC fitted with a flame ionization detector.

Chemical analysis

Samples of feed offered, orts and faeces were analysed according to AOAC, (2000) for dry matter (DM; ID 934.01), ash (ID 942.05) and crude protein (ID 968.06) using the Leco analyser (Leco TruMac N determinator Leco Corporation, St. Joseph, USA.). Urine-N was also analysed using the Leco analyser. Neutral detergent fibre (NDF) and acid detergent fibre (ADF) was determined according to the method of Van Soest et al., (1991). Heat stable alpha amylase and sodium sulphite were incorporated in the NDF assay following the ANKOM filter bag technique. Acid detergent fibre (ADF) was also determined using the ANKOM filter bag technique and both NDF and ADF expressed exclusive of residual ash.

Rumen fluid samples were analysed for pH (model PB-10/c, Sartorius, Germany), while samples for VFA analysis was centrifuged at 15,000 × g for 10mins at 4°C (MIKRO 220R Centrifuge; Hettich FurnTech Division DIY, Kirchlengern, Germany). The supernatant was analysed by gas chromatography (Shimadzu GC-2010 Tracera; Shimadzu corp., Kyoto, Japan). The GC is fitted with a 30m Inert Cap Pure Wax column (df = 0.25µm, I.D. = 0.25 mm). Using external standards (Sigma-Aldrich Inc., St. Louis, MO, USA), volatile fatty acid concentrations were quantified by comparison of peaks and retention times (Calabrò et al., 2012). Ammonia nitrogen was analysed by using the phenol-hypochlorite reagent spectrophotometric procedures according to Broderick and Kang, (1980).

Statistical analysis

Weekly body weight measurements of individual animals were used to regress average daily gain (ADG) and average feed consumption within a pen and was used for the analysis of daily dry matter intake of each animal during the growth study. The statistical model used included a block effect and treatment effect, which was further partitioned into the effects of NPN sources and tannin inclusion, and an interaction between NPN source and tannin inclusion. The model used for the analysis was as follows:

$$y_{ijk} = \mu + Block + A_i + B_j + (AB)_{ij} + \varepsilon ijk$$

where y_{ijk}= observation k at different nitrogen source (i; urea or nitrate) and level j of tannin inclusion (j; no-tannin, with tannin); μ= overall mean; Block= effect of blocking (initial weight × sex); A_i= the effect of nitrogen source; B_j= the effect of level of tannin extract inclusion; $(AB)_{ij}$= the effect of the interaction of nitrogen source with or without tannin inclusion, and εijk= random error with mean of 0 and variance σ^2. Data was analyzed as a 2 × 2 factorial treatment combination in a randomised complete block design with replicates. Statistical analyses were performed using the GLM procedure of SAS (SAS Inst. Inc.; Cary, NC, USA). Means were compared using Tukey's test and reported as least significant means and standard error of means. Significant differences were declared when $P< 0.05$.

Table 4.1. Ingredients and composition of experimental diet

Ingredient (%)	Urea-based diet	Urea + tannin diet	Nitrate diet	Nitrate+ tannin diet
Sunflower oilcake meal	16.8	16.1	17.1	16.4
Fine milled maize	27.6	26.4	28.2	27.0
Wheat bran	4.9	4.7	3.4	3.3
Molasses	5.9	5.7	5.1	4.9
Lucerne meal	19.70	18.9	19.0	18.2
Eragrotis hay	23.2	22.2	23.1	22.1
Urea	1.0	1.0	0	0
Coarse salt	0.5	0.5	0.50	0.5
Premix[1]	0.4	0.4	0.40	0.4
Nitrate Source[2]	0	0	3.2	3.1
Tannin extract[3]	0	4.2	0	4.2
Total, %	100	100	100	100
Chemical composition				
Dry matter, g/kg	912	909	910	910
Organic matter, g/kg DM	930	934	920	923
Crude protein, g/kg DM	203.8	209.4	188.5	183.9
Neutral detergent fibre, g/kg DM	333.7	364.4	377.0	354.0
Acid detergent fibre, g/kg DM	196.1	218.9	215.3	195.4

[1]supplied in g/kg the following: vit A, 18,000 iu; vit D, 3920 iu; vit E, 2.45 iu; Zn, 5.0 mg; Mn, 4.1 mg; Cu, 0.5 mg; Se, 0.2 mg; Mg, 28 mg; and Co, 0.3 mg. [2]$5Ca(NO3)_2.NH_4.NO_3.10H_20$; 155 g/kg N, 75% NO_3 in DM.
[3]Contains 350 g/kg condensed tannin (leucocyanidin equivalent)

4.3 Results

The ingredients and chemical composition of the experimental diets are presented in Table **4**.1. All diets contain CP ranging from 183.9 g/kg to 213.8g/kg DM. There was a minor difference in the CP content of the urea-based concentrate and the nitrate-based concentrate. However, the NDF and ADF contents of the diets were similar.

There was no significant interaction effect between the nitrogen source and tannin inclusion level on intake and digestibility of DM, OM, CP, NDF and ADF (Table 4.2). Nitrogen source generally did not affect DM, OM, NDF, ADF and ADL intake of sheep ($p > 0.05$). However, nitrogen source influenced the CP intake. Animals on urea-based TMR diets consumed higher CP (in g per head per day) compared to animals on nitrate-based TMR diets ($p \leq 0.05$). Tannin inclusion, however, did not affect nutrient intake (DM, CP, NDF and ADF) in the experimental animals ($p > 0.05$). Nitrogen source had no effect on digestibility of DM, OM, CP and ADF, but the use of nitrate-based TMR diet improved the NDF digestibility ($p < 0.05$) when compared

Table 4.2. Intake and apparent nutrient digestibility in growing lambs fed urea or nitrate-based total mixed ration with or without acacia tannin extract

	Urea-based diet		Nitrate-based diet			*P*-values		
	No tannin	With tannin	No tannin	With tannin	SEM[1]	N	T	N*T
Nutrient intake								
Dry matter (DM), g/d	1595	1484	1489	1419	57.7	0.459	0.432	0.855
DM, g/kg $BW^{0.75}$	86.5	80.2	75.6	76.9	2.96	0.269	0.689	0.549
Organic matter (OM), g/d	1481	1381	1370	1309	53.9	0.432	0.488	0.869
OM, g/kg $BW^{0.75}$/d	80.3	74.6	69.6	70.9	2.78	0.229	0.712	0.555
Crude protein (CP), g/d	335	330	282	266	13.19	0.005	0.369	0.875
Neutral detergent fibre (NDF), g/d	499	489	565	486	23.98	0.504	0.342	0.465
Acid detergent fibre (ADF), g/d	296	298	321	265	14.04	0.886	0.330	0.279
Apparent nutrient digestibility (%)								
DM	67.3	59.8	65.4	62.6	0.87	0.727	0.001	0.088
OM	68.3	60.4	67.0	63.8	0.90	0.421	0.001	0.091
CP	79.9	74.5	78.9	74.0	0.78	0.495	0.0002	0.817
NDF	38.8	29.4	45.9	37.4	1.86	0.007	0.002	0.850
ADF	35.8	23.5	40.5	23.4	2.19	0.448	0.0001	0.412

[1]SEM, standard error of mean; N, effect of nitrogen source; T, effect of tannin inclusion; N*T, interaction effect of nitrogen source and tannin inclusion.

to the urea-based diet. In contrast, the inclusion of ATE in the TMR diets reduced DM, OM, CP, NDF and ADF digestibility of the TMR diets ($p < 0.05$).

The interaction effect of nitrogen source and tannin inclusion was not significant for total-N excretion, faecal-N and urinary-N excretion. Nitrogen source influenced nitrogen intake as well as total nitrogen excretion both in terms of g/head/d and g/kg $BW^{0.75}$/d, with animals fed with urea-based TMR diets consuming as well as excreting higher nitrogen than animals fed with the nitrate-based TMR diets ($p < 0.05$) (Table 4.3). In contrast, tannin inclusion did not show any effect on N intake in g/kg $BW^{0.75}$/d ($p > 0.05$) and total N-excretion in g/kg $BW^{0.75}$/d ($p > 0.05$). However, faecal-N excretion in g/kg N-intake increased due to tannin inclusion in the TMR diets ($p < 0.05$), but the faecal-N excretion was not influenced by nitrogen source ($p > 0.05$). Urinary-N excretion (g/kg N-intake) was neither influenced by NPN sources, nor tannin inclusion ($p > 0.05$). Retained-N in g/kg N-intake was not affected by NPN sources

Table 4.3. Nitrogen balance in growing lambs fed urea or nitrate-based total mixed ration with or without acacia tannin extract

	Urea-based diet		Nitrate-based diet		SEM[1]	[2]*P*-values		
Parameters	No tannin	With tannin	No tannin	With tannin		N	T	N*T
n	5	5	5	5				
Nitrogen (N)-intake, g/head/d	53.6	52.8	45.1	42.5	2.11	0.005	0.369	0.875
N-intake, g/head/kg $BW^{0.75}$/d	2.90	2.85	2.29	2.30	0.11	0.002	0.613	0.559
N-excretion, g/head/d	39.2	37.6	32.6	30.4	1.97	0.004	0.354	0.876
N-excretion, g/head/kg $BW^{0.75}$/d	2.13	2.05	1.66	1.66	0.08	0.004	0.779	0.765
Faecal-N proportion, g/kg N-intake	201	255	211	260	7.79	0.495	0.0002	0.807
Urinary-N proportion, g/kg N-intake	497	472	516	459	32.17	0.923	0.177	0.590
Retained-N proportion, g/kg N-intake	302	273	273	282	31.79	0.712	0.733	0.505

[1]SEM, standard error of mean; N, effect of nitrogen source; T, effect of tannin inclusion; N*T, interaction effect of nitrogen source and tannin inclusion; $P < 0.05$.

or tannin inclusion ($p > 0.05$). The proportion of dietary nitrogen retained ranged between 273-302 g/kg N-intake across the treatments.

The effect of NPN source and tannin inclusion on rumen fermentation characteristics of South Africa Mutton Merino lambs is shown in Table 4.4. There was no interaction effect of nitrogen sources and tannin inclusion on pH, NH_3-N and total VFA composition of rumen fluid from the experimental animals. Neither nitrogen sources, nor tannin inclusion had any effect on rumen pH of the lambs ($p > 0.05$). While nitrogen sources did not affect rumen ammonia-N concentration ($p > 0.05$), tannin inclusion reduced rumen ammonia-N concentration in the experimental animals fed with the tannin added TMR diets compared to the TMR diets without tannin ($p < 0.05$). On the other hand, nitrogen source affected the total VFA concentration in the experimental animals, with lambs feeding on the urea-based TMR diet having a higher concentration of total VFA compared to lambs feeding on the nitrate-based TMR diets ($p \leq 0.05$). Tannin inclusion did not elicit any effect on ruminal total VFA concentration in the experimental lambs ($p > 0.05$). The molar proportions of the individual VFAs revealed that

there was a strong interaction effect between nitrogen sources and tannin inclusion on acetate, propionate, butyrate, isovalerate, and valerate proportions of total VFA as well as on the acetate: propionate ratio. Tannin inclusion reduced the acetate proportion in the animals consuming the urea-based TMR diet, whereas it slightly increased its proportion in animals consuming the nitrate-based diets. Tannin inclusion resulted in an increase in propionate proportion in animals consuming the urea-based TMR diets while it had no effect on propionate proportion in animals consuming the nitrate-based TMR diet. While valerate proportion was higher in the animals consuming the urea-based diet, tannin inclusion increased valerate concentration in animals receiving the urea-based diet, but did not elicit any effect in the nitrate-based TMR diet. Tannin inclusion also tended to result in a decreased A/P ratio in animals consuming the urea-based diet but did not seem to have any effect in animals consuming the nitrate-based diet. Nitrogen sources and tannin inclusion did not affect Isobutyrate concentration in the animals ($p > 0.05$).

Table 4.4. Ruminal characteristics of growing lambs fed a urea or nitrate-based total mixed ration diet supplemented with or without acacia tannin extract

	Urea-based diet		Nitrate-based diet			[2]*P*-values		
[3]Parameter	No tannin	With tannin	No tannin	With tannin	SEM[1]	N	T	N*T
n	5	5	5	5				
pH	6.11	5.96	6.22	6.02	0.06	0.469	0.163	0.847
NH_3-N, mg/dL	29.7	22.9	26.8	18.9	1.80	0.209	0.012	0.819
Total volatile fatty acid (VFA), mmol/L	117	130	111	112	2.97	0.05	0.23	0.26
VFA molar proportion, mol/100 mol								
Acetate	58.4[a]	51.5[b]	54.2[a]	55.7[a]	0.71	0.864	0.015	<0.0001
Propionate	20.5[b]	23.3[a]	22.5[a]	23.2[a]	0.39	0.064	0.002	0.05
Butyrate	15.5[b]	18.8[a]	17.5[a]	15.8[a]	0.37	0.404	0.186	<0.0001
Isobutyrate	1.45	1.43	1.49	1.20	0.06	0.457	0.225	0.292
Isovalerate	2.21[a]	2.54[a]	2.32[a]	2.10[a]	0.06	0.160	0.636	0.025
Valerate	1.95[b]	2.39[a]	1.98[b]	2.00[b]	0.06	0.024	0.004	0.009
Acetate:propionate ratio	3.03[a]	2.25[b]	2.46[b]	2.43[b]	0.09	0.175	0.007	0.011

[1]SEM, standard error of mean; N, effect of nitrogen source; T, effect of tannin inclusion; N*T, interaction effect of nitrogen source and tannin inclusion. [3]NH_3-N, ammonia nitrogen. Means with different superscript across a row are significantly different ($P < 0.05$).

The average body weights of the lambs were slightly different across treatments at the start of the experiment (Table 4.5). No significant interaction effect was observed between nitrogen sources, and tannin inclusion on dry matter intake, average daily gain and feed conversion ratio in the experimental animals. Regardless of nitrogen source, tannin inclusion appeared to have reduced ($p< 0.074$) daily dry matter intake of lambs feeding on TMR with tannin compared to lambs feeding on TMR without tannin. The average body weight of the lambs at the end of the growth trial was not affected by nitrogen source ($p> 0.05$) while tannin inclusion tended to reduce ($p< 0.096$) the average final body weights of the animals fed on TMR with tannin diet compared to those lambs fed on TMR without tannin. The average daily gain (ADG) was significantly affected by both nitrogen source ($p< 0.05$) and tannin inclusion ($p< 0.05$). Animals on nitrate-based TMR diets recorded higher ADG compared to animals on the urea-based TMR diets while animals consuming TMR without tannin recorded higher ADG compared to animals receiving TMR diet with tannin. Lambs fed on TMR with tannin, utilised feed less efficiently ($p< 0.05$) compared to those lambs fed on TMR without tannin extract ($p< 0.05$) based on the feed conversion ratio.

Table 4.5. Body weight, average daily gain (ADG), and intake of growing lambs fed urea or nitrate-based total mixed ration supplemented with or without acacia tannin extract

	Urea-based diet		Nitrate-based diet			[2]*P*-values		
Item	No tannin	With tannin	No tannin	With tannin	SEM[1]	N	T	N*T
n	10	10	10	10				
Dry matter intake, g/head/d	1121	1074	1194	1086	22.7	0.322	0.074	0.471
Initial body weight, kg	34.7	34.5	34.7	34.9	0.63	0.836	0.977	0.874
Final body weight, kg	47.0	43.8	49.3	45.7	0.98	0.246	0.096	0.922
Average daily gain, g/head/d	173	125	197	168	9.39	0.043	0.020	0.533
Feed conversion ratio	5.8	7.3	4.9	6.5	0.38	0.235	0.029	0.930

[1]SEM, standard error of mean. [2]N, effect of nitrogen source; T, effect of tannin inclusion; N*T, interaction effect of nitrogen source and tannin inclusion; $P < 0.05$.

The result of *in vivo* methane emission by experimental sheep are shown in Table 4.6. There was no significant interaction effect between nitrogen source and tannin inclusion on methane

(CH_4) production in the sheep when it is expressed in L of CH_4/head/d, L of CH_4/kg $BW^{0.75}$/d, g of CH_4/kg DMI or g CH_4/kg NDF intake. Tannin inclusion did not show any reduction in methane production ($p > 0.05$). However, animals on nitrate-based TMR diets produced lower methane in L/kg $BW^{0.75}$/d, compared to animals on the urea-based TMR diets ($p < 0.05$). Based on the amount of feed consumed, lambs fed with the nitrate-based diets produced lower methane per unit of DM ($p < 0.05$) or per unit of NDF consumed ($p < 0.05$), compared to animals feeding on the urea-based diets.

Table 4.6. Methane emissions of growing lambs fed a urea or nitrate-based total mixed ration diet supplemented with or without acacia tannin extract

[3]Parameter	Urea-based diet		Nitrate-based diet			[2]*P*-values		
	Without tannin	With tannin	Without tannin	With tannin	SEM[1]	N	T	N*T
n	5	5	5	5				
Methane, L/d	62.4	61.7	51.9	47.6	1.87	0.006	0.355	0.509
Methane, L/kg $BW^{0.75}$/d	3.34	3.35	2.60	2.58	0.12	0.005	0.895	0.848
Methane, g/kg DMI	31.2	29.8	26.3	24.0	1.14	0.018	0.368	0.827
Methane, g/kg NDF-intake	93.4	81.7	69.8	67.9	3.56	0.005	0.147	0.286

[1]SEM, standard error of mean. [2]N, effect of nitrogen source; T, effect of tannin inclusion; N*T, interaction effect of nitrogen source and tannin inclusion; $P < 0.05$. [3]DMI, dry matter intake; NDF, neutral detergent fibre.

4.4 Discussion

The astringency properties of condensed tannins are well documented (Bhatta et al., 2002; Landau et al., 2000a; Reed, 1995). However, the extent to which it affects dry matter and other nutrient intake in sheep and other ruminants is variable (Animut et al., 2008b; Bhatta et al., 2002). Tannin-containing additives have been suggested as supplements in the strategic management of nitrogen utilization in ruminants, especially in feeds containing high nitrogen content (Fernández et al., 2012). Nevertheless, studies exploring the relationship between rumen degradable nitrogen source and dietary tannin inclusion are scarce. In our study, both urea and nitrate-based diets had a high amount of non-protein nitrogen content that is easily soluble and thus expected to meet the rumen ammonia nitrogen requirements of the rumen microbes. The result of the trial showed that there was no significant effect of nitrogen source and tannin inclusion on dry matter intake. However, crude protein intake, g/head/day was significantly lower in the animals consuming nitrate-based diets. In contrast to our findings,

Van Zijderveld et al. (2011) did not observe any significant difference in nitrogen intake between sheep feeding on a urea-based diet and those on dietary nitrate. Reasons for significantly lower N-intake in sheep consuming the nitrate-based TMR in the current experiment may include the slightly lower CP content of the nitrate-based TMR compared to the urea-based TMR (207 g/kg vs 186 g/kg). In Kertz et al. (1982) study, a high ammonia nitrogen builds up in the rumen has been cited partly for lower crude protein intake in ruminants. Sheep have been observed to increase their consumption of a low quality, tannin-containing diet, following the consumption of a high crude protein diet, ostensibly to attenuate the high ammonia nitrogen build-up in the rumen (Fernández et al., 2012). In the current study, rumen ammonia concentration was not different between animals consuming the urea-based TMR diets and the nitrate-based TMR diets, at the time of rumen sampling. Elevated levels of nitrite in the rumen or blood can also be an explanation for lower crude protein intake in the ruminant animals as suggested by Leng (2008). There is a possibility that animals consuming the nitrate-based diet might select for a portion of the ration that is lower in nitrogen, in response to elevated nitrite levels in the rumen. This can partly explain the observed significantly lower N-intake which does not mirror the dry matter intake of the same group of animals consuming the nitrate-based TMR diets.

While the prolonged consumption of the acacia tannin extract (ATE) in their TMR diets did not result in any significant reduction in feed and nutrient intake, the reduction in DM, OM, CP, NDF and ADF digestibility was significant. According to Silanikove et al. (1996), the effect of tannin on nutrient digestibility may be stronger than the effect on feed intake. The experimental animals may have successfully adjusted to the bitter taste of the tannins, a trait that relates to reduced feed intake, while the protein-binding properties continue to manifest in the rumen. In the current study, tannin inclusion significantly reduced nutrient digestibility regardless of the non-protein nitrogen source. This trend is similar to previous reports on the impact of condensed tannins on rumen fermentation and nutrient degradability (Hariadi and Santoso, 2010; Hassen et al., 2016; Kamra et al., 2012; Lascano et al., 2003). Similar to the finding of this study, Beauchemin et al. (2007) observed significant reduction in crude protein digestibility, but contrary to our findings, no reduction in NDF and ADF digestibility was observed in cattle consuming quebracho condensed tannins up to 20 g/kg feed DM. Likewise, Carulla et al. (2005) found a significant reduction in CP, NDF and ADF in animals consuming *Acacia mearnsii* condensed tannin at 25 g/kg DM. Condensed tannins are known to have varying affinity levels for feed protein, microbial protein and fibre (Beauchemin et al., 2007;

Makkar, 2003a). For example, proline-rich proteins in the saliva of browsers such as goats are known to inactivate tannins to a greater extent than dietary proteins (Goel et al., 2005). Similarly, the dissociation of a protein-tannin complex post ruminally can be affected by the nature of the tannin and its affinity for dietary protein, endogenous protein and the presence of other macromolecules (Makkar and Singh, 1995), which will affect digestibility values.

Nitrogen balance was positive in all of the treatments, and differences in N-retention were neither associated with nitrogen source nor with tannin inclusion. This agrees with the report of Silivong et al. (2011) who observed a lack of differences in CP digestibility and N-retention as a result of urea or nitrate inclusion. Many studies have reported improved utilization of N with dietary tannin inclusion, associated with reduced rumen degradation of proteins and higher bypass protein (Grainger et al., 2009; Hristov et al., 2013), how well this bypass protein is digested and absorbed in the hindgut may vary widely (Waghorn, 2008). While this result showed that tannin inclusion resulted in a decrease in apparent total tract digestibility of crude protein (CP), the effect on total-N retention was not significant. This agrees with the findings of Carulla et al. (2005) and Norton (1999) who reported that dietary tannins did not significantly improve N-retention and overall N-balance in sheep despite a significant reduction in crude protein digestibility. According to Carulla et al. (2005) apparent CP digestibility, which influences Faecal-N loss, is known to depend on the fermentable organic matter rather than the absolute dietary-N content of feedstuff. Therefore, a reduced CP digestibility confirms the impact of tannin on the fermentable OM content of the ingested feed. This also reflects in the reduced performance of the animals on tannin TMR diets.

Lower N-Excretion observed in nitrate diets compared to urea diets in this study agrees with the findings of Sophal et al. (2013). However, while that study also reported significantly higher N-retention in nitrate diets compared to the urea diet, the current study did not find any significant differences in N-retention associated with the nitrogen sources. Sophal et al. (2013) also observed that while nitrate treatment resulted in significantly lower urinary-N, the current study did not observe any significant differences associated with nitrogen source or tannin inclusion. The lack of differences in urinary-N excretion as a result of NPN source or tannin inclusion may be as a result of the generally high amount of non-protein nitrogen in the TMR diets. High rumen ammonia-nitrogen concentration is often related to higher urinary-N excretion as a result of increased clearance of rumen ammonia and excretion in urine (Takahashi et al., 1998).

Endogenous-N losses, arising from the wear and tear of the GIT and other cells, also contribute to faecal N-losses and therefore dietary-N retention may be underestimated (Makkar, 2003a). One of the negative consequences of high dietary condensed tannin includes damage to the mucosal lining of the small and large intestine, and a high amount of mucous in the faecal material (Reed, 1995). No obvious change in the consistency of faeces such as scouring was observed in the lambs consuming the tannin extracts as part of the TMR diets.

Dietary nitrate or urea did not affect rumen pH as was previously reported by Nolan et al. (2010) and Silivong et al. (2011) in previous *in vivo* and *in vitro* studies, respectively. Similarly, Calabrò et al. (2012) showed that tannin inclusion did not affect rumen pH and only a slight increase in pH was reported by Hassanat and Benchaar (2013). However, tannin inclusion reduced ammonia-N concentration in the current study, and this is consistent with previous reports that condensed tannin may elicit reduced proteolytic activity in the rumen as a result of its binding to dietary protein, thus making them undegradable by the rumen microbes (Carulla et al., 2005; Cieslak et al., 2012). The ammonia concentration in rumen fluid was not different between animals consuming urea-based TMR diets and nitrate-based TMR diets. This result is consistent with the report of Van Zijderveld et al. (2010) in sheep supplemented with urea or nitrate. Wang et al. (2018) however reported lower ammonia concentration in lactating cows consuming nitrate compared to those consuming urea. However, the CP content of the diets in that study was lower (11%) compared to the study by Van Zijderveld et al. (2010) (15%) or the current study (19%). Wang et al. (2018) noted that the reduced ammonia concentration was because of increased clearance of rumen ammonia and their incorporation into microbial protein synthesis largely because of the low protein content of the diet. Generally, when nitrate is administered in the diet of ruminants, the efficiency of nitrate utilization and rumen fermentation is expected to depend, among other things, on the CP content of the diet and requirements of the animal (Hulshof et al., 2012; Olijhoek et al., 2016). These would affect the rate of incorporation of ammonia into microbial protein and the rate of ammonia excretion as urine (Wang et al., 2018).

Nitrogen source did not significantly affect propionate concentration and this is in agreement with some previous findings reported for sheep (Nolan et al., 2010; Van Zijderveld et al., 2010) and cattle (Van Zijderveld et al., 2011). In contrast to our findings, Hulshof et al. (2012) reported reduced propionate following dietary nitrate inclusion as a replacement for urea in the diet of beef cattle. Time of rumen fluid sampling may have a profound effect on rumen fermentation parameters studied. In the current study, lambs were fed *ad lib* at 110% of

previous day feed intake, and prior to slaughtering, animals only had access to the remnant of the previous day's feed. A pattern of a significant shift in VFA proportion from propionate to acetate has been reported to wane after 12 h of feeding as this shift was not observed at 24 h after feeding (Van Zijderveld et al., 2010). According to Bannink et al. (2008) sheep have a lower feed intake relative to body weight compared to cattle, and coupled with generally higher pH; they tend to produce relatively less propionate and more acetate. This generally lower propionate consequently enables nitrate to compete for reducing equivalents more with methanogenesis than with propiogenesis unlike in high producing dairy cows for example, where propionate concentrations are generally high (Van Zijderveld et al., 2011). Large fluctuations may, therefore, occur in propionate concentration due to small variation in rumen fluid sampling or handling. However, tannin inclusion increased the proportion of propionate, thus partly resulting in a reduced A/P ratio. However, at constant TVFA concentration, the reduced A/P ratio might also have been associated with reduced fibre digestion (Carulla et al., 2005). This corresponds well with the observed significantly reduced NDF and ADF digestibility associated with tannin inclusion irrespective of the NPN source.

Animals on nitrate-based TMR had higher ADG compared to those on urea-based TMR diets even though dry matter intake (DMI) and FCR was not different. Newbold et al. (2014) however observed reduced DMI in Nellore bulls with increasing levels of nitrate intake. This may be as a result of the palatability of the nitrate salt. The degree to which feed intake is decreased following nitrate supplementation may depend on the feed type. Generally, reduced DMI in nitrate diets have been associated with the suppressive effect of nitrite on NDF digestibility or a reduced palatability of nitrate-containing diets (Bruning-Fann and Kaneene, 1993; Newbold et al., 2014). Wider variability in the effect of nitrate on DMI may be expected as the forage to concentrate ratio of the animal's diet increases. Animals on the nitrate-based TMR diets, on the other hand, produced significantly reduced methane with higher ADG compared to those on urea-based TMR diets. Significant methane reduction was similarly observed in the trials of Hulshof et al. (2012) and Van Zijderveld et al. (2011) but this reduced methane was not accompanied with improved performance, in terms of growth and milk production respectively. However, Hao Phuc et al. (2009) reported significantly higher ADG and FCR in ammonium nitrate supplemented goats compared to urea supplemented goats. Guo et al. (2009) equally reported that more efficient microbial protein synthesis is likely to occur when nitrate replaces urea in ruminant diets. This may partly explain the observed improved ADG associated with nitrate-based TMR over the urea-based TMR diets.

The significant reduction in enteric methane production associated with nitrate diets confirm earlier findings reported in nitrate-fed sheep (Nolan et al., 2010; Sar et al., 2004a) and cows (Sophal et al., 2013). The result also confirms the persistence of nitrate to reduce methane over prolonged feeding time as observed in dairy cows (Van Zijderveld et al., 2011). The inhibition of methane production can be attributed to the more favourable use of available hydrogen in nitrate reduction to ammonia, competing with the process of methanogenesis, where CO_2 is reduced to methane by methanogenic archaea among other processes. Several factors, however, may affect the nitrate reduction process in the rumen. A significant amount of dietary nitrate can be converted to ammonia and absorbed through the rumen wall and into the blood circulation where it is subsequently lost in the urine (Takahashi et al., 1998). The solubility of the particular nitrate salt in the rumen may also affect how much methane reduction is achieved with nitrate supplementation (Van Zijderveld et al., 2010). Similarly, the concentration of rumen ammonia-N, as affected by dietary characteristics, may affect nitrate clearance through the rumen wall, versus nitrate-reduction to ammonia in the rumen. In our study, we used the prolonged and gradual adaptation strategy proposed by Nolan et al., (2010) to increase the rumen capacity of the experimental animals, to adapt to nitrate and nitrite reduction as well as elevated ammonia-N concentration.

4.5 Conclusions:

Adding Acacia tannin extracts to the diets of growing lambs did not significantly improve total –N retention or reduce methane emission regardless of the NPN source, although it helped in shifting nitrogen loss from urine to faeces and reduced crude protein degradability in the rumen. Calcium nitrate instead of urea consistently reduced methane emissions and increased lamb growth performance with or without the inclusion of tannin extract in the TMR diet of South African Mutton Merino lambs.

CHAPTER FIVE

Effect of different non-protein nitrogen sources and tannin supplementation on haematology and serum biochemical traits in growing merino lambs

Abstract

The aim of this study was to determine the effect of NPN sources and tannin inclusion (Acacia tannin extract, ATE) on the haematology and serum biochemical parameters of growing Merino lambs. Twenty-four ram-lambs and sixteen ewe-lambs (34.7±4.0 kg) were blocked for sex, stratified and allotted as two animals per pen in a randomised complete block design with 5 blocks and 4 dietary treatments: urea-based TMR, urea-based TMR+42 g/kg DM ATE, calcium nitrate-based TMR, calcium nitrate-based TMR+42 g/kg DM ATE. Diets were formulated to be isonitrogenous with about 60:40 concentrate: forage ratio comprising of Lucerne and Eragrostis hay. The experiment lasted for 81 d, consisting of 21 d for gradual adaptation and 60 d for continuous feeding and growth performance monitoring. Thereafter, 5 mL of blood was collected from each animal via the jugular vein to determine the haematological profile and serum biochemical variables. Data obtained was analysed using the GLM procedure of SAS and treatment means separated with Tukey's test. No clinical or sub-clinical signs of morbidity and tannin intoxication symptoms were observed across the treatments. Animals on nitrate-based TMR had higher haematocrit, RBC count and haemoglobin concentration than animals on urea-based TMR. Similarly, ATE reduced plasma LDH and cholesterol concentration. Compared to reference standards, there was slightly elevated BUN levels, while creatinine, total protein, ALT, AST and LDH levels were within normal range in all treatments. The result suggests that Acacia tannin extract could be added at the current dose to the diets of animals consuming either urea or calcium nitrate as NPN source without any serious negative consequences to the animal. No significant interaction effect between NPN source and tannin inclusion was observed in all the parameters evaluated.

Keywords: Haematology, Nitrate, Non-protein nitrogen, Serum biochemistry, Tannin, Urea.

5.1 Introduction

The restriction on the use of antibiotics as dietary supplement across the EU has elicited wide interest in the use of plant secondary metabolites in sustainable animal production activities (Akanmu and Hassen, 2017; Patra and Yu, 2014). As a dietary additive, their utilization continues to generate much interest especially in their ability to help increase bypass protein flow to the small intestine, control of bloat and intestinal nematode, as well as methane mitigation (Huang et al., 2011; Pathak et al., 2013; Patra and Saxena, 2011).

Even though it is understood that condensed tannins do not directly constitute any physiological risk to vital organs of ruminants under normal physiological conditions (Terrill et al. 1994), a very high concentration of condensed tannin could lead to intestinal damage (Degen et al., 1995; Makkar and Singh, 1995). The resulting higher permeability of condensed tannin into the bloodstream, may result in toxicity symptoms particularly in the presence of saponins (Makkar, 2003a). Equally, the presence of hydrolysable tannin and other low molecular weight phenols in the crude tannin extract, makes their impact on the optimal physiological state of the animal become an important consideration. Tannic acid at a dose exceeding 0.4 g/kg BW per day has been noted to result in toxicity symptoms in Sheep (Murdiati et al., 1992).

Urea which is widely included in the diets of ruminants serves as a cheap source of non-protein nitrogen (NPN) utilisable by rumen microbes for their microbial protein synthesis (Adejoro and Hassen, 2017; Mapato et al., 2010). Recent interest in the use of nitrate salts has been due to its potential in mitigating enteric methane, apart from also being a NPN source (Cottle et al., 2011). Most studies on tannin and nitrogen interaction in ruminants have focused on protein digestibility and growth performance, while their impact on the physiology and health status of animals have not been widely reported. High blood urea nitrogen can arise as a result of supplementation of urea or nitrate and can alter hepatic metabolism through increased ureagenesis, and glucose metabolism occurring in the liver and other tissues (Huntington et al., 2006; Taylor-Edwards et al., 2009). High ammonia can therefore elicit serious effects on growth, as well as on the quality of animal products such as milk, and meat. Even when nitrate toxicity symptoms are not observable, increased production of indicators of oxidative stress in blood and tissue of animal may affect the optimal physiological wellbeing of animals (Vatassery et al., 2004). When reactive oxygen species (ROS) production exceeds the antioxidant defence mechanisms in the body of the animal, oxidative stress occurs (Cigerci et al., 2009) and this may exert negative effects on meat quality.

Certain phytochemicals have been noted to stimulate the production of certain enzymes and hormones capable of affecting the physiological processes of animals (Adedapo et al., 2005; Longstaff and McNab, 1991). This has been reported to ameliorate disease conditions and prevent morbidity (Athanasiadou et al., 2001; Min et al., 2004). Haematological parameters, as well as serum indexes, are reliable tools to evaluate the immune status and systemic toxicity in animals, especially serum enzymes which are known to be sensitive indicators of liver or kidney damage (Silanikove et al., 1996b). It is hypothesised that the choice of NPN, either urea or calcium nitrate, with or without tannin extract supplementation, does not have any negative effect on the selected haematological and serum biochemical parameters of the experimental animals. This will indicate that the additives do no impose any toxicity symptom or alter the physiological state of the animals after long-term consumption of the experimental diets. Therefore, this study was designed to investigate the effect of NPN source and tannin inclusion on the haematology and serum biochemical parameters of growing Merino lambs.

5.2 Materials and methods

Animal management protocols were carried out in accordance with the University of Pretoria Animal Ethics Committee (AEC) guidelines as stipulated in the approval number EC061-14. The experiment was conducted at the small stock unit of the University of Pretoria experimental farm, Hatfield Pretoria, South Africa.

Animal, experimental design and adaptation

The experiment was designed as a randomised complete block design. Twenty-four ram-lambs were stratified into three blocks while sixteen ewe-lambs were stratified into two blocks, based on body weight at the start of the experiment, making a total of forty animals in five blocks. Two animals from each block were then randomly allocated to each pen. Five pens, one from each block was therefore allocated to each dietary treatment. Within each treatment, the average body weight was 34.7±4 kg. Dietary treatment, comprising an iso-nitrogenous and iso-caloric TMR contain urea or calcium nitrate as NPN sources with or without tannin extract. The treatment I, urea-based TMR; treatment II, urea-based TMR + tannin, treatment III, nitrate-based TMR and treatment IV, nitrate-based TMR + tannin. Acacia tannin extract (ATE) supplied by UCL Company (Pty) Ltd. Dalton, South Africa was included at 42 g/kg feed DM, and equivalent to 1.7 g/kg (leucocyanidin equivalent) CT inclusion. Animals were fed the respective diets for a total period of 81 days comprising of an initial 21 day gradual adaptation and 60 d of continuous feeding and monitoring of growth performance. At the end of the 81 d,

blood sampling was done for individual animals. The ingredient and chemical composition of the experimental diets are as shown in Table 4.1 in the previous chapter.

Blood sampling and analysis

At the end of the growth period, 5 ml blood samples were drawn from the jugular vein of each animal into separate serum bottles, for the analysis of serum biochemical and haematological parameters. Samples were subsequently analysed at the Clinical pathology laboratory, Faculty of Veterinary Science, University of Pretoria, Pretoria. South Africa. Complete blood count was done on whole blood collected in bottles containing EDTA while a second sample was analysed for the following serum parameters: blood urea nitrogen, creatinine, glucose, total protein, albumin, globulin, cholesterol, total triglycerides, aspartate aminotransferase, alkaline phosphatase, alanine aminotransferase, and lactate dehydrogenase using standard protocols.

Statistical analysis

Values obtained from blood sample analysis was analysed as a 2 × 2 factorial treatment combination in a randomised complete block design with replicates. The statistical model used included a block effect and treatment effect, which was further partitioned into the effects of NPN sources and tannin inclusion, and an interaction between NPN source and tannin inclusion, using the GLM procedure of SAS (SAS Inst. Inc.; Cary, NC, USA). The statistical model used for the analysis include:

$$y_{ijk} = \mu + \text{Block} + A_i + B_j + (AB)_{ij} + \varepsilon ijk$$

where y_{ijk}= observation k at different nitrogen source (i; urea or nitrate) and level j of tannin inclusion (j; no-tannin, with-tannin); μ= overall mean; Block= effect of blocking (body weight × sex); A_i= the effect of nitrogen source; B_j= the effect of level of tannin inclusion; $(AB)_{ij}$= the effect of the interaction of nitrogen source with or without tannin inclusion, and εijk= random error with mean of 0 and variance σ^2. Treatment means were compared using Tukey's test and reported as least significant means and standard error of means. Significant differences were declared when $P < 0.05$.

5.3 Result

The result of haematology analysis is presented in Table 5.1. The interaction effect between nitrogen source and tannin inclusion was not significant for all the haematological parameters studied which include Haemoglobin (Hb), Haematocrit (HCT), corpuscular haemoglobin (MCH), Mean corpuscular haemoglobin concentration (MCHC), Mean corpuscular volume (MCV), Red blood cell (RBC) count, White blood cell (WBC) count, RBC distribution width, segmented neutrophil, Lymphocyte count, Monocyte count, Eosinophil count, Basophil count and Platelet count. Animals on nitrate-based TMR diet showed a tendency for higher plasma haemoglobin ($p< 0.094$), RBC count ($p< 0.089$) and haematocrit concentrations ($p< 0.083$) than animals fed on urea-based TMR diets. Tannin inclusion did not seem to have any effect on most of the haematological parameters. However, there appears to be a slight reduction in MCV ($p< 0.077$), and an increase in RBC distribution width ($p< 0.077$) and monocyte count ($p< 0.081$) associated with tannin inclusion in the TMR diet compared to animals fed TMR without tannin extract. For most of the haematological parameters, recorded values were within the reference range quoted for ovine species in South Africa (Jain, 1986; Van Zyl, 1967).

Table 5.1 Haematological parameters of growing lambs fed a total mixed ration diet containing urea or nitrate with or without acacia tannin extract

[1]Parameter	[2]Ref. range	Urea diet		Nitrate diet		SEM[3]	p-values		
		No tannin	With tannin	No tannin	With tannin				
N		10	10	10	10		N	T	N*T
Haemoglobin (HB; g/L)	80-140	116.9	119.9	122.5	125.0	1.79	0.094	0.629	0.895
RBC count ($\times 10^{12}$ cells/L)	5.5-9	9.97	10.6	10.6	10.8	0.17	0.089	0.348	0.518
Haematocrit (HCT; L/L)	0.22-0.44	0.33	0.34	0.35	0.34	0.01	0.083	0.877	0.372
MCV (fL)	23-48	33.1	32.1	33.2	32.1	0.27	0.869	0.077	0.80
MCH (pg)	Nr	11.8	11.4	11.6	11.6	0.09	0.716	0.279	0.258
MCHC (g/dL)	31-38	35.6	35.4	34.8	36.2	0.26	0.833	0.341	0.147
RBC Distr. Width (%)	Nr	17.3	17.4	16.7	17.8	0.19	0.589	0.077	0.608
WBC count ($\times 10^{9}$ cells/L)	4-10	7.56	7.46	7.11	7.43	0.23	0.721	0.668	0.222
Segmented Neutrophil	0.4-5	2.84	2.77	2.59	2.51	0.19	0.383	0.935	0.966
Lymphocyte count ($\times 10^{9}$ cells/L)	1.6-7.5	4.36	4.19	4.13	4.50	0.18	0.653	0.649	0.950
Monocyte count ($\times 10^{9}$ cells/L)	0-0.6	0.29	0.38	0.28	0.36	0.02	0.872	0.081	0.853
Eosinophil count ($\times 10^{9}$ cells/L)	0-1	0.05	0.08	0.09	0.06	0.01	0.765	0.839	0.419
Basophil count ($\times 10^{9}$ cells/L)	0-1	0.004	0.001	0.020	0.004	0.004	0.232	0.184	0.518
Platelet count ($\times 10^{9}$ cells/L)	250-750	462.4	489.9	490.8	481.8	15.2	0.841	0.737	0.682

[1]RBC, Red blood cell; MCV, . [2]Ref, Reference range (Van Zyl, 1967); nr, no reference value available. [3]SEM, standard error of mean.

The results of the serum biochemistry analysis of experimental animals is shown in Table 5.2. The interaction effect of nitrogen source and tannin inclusion was significant ($p< 0.05$) for the blood urea nitrogen (BUN) parameter. Compared to urea, the use of nitrate in the TMR diet increased the BUN concentration of animals fed TMR diet without tannin while nitrate has no effect on BUN concentration of animals in the presence of tannin. There was no interaction between nitrogen source and tannin inclusion on creatinine, blood glucose, serum protein, albumin, globulin and cholesterol concentration in the animals. Tannin inclusion reduced blood glucose concentration in animals consuming the TMR diet with tannin when compared with animals consuming the TMR diet without tannin extract inclusion ($p< 0.05$). Nitrogen source tends to affect plasma creatinine concentration ($p< 0.054$) in the growing lambs, with lambs on the nitrate-based TMR diets generally having elevated creatinine concentrations compared to lambs on the urea-based TMR diets. However, tannin inclusion did not show any effect on creatinine concentration in experimental animals ($p> 0.05$). Neither nitrogen source, nor tannin inclusion had any effect on total serum protein, albumin and globulin concentrations in the plasma of the lambs. Even though nitrogen source did not affect blood glucose and cholesterol concentration, the inclusion of tannin in the TMR diet reduced blood glucose and plasma cholesterol levels of lambs consuming the TMR diet with tannin, compared to those lambs that received TMR diet without tannin ($p< 0.05$). Nitrogen source and tannin inclusion did not show any effect on Aspartate aminotransferase and Alkaline phosphatase concentrations of the blood of the lambs ($p> 0.05$). However, the Alanine aminotransferase level was higher in those lambs fed on the nitrate-based TMR diets compared to those lambs fed on the urea-based TMR diets ($p< 0.05$). Similarly, Lactate dehydrogenase concentration in the lambs that received TMR diet with tannin was higher than those lambs fed TMR diets without tannin ($p< 0.05$). The total triglyceride concentration in the lambs was not affected neither by nitrogen source nor by the inclusion of tannin.

Table 5.2 Effects of a total mixed ration diet containing urea or nitrate with or without acacia tannin extract on serum biochemical parameters of growing Merino lambs

		Urea diet		Nitrate diet					
Parameter	[1]Ref. range	No tannin	With tannin	No tannin	With tannin	SEM[2]	P-value		
N		10	10	10	10		N	T	N*T
BUN, mmol/L	4.7-7.6	8.79[b]	9.09[b]	10.2[a]	8.89[b]	0.18	0.015	0.128	0.018
Creatinine, µmol/L	44-150	57.9	58.0	67.3	65.1	1.23	0.054	0.692	0.99
Blood Glucose, mmol/L	2-3.7	4.57	3.77	4.30	3.60	0.13	0.834	0.01	0.855
Total serum protein, g/L	60-75	62.9	60.5	63.1	62.1	0.48	0.759	0.272	0.852
Albumin, g/L	28-34	35.6	35.7	37.6	37.6	0.39	0.290	0.775	0.768
Globulin, g/L	32-43	27.3	24.8	25.6	24.5	0.52	0.311	0.235	0.719
Cholesterol, mmol/L	nr	1.34	1.10	1.41	1.31	0.04	0.818	0.029	0.511
Aspartate amino-transferase (AST), U/L	10-125	106.8	105.1	97.7	107.6	2.81	0.388	0.677	0.660
Alkaline phosphatase (ALP), U/L	33-246	297.2	273.0	302.8	274.6	14.8	0.871	0.913	0.982
Alanine amino-transferase (ALT), U/L	9-45	14.0	15.6	17.9	18.8	0.58	0.001	0.351	0.656
Lactate dehydrogenase (LDH), U/L	nr	917.8	805.9	946.3	872.1	18.6	0.204	0.012	0.781
Total triglyceride, mmol/L	nr	0.229	0.246	0.217	0.217	0.01	0.899	0.615	0.627

[1]Ref, Reference (Van Zyl, 1967); nr, no reference value available. 2SEM, standard error of mean.

5.4 Discussion

There was no obvious sign of disease condition such as scouring, diarrhoea, anorexia, bloat or anaemia in the experimental animals. However, sub-clinical disease conditions like mild methemoglobinemia may occur undetected, especially under the prevailing nutritional management system. Most of the haematological parameters were within the normal reference range for ovine species. The observed tendency for elevated levels of Hb and RBC count associated with nitrate supplementation in this study is consistent with the report of El-Zaiat et al. (2014) on nitrate supplementation. Although, Cherdthong et al. (2014) did not observe any significant increase in Hb and RBC count due to nitrate supplementation. Haemoglobin (Hb) is the iron-containing conjugated protein that transports oxygen and carbon dioxide across cells and tissues in the body. Therefore, increased Hb and RBC count in animals consuming nitrate diets can be associated with a physiological response to mitigate the effect of nitrite on the oxygen-carrying capacity of the blood, by increasing the cell numbers to meet reduced capacity due to methaemoglobin. Unfortunately, the methaemoglobin level in the lambs was not determined in this study.

Mahgouba et al. (2008) observed higher leukocyte, alanine aminotransferase (ALT) and lower serum iron in growing lambs due to negative consequences of phenolics and tannins. In contrast, the white blood cell count (leukocyte) and ALT concentration in the current study were not affected by tannin supplementation. Rivero et al. (2012) similarly did not observe any negative effect on these serum parameters due to the supplementation of tannin. The urea-treated palm fronds consumed by the animals in Mahgouba et al. (2008) contained total extractable phenol concentration of 112.6g/kg and condensed tannin concentration of 12.8g/kg DM, indicating that high amount of other phenolic compounds like hydrolysable tannins or saponins may be present. High saponin concentration is known to have a very strong haemolytic activity because of the affinity of aglycone saponins for the cell membrane cholesterol, forming complexes, and increasing cellular permeability and loss of haemoglobin (Wang et al., 2007). According to Terrill et al. (1994), condensed tannins are not absorbed into the portal circulation. However, the presence of other phenolics in the plant extract may instigate symptoms other than those associated with CT.

In the current study, the level of lymphocyte, monocyte and eosinophil recorded for the lambs are within a normal range of values reported for healthy animals indicating a healthy immune status of the experimental animals. Monocytes, being precursors of macrophages and

lymphocytes, are essential for humoral and cell-mediated immunity (Mahgouba et al., 2008). In this study, only a slight reduction in MCV, coupled with a slight increase in RBC distribution width and monocyte count were observed to be associated with tannin inclusion. Nitrogen source or tannin inclusion did not have any significant effect on WBC and lymphocyte count in the current study. This is an indication that underlying pathophysiological conditions such as leucopenia, lymphocytosis or lymphopenia are not likely to have occurred in the experimental animals (Olafadehan et al., 2014) and serving as an indication of the health status of the experimental animals. MCV is an indication of high cell turn over, often increasing the probability of releasing immature RBC into the circulatory system.

There was a significant interaction effect between nitrogen source and tannin inclusion in terms of BUN levels. Nitrate addition increased BUN levels when used in a TMR diet without tannin extract while the effect of nitrate was not significant when used in a TMR diet with tannin extract. Nevertheless, the BUN concentration across the treatments was generally higher than the reference values for Merino Sheep in South Africa (4.7-7.6 mmol/L) (Van Zyl, 1967). This may be partly attributable to the high concentration of non-protein nitrogen or high crude protein content of the TMR diets of the lambs. Similarly, Blood glucose concentration across the treatments was higher than the reference range (2-3.7 mmol/L). The high BUN and total serum protein could be due to high ammonia concentration diffusing from the rumen, and entering portal circulation. Kohn et al. (2005) reported that BUN has a linear relationship with total N-excretion rate. In the current study, the high BUN concentration could be related to the generally high N-excretion (as proportion of N-intake) rate reported in the experimental lambs (Adejoro and Hassen, 2018 Chapter 4; unpublished). The impact of tannin extract in reducing BUN in lambs consuming nitrate based TMR diets may be indicative of tannin counteracting elevated ammonia levels in the rumen through its protein binding properties. The generally high BUN concentration observed in this study also correlates with high plasma glucose concentration, irrespective of the nitrogen source and tannin inclusion, although tannin inclusion led to a reduction in glucose concentration. This result agrees with the observation of Taylor-Edwards et al. (2009) who observed that high BUN was associated with an increased serum glucose concentration. This has been attributed partly to either a reduction in the rate of glucose utilization, net increase in hepatic glucose production or an overall increase in hepatic glycogenolysis.

Most ruminant species normally absorb only a small amount of glucose from their feed because most of the carbohydrates are fermented to volatile fatty acids. Glucose requirements are

usually met by synthesis in the liver, from glucose precursors such as propionate from rumen fermentation. This is particularly important in lactating dairy cows, where more than 70% of the serum glucose supply is used in milk synthesis (Reynolds, 2005). Diet characteristics may play an important role in serum glucose and BUN levels. For example, the improved utilization of rumen degradable protein when an adequate amount of water-soluble carbohydrates are present have been observed to increase serum glucose, microbial protein synthesis and milk production in cows (Miller et al., 2001). Creatinine and BUN are indicators of kidney functionality in animals (Garg et al., 1992; Olafadehan, 2011). Creatinine levels in this study were within normal range of the reference values, although slightly higher values were observed in lambs consuming the nitrate based TMR diet. However, tannin extracts reduced BUN levels in animals consuming the nitrate-based TMR diets. While hepatic and renal damage has been reported for animals consuming tannins in certain studies, the reported cases related mainly to plants with high hydrolysable tannin content as observed in goats consuming soapbush (Murdiati et al., 1990) and Cattle consuming oak leaves (Garg et al., 1992) as opposed to condensed tannin.

Total serum protein, albumin and globulin, although not significantly different across the treatments, are indicators of the protein status of animals (Tennant and Center, 2008). Total protein and albumin are also useful indicators of infection, inflammation or hydration status of animals (Knowles and Warriss, 2000). Regardless of the nitrogen source and with or without tannin inclusion, these parameters were within normal range. The significant increase in ALT in animals consuming the nitrate-based TMR compared to animals consuming the urea-based TMR diet may relate to the production of nitric oxide and reactive oxygen species that are capable of promoting lipid peroxidation (Salvemini and Cuzzocrea, 2002). The ALP and cholesterol are conventionally used to detect bile obstruction in hepatic damage diagnosis, both in the human and domestic animal while ALT is a liver-specific hepatocellular enzyme that indicates liver cell damage (Mahgoub et al., 2008; Olafadehan, 2011). In this study, the ALT levels in all the treatments were within a normal range. It is unclear what factors could lead to slightly higher ALP levels in the experimental animals compared to the reference values but the age of animals have been reported to significantly affect ALP concentrations (Lepherd et al. 2009), with younger animals usually having higher ALP concentrations in their blood than older animals. This may explain the higher values recorded in this study.

In the current study, tannin inclusion significantly reduced lactate dehydrogenase (LDH) concentrations in the experimental lambs. In contrast to the findings of this study, a higher LDH

concentration was reported in cattle consuming oak leaves (Garg et al., 1992). This may be attributed to variation in the nature of tannins (proportion of hydrolysable tannin versus condensed tannin). Normal to moderately low LDH levels are regarded as positive signs of an animal's health and welfare (Karakilcik et al., 2004; Salimeh et al., 2010). Significantly low LDH levels have been reported in rabbits as a result of ascorbic acid supplementation (Karakilcik et al., 2004) and therefore this low LDH levels may imply that the tannin extract exerts some antioxidant properties on the animals. Nitrogen source and tannin inclusion did not affect total triglyceride concentration in the animals. The stepwise introduction of the diets and gradual adaptation allows the rumen microbes to stimulate the adequate multiplication of nitrate and nitrite reducing bacteria thus potentially reducing the animal's susceptibility to any clinical or subclinical intoxication (Alaboudi and Jones, 1985; Leng, 2008). These may have also played a significant role in ensuring that the physiological wellbeing of the animals was sustained throughout the experiment.

5.5 Conclusions

The absence of any clinical or sub-clinical signs of morbidity and tannin intoxication symptoms and the physiological values from the haematology and biochemical parameters recorded on the animals suggest that Acacia tannin extract or calcium nitrate at the current dose can be fed to growing lambs, without any serious negative consequences. A slight increase in haemoglobin, haematocrit and RBC count was associated with the use of calcium nitrate instead of urea as a NPN source.

CHAPTER SIX

Characterization of starch and maltodextrin microparticles encapsulating Acacia tannin extract and evaluation of their potential for use in ruminant nutrition

Abstract

The use of tannin extract and other phytochemicals as a dietary additive in ruminants is becoming more popular due to their wide biological uses such as methane mitigation, bypass of dietary protein, control of intestinal nematodes, among others. Unfortunately, some have strong astringency, low stability and bioavailability, and negatively affecting dry matter intake and digestibility. To circumvent these drawbacks, an effective delivery system may offer a promising approach to administer these extracts to the site where it is required. The objectives of this study were to encapsulate Acacia tannin extract (ATE) with native starch and Maltodextrin-gum Arabic and to test the effect of encapsulation parameters on encapsulation efficiency, yield and morphology of the microparticles obtained as well as the effect on rumen *in vitro* gas production. Acacia tannin extract was encapsulated with the wall materials, and the morphological features of freeze-dried microparticles were evaluated by scanning electron microscopy. The *in vitro* release pattern of microparticles in acetate buffer, simulating the rumen, and its effect on *in vitro* gas production was evaluated. The morphological features revealed that microparticles encapsulated with maltodextrin/gum-Arabic were irregularly shaped, glossy and smaller, compared with those encapsulated with native starch, which was bigger, and more homogenous. Maltodextrin-gum Arabic could be used up to 30% loading concentration compared with starch, which could not hold the core material beyond 15% loading capacity. Encapsulation efficiency ranged from 27.7%±6.4– 48.8%±5.5 in starch and 56.1%±4.9– 64.8%±2.8 in maltodextrin-Gum Arabic-encapsulated microparticles. Only a slight reduction in methane emission was recorded in encapsulated microparticles when compared with the samples containing only wall materials. Both encapsulated products exhibited the burst release pattern under the pH conditions, and methane reduction that is associated with tannin was marginal. This is attributable to small loading percentages, therefore other wall materials or encapsulation methods should be investigated.

Keywords: Encapsulation; Gum-Arabic; *In vitro* release; Maltodextrin; Microparticles; Starch; Rumen fermentation; Tannin extract.

6.1 Introduction

The utilization of tannins has become very important in ruminant nutrition studies as a result of their wide application in ruminant animal production. Their biological significance has revealed that their protein binding properties can be harnessed in many applications to improve ruminant animal performance (Hassen et al., 2016; Hristov et al., 2013). Dietary condensed tannin extracts have shown a significant reduction in enteric methane production both *in vitro* and *in vivo* (Huang et al., 2010). This has been related to both direct inhibition of the growth of methane-producing archaea community (methanogens) through tannin action of their functional proteins, resulting in bacteriostatic and bactericidal effects or indirectly by the defaunating action on methanogen-associated protozoa populations (Animut et al., 2008a; Bhatta et al., 2009). Condensed tannins have also been found very effective in the control of intestinal parasites such as *Haemancorchotus conchortus* nematode and larva, both in ruminant species consuming tannins as part of their natural browse forage or by direct administration of the extract (Naumann et al., 2014; Ribeiro et al., 2013). Other important applications of condensed tannins include the enrichment of conjugated linoleic acid (CLAs) in meat and milk via ruminal bio-hydrogenation, control of bloat and improving the efficiency of protein digestion via the bypass of a good quality dietary protein (Patra and Saxena, 2011).

Animal responses to dietary tannin have however been noted to be dependent on dose and other chemical characteristics of the tannin source (Kamra et al., 2012). Generally, increasing dietary condensed tannin concentration has resulted in a decrease in methane production per unit of digestible organic matter during rumen fermentation (Hassen et al., 2016). However, some of the limitations to the use of condensed tannins relates to its astringency and bitter taste, which among other negative consequences, leads to reduced voluntary dry matter intake in the animals (Dschaak et al., 2011). The astringency of tannins is associated with the interactions between polyphenols and salivary proteins, which obstructs palate lubrication and precipitation of insoluble aggregates in the mouth (de Freitas et al., 2003). The administration of polyphenolic compounds like tannin extracts can be improved by the formulation of a finished product that is able to mask their taste while retaining their structural integrity until consumption, increase their bioavailability, and then deliver as well as release them precisely at the target site (Munin and Edwards-Lévy, 2011). This can be attained through the various encapsulation techniques (Gibbs et al., 1999).

The microencapsulation process relies on the use of wall materials that are biological polymers (Munin and Edwards-Lévy, 2011). Various wall materials can be utilised in encapsulating plant

extracts and various polyphenolic substances in the food/feed industry. These wall materials include among others, starch, maltodextrin, gelatine etc. or combination of polymers such as maltodextrin and inulin, maltodextrin and gum arabic, gum Arabic and tapioca starch etc. (Tonon et al., 2010; Zhang et al., 2007) depending on the characteristic properties of the active ingredient. Additives for ruminant animals that have been widely encapsulated include rumen-protected amino acids (methionine, lysine), multivitamin products, fumaric acid and slow release urea products (Huntington et al., 2006; Ni et al., 2009; Wood et al., 2009). However, the limitations of cost and suitability of many of the common polymers used in the food industry have been noted (Krishnan et al., 2005a) and may hinder their commercial use in livestock applications. Their pattern of release of the active ingredient in the ruminant digestive system has also not been extensively evaluated. Therefore, the selection of a suitable wall material is critical to the success of the encapsulation process in terms of efficiency, yield and retention of the biological activity of the core material (Fernandes et al., 2014).

The effectiveness of Gum-Arabic in the encapsulation of polyphenolic extracts has been documented in literature but remains an expensive choice in most food applications (Kanakdande et al., 2007). Starch is a common wall material that is very cheap and accessible but has very low emulsifying properties. Maltodextrin is a hydrolysed starch product that also offers the advantage of being cheap, has low viscosity at high solid concentrations and is capable of protecting the core material against oxidative damage (Goula and Adamopoulos, 2012). Encapsulation of tannin extract has the potential of reducing the impact of tannin consumption on dry matter intake. A sustained release of tannin in the rumen will also improve its utilization significantly. It is hypothesised that encapsulated-ATE will result in microparticles that exhibit slower *in vitro* release, compared with the crude extract, while retaining the attributes of tannin such as reduction in methane gas *in vitro*. This study aimed to encapsulate Acacia tannin extract (ATE) with native starch or maltodextrin-gum Arabic and then characterize the microparticles based on their morphology, encapsulation efficiency and yield. Furthermore, the *in vitro* release profiles of the microparticles in buffer solutions that simulate ruminant GIT, as well as effect of their dietary supplementation on *in vitro* gas production were studied. To the best of our knowledge, there are no published works reporting the encapsulation of tannin extract for ruminant animal applications, specifically using these wall materials.

6.2 Materials and Method

This study was carried out in accordance with the guidelines stipulated by the National Health Research Ethics Council of South Africa and approved by the University of Pretoria Animal Ethics Committee (AEC) with the approval number EC061-14.

Materials

Acacia tannin extract (ATE), a highly water-soluble extract from the *Acacia mearnsii* tree bark, was obtained from UCL Company Pty (Ltd), South Africa, and used as the active ingredient or core material in the current study. Gum arabic, maltodextrin (DE 16.5), native potato starch and tannic acid were obtained from Sigma Aldrich Inc. (USA); the F57 fibre filter bags were purchased from ANKOM Inc (NY, USA). All chemicals and reagents were of the analytical grade in purity.

Properties of acacia tannin extract

Acacia tannin extract (ATE), obtained from UCL Company Pty (Ltd). Dalton, South Africa, was extracted from the bark of black wattle (*Acacia mearnsii*) trees by steam distillation and then concentrated into powdered form. The extract has a molecular weight that ranges from 500 to 3000, with an average of 1250 and it contains other non-tannins (including low molecular weight polyphenols, salts, sugars, and organic acids). The result of our laboratory analysis showed that the sample had total phenol and total tannin concentrations of 65.8% and 58.5% respectively (as tannic acid equivalent) according to the procedure of (Makkar et al., 1993) and condensed tannin concentration of 30.5% (as leucocyanidin equivalent) according to the procedure of Porter et al., (1985). Due to possible variation in extracts' characteristics from the company, a single batch of extract stored at 4°C was used for all preparations and analysis throughout this experiment.

Microparticles preparation

The microparticles encapsulating ATE were prepared using a procedure similar to that of Zhang et al., (2007) with slight modifications. Briefly, wall materials were dissolved in water and homogenised. Acacia tannin extract was added to the solution under continuous stirring and homogenization using an IKA overhead homogeniser for 180 seconds. For the maltodextrin-Gum Arabic microparticles, the following parameters were fixed for all preparations: Gum-Arabic and Maltodextrin were added at the ratio of 40: 60 (w/w); solute concentration in water was 1% (w/v) (Zhang et al., 2007). For the starch microparticles, the following parameters were fixed: Native starch was suspended in ethanol: water (10:90 v/v), and heated under continuous

mixing at 65-70 °C until a gelatinised paste was obtained. Gradual cooling was done with the addition of tannin extract under continuous homogenization as described by (Fernandes et al., 2014). The final mix was freeze-dried, and thereafter stored away in an airtight container. The ratio of the wall material to the core material in both preparations was varied from 85:15 to 65:35 in order to evaluate optimum loading conditions. The encapsulation procedure was carried out in three repeat batches for each wall material-core material combination. The different preparation concentration of the wall material and ATE are designated as MG-TE_{25-35} and S-TE_{15-30}.

Spectrophotometric procedure

The total phenol content of the acacia tannin extract was evaluated using the Folin-Ciocateau colourimetric method and values were expressed as tannic acid equivalents (Makkar et al., 1993) while the total condensed tannin content was evaluated using the butanol-HCl method and expressed as leucocyanidin equivalent (Porter et al., 1985). For assaying the total phenol content, about 0.5 mL of extract was vortex mixed with 0.25 mL of the Folin-Ciocateau reagent and 1.25 mL of 20% sodium carbonate. The absorbance of the resultant solution was measured at 725 nm after 40 min and concentration estimated using a standard absorbance curve from tannic acid. Total polyphenolic contents were expressed as mg/g tannic acid equivalent. For the butanol-HCL method, 0.5 mL of sample containing extract, 3.0 mL butanol HCl (95:5 v/v) and 0.1 mL ferric reagent was added. Samples were vortexed, heated at 100°C for 60 min and absorbance read at 550 nm. A suitable blank was subtracted containing the unheated sample. For the quantification of encapsulation efficiency, and rate of release of the active ingredient from microcapsules, the Folin-Ciocateau method was used as it allows for easy reference with a known standard, in this case, tannic acid. Blank microcapsules were used to correct for the effect of wall material on the absorbance readings. From freeze-dried microparticles, the amount of the bioactive compound on the surface of microparticles and the total amount of the bioactive compound loaded as described by Zhang et al., (2007) and Robert et al., (2012) was evaluated in triplicates.

Surface tannin content

Surface tannin was estimated as the amount of un-trapped ATE in the microparticles. Briefly, starch microparticles (200 mg) was dispersed in 20 mL of an ethanol: methanol solution (1:1) and filtered through a 0.22 µm Millipore filter membrane while for gum Arabic/maltodextrin matrix, 200 mg of microparticles was washed several times with a total of 45 mL anhydrous

ethanol, filtered and the filtrate made to 50 mL mark with distilled water. An aliquot was taken to determine the surface tannin content of the encapsulated product.

Total tannin content

The structure of the coating material for each microparticle was completely destroyed for the evaluation of the total bioactive compound loaded as against the theoretical amount added. For starch microparticles, 200 mg of the samples were dispersed in 20 mL 52% (aqueous) perchloric acid and ultra-sonicated for 15 minutes and aliquots were taken for analysis (Robert et al., 2012). For the maltodextrin/Gum-Arabic matrix, 200 mg sample was macerated with pestle and mortar with 5 mL of distilled water and thereafter washed with anhydrous ethanol and filtered. Filtrate collected was made to 50 mL volume and aliquot sampled for analysis of tannin content.

Determination of core loading and encapsulation efficiency

Surface tannin percentage (ST %) and the encapsulation efficiency (EE) were calculated according to equations 1 and 2, respectively (Robert et al., 2012).

$$ST\ (\%) = \left(\frac{surface\ tannin\ concentration}{total\ tannin\ recovered}\right) X\ 100 \qquad (1)$$

$$EE\ (\%) = 100 \times \left(\frac{total\ tannin\ recovered - surface\ tannin\ concentration}{Theoretical\ loaded\ tannin\ concentration}\right) \qquad (2)$$

Morphological analysis of microparticles using scanning electron microscopy

Morphology of microparticles and particle sizes were evaluated by scanning with an electron microscope. Preparations of microparticles were mounted on a slide with double-sided tape and coated with carbon before sputtering with gold under an argon atmosphere using an Emitech K950X (Ashford, UK) vacuum carbon evaporator. The gold sputtered microparticles were then viewed under a Field Emission Scanning Electron Microscope (FE-SEM), ZEISS ULTRA PLUS (JEOL, Tokyo, Japan). The size of the microparticles was determined by comparing the electron images of at least 50 microparticles per treatment, with those of a scale bar of the same magnification (Taylor et al., 2009).

In vitro release kinetics of encapsulated Acacia tannin from microparticles

The *in vitro* release of tannin extract from the encapsulation matrix in the gut of ruminant animals was simulated using product solubility in various pH media following the procedure of Rossi et al. (2003) and Sanna et al. (2004). Elution media used were: acetate buffer (pH 5.6), HCl buffer (pH 2.2) and phosphate buffer (pH 7.4) to simulate rumen, abomasal and intestinal

conditions, respectively. Microparticles (200 mg) were placed in fibre filter bags and sealed using bag sealer, thereafter suspended in 50 mL of elution media, and agitated at 50 rpm inside an incubator at 39°C (Robert et al., 2012). Aliquots of 1 mL were removed at 30 mins, and at 1, 2, 4, 8, and 24 hours after incubation for analysis. The initial volume was maintained by the addition of fresh media. Aliquots taken were frozen immediately for subsequent analysis. The release of tannin was monitored by UV spectrophotometry as described above. The cumulative amount of tannin released at each time interval was corrected with the volume of the dissolution media. The data obtained for acetate was further fitted into zero order, first order, and Higuchi *in vitro* release kinetic models to find the best model. that could predict the release of tannin extract in the rumen (Chime et al., 2013).

In vitro gas production

In vitro gas production studies were performed following the procedure of Menke et al. (1979) with the modifications detailed in Adejoro and Hassen, (2017). Rumen liquor was collected from two rumen-cannulated merino wethers fed lucerne hay (Medicago sativa) ad libitum, strained through four layers of cheesecloth into a pre-warmed thermos flask, and transported quickly to the laboratory. This rumen fluid was mixed with buffer-mineral solution and added to each serum bottle, which already contained approximately 400 mg of substrate, under a continuous stream of CO_2 and sealed with rubber stoppers. All bottles were placed inside an incubator at 39°C and 120 rpm. Using a semi-automated gas pressure transducer and digital tracker, gas pressure was recorded at 2, 4, 8, 12 and 24 hour after incubation, and subsequently converted to volume. Gas samples were collected using a syringe and analyzed for methane concentration using gas chromatography (8610C BTU Gas analyser GC System, SRI Instruments, Germany). *Eragrostis curvula* hay (Crude protein (CP), 55 g/kg; Neutral detergent fibre (NDF), 784 g/kg; Acid detergent fibre (ADF), 492 g/kg) and a total mixed ration (TMR) diet (CP, 180 g/kg; NDF, 301 g/kg; ADF, 214 g/kg) were used as substrates, and incubated with the crude acacia tannin extract, or encapsulated acacia tannin extract. The incubations containing an equivalent amount of the wall materials only (starch or maltodextrin/gum-arabic) were also included for comparison. For each substrate, treatments include i) diet only (control), ii) diet plus crude ATE iii) diet plus starch-encapsulated ATE iv) diet plus gum arabic-maltodextrin-encapsulated ATE v) diet plus starch only vi) diet plus gum Arabic-maltodextrin only. To each treatment containing ATE or encapsulated-ATE ,an equivalent of 25 mg ATE was added, which corresponds to 2.33% CT (leucocyanidin equivalent). Gas production and methane concentration were measured at 2, 4, 8, 12 and 24 hours after incubation. Gas

production data was fitted into the Ørskov and McDonald, (1979) equation $y = a + b\ (1 - e^{-ct})$ to predict the rate and extent of fermentation. (y= gas production at time t; b= slowly fermentable fraction, mL g-1 DM; and c= rate of fermentation of fraction 'b', mL h-1). Rumen fluid pH was measured after 24h of incubation using a pH meter (Mettler Toledo 230 pH meter) while ammonia-nitrogen concentration was analysed as described by Broderick and Kang, (1980).

Statistical analysis

Results of the microparticles size, yield and encapsulation efficiency were expressed as the mean of at least four repeat batches. For the *in vitro* gas production, individual bottles within each run served as analytical replicates while each run served as a statistical replicate. Gas volume was plotted against incubation time using the non-linear equation to predict fermentation kinetics (Ørskov and McDonald, 1979). Data on morphological parameters and gas production were analysed using the GLM procedure of SAS, (2013) with the model:

Yij = μ+ Bi+ Tj + + eij

Where, μ= overall mean, Bi = block effect (replicate), Tj = treatment effect, Yij= mean of individual observation and eij= residual error. Mean separation was done using Tukey's test and significance was declared at $p < 0.05$.

6.3 Results

Characterization of encapsulated Acacia tannin microparticles

Scanning electron microscopy showed that the unencapsulated-ATE particles were irregular in shape and had shiny or glossy surfaces with numerous impregnations (Figure 6.1). Their particle size ranged from 15-40 μm diameter, with most particles within the 20-25 μm range. Native starch (S-TE) and maltodextrin-gum Arabic (MG-TE) encapsulated-ATE ranged in size from 20-65 μm in diameter, with S-TE having more particles within 30-45 μm while MG-TE particles were mostly within 25-40 μm. The native S-TE microparticles were slightly bigger than the MG-TE microparticles. The S-TE microparticles were generally spherical or ovoid shaped, and dull in appearance while the MG-TE microparticles appeared in various flake shapes with mostly flat surfaces. There was a discernible effect of encapsulation on microparticle morphology, with a majority of them having a diameter of 25-40 μm, and the outer surfaces showing obvious modifications and evidence of adherence of the wall materials to the ATE particles in form of several layers of coating.

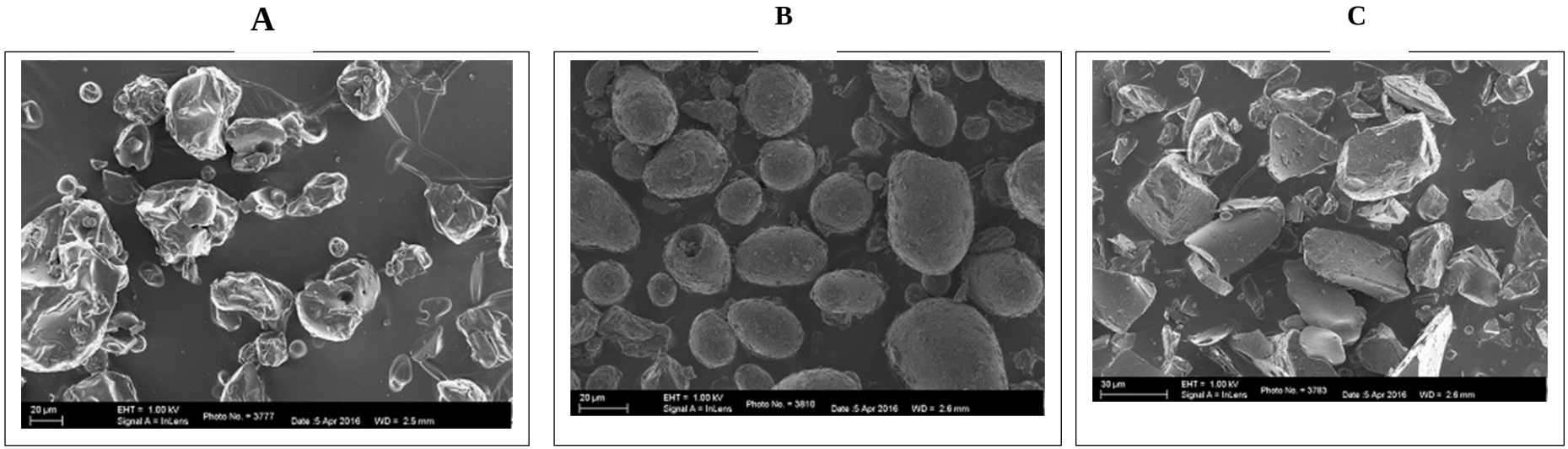


Figure 6.1 Scanning electron micrographs showing the external morphological characteristics of Acacia tannin extract (A) un-encapsulated, encapsulated with (B) native starch and (C) maltodextrin-Gum-Arabic

Yield and encapsulation efficiency of microparticles

In this study, the core (ATE) to wall material ratio was evaluated as the independent variable, capable of affecting the yield and encapsulation efficiency of ATE. There were significant differences in surface and total tannin contents among the wall materials used, and within each wall material (Table 6.1). The ratio of core to wall material also significantly influenced the surface and total tannin contents of microparticles. Surface tannin contents of the microparticles ranged from 35.0% to 63.9% for S-TE and 8.43-11.2% for MG-TE. The total tannin recovered varied from 70.9-75.8% and 63.2 – 70.7% for S-TE and MG-TE microparticles, respectively (Table 6.1). Surface tannin content in MG-TE microparticles was generally lower than that in S-TE. Within each wall material, when the concentration of ATE was varied, total tannin recovered was not significantly different in both MG-TE and S-TE microparticles. The result of the encapsulation efficiency for MG-TE showed values of 64.8% at 25:75 (core to wall material ratio), 60.5% at 30:70 and 56.1% at 35:65. There was a decrease in encapsulation efficiency with increasing concentration of ATE. For S-TE, the result showed that at 15% ATE concentration, encapsulation efficiency was 48.8% while 33.4%, 27.7% and 31.1% were recorded at 20%, 25% and 30% ATE concentrations, respectively (Table 6.1).

Table 6.1 Effect of variation in processing parameters on encapsulation efficiency and recovery of Acacia tannin extract in starch and maltodextrin/Gum-Arabic microparticles

[1]Sample	Wall material (g 100g^{-1} solution)			Core material (g 100 g^{-1} wall material)	Surface tannin (g 100 g^{-1})	Total tannin recovery (g 100 g^{-1})	Encapsulation efficiency (g 100 g^{-1})
	GA	MD	NS				
MG-TE$_{25}$	30	45	-	25	8.43^{d}	70.7^{ab}	64.8^{a}
MG-TE$_{30}$	28	42	-	30	10.7^{d}	66.4^{ab}	60.5^{ab}
MG-TE$_{35}$	26	39	-	35	11.2^{d}	63.2^{b}	56.1^{b}
S-TE$_{15}$	-	-	85	15	35.0^{c}	75.1^{a}	48.8^{c}
S-TE$_{20}$	-	-	80	20	53.4^{b}	70.9^{ab}	33.4^{d}
S-TE$_{25}$	-	-	75	25	63.9^{a}	75.6^{a}	27.7^{d}
S-TE$_{30}$	-	-	70	30	58.8^{ab}	75.8^{a}	31.1^{d}
SEM					4.21	1.30	2.67
p-value					<0.0001	0.03	<0.0001

[1]MG-TE, maltodextrin+ Gum-Arabic encapsulating acacia tannin extract (at 25, 30, 35% w/w); S-TE, native starch encapsulating acacia tannin extract (at 15, 20, 25, 30% w/w). [2]GA, gum Arabic; MD, maltodextrin; NS, native starch; Means with different superscripts across a column are significantly different ($p < 0.05$); Treatments are expressed as mean and values are calculated from a minimum of four repeat batches.

In vitro release of Acacia tannin from microparticles in dissolution media

The release profile of tannin from the unencapsulated-ATE, S-TE and MG-TE under optimal loading conditions, in acetate, phosphate and HCl buffer media at 39°C are shown in Figure 6.2a-c. In each dissolution media, a burst release pattern was observed within the first 4 h of incubation for all microparticles across the different pH media. This phase was followed by a phase of gradual release from 4-8 h after dissolution. Beyond 8 hours most of the tannin in the microparticles had been released into the dissolution media. For the unencapsulated-ATE, about 75.5% of ATE was released within 2 h and about 90.2% within 4 h after dissolution at 39°C. The release profile indicates that 58.7% and 75.8% of tannin was released from the MG-TE microparticles at 2h and 4h, respectively while 65% and 78.7% were released from S-TE after the same incubation times respectively. The *in vitro* release data obtained in acetate buffer, simulating rumen pH, showed that the release patterns of tannin from microparticles fitted the Higuchi and first-order models better than the zero-order model (Table 6.2).

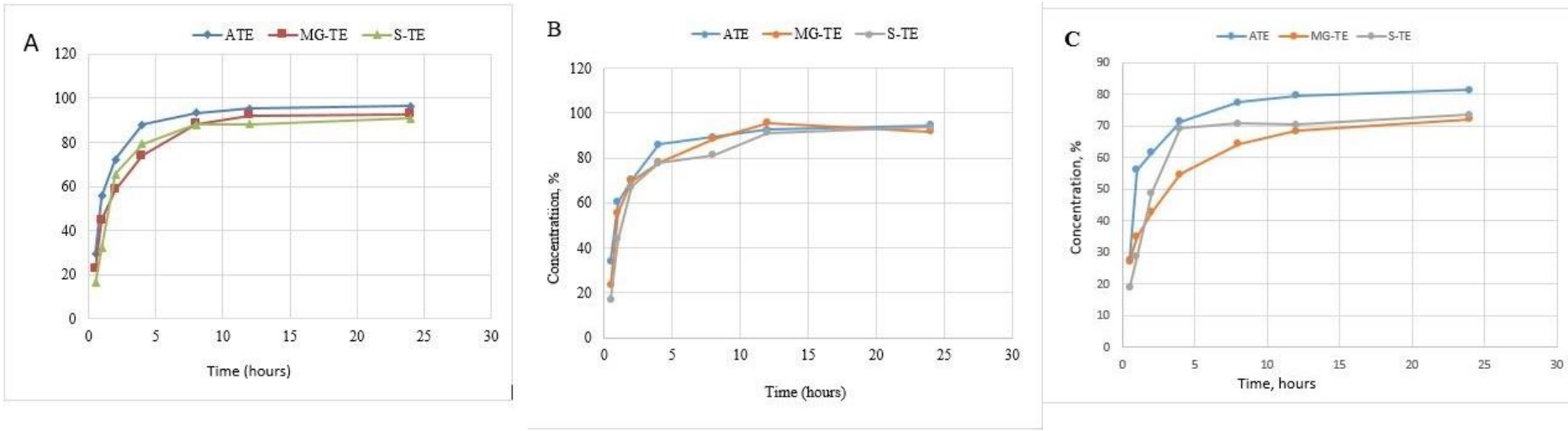


Figure 6.2 *In vitro* release profile of Acacia tannin extract microparticles in (A) Acetate buffer, pH 5.6 (B) phosphate buffer, pH 7.4 (C) HCl buffer, pH 2.2, at 39 °C; unencapsulated-tannin extract (ATE), ATE encapsulated in starch (S-TE) and ATE encapsulated in maltodextrin/gum-arabic (MG-TE)

Table 6.2 *In vitro* release kinetic parameters in acetate buffer media (pH, 5.6), of Acacia tannin extract (ATE) encapsulated with maltodextrin/gum-Arabic (MG-TE) and native starch (S-TE)

	Zero Order, Q vs. *t*	First Order, ln (Q_0-Q) vs. *t*	Higuchi, Q vs $\sqrt{t}$	R^2		
				Zero Order	First order	Higuchi
ATE	Y = 2.861x+47.9	Y = -0.106x+1.78	Y = 27.1x+17.9	0.439	0.874	0.836
MG-TE	Y = 3.073x+39.4	Y = -0.090x+1.87	Y = 26.9x+10.5	0.543	0.952	0.924
S-TE	Y = 3.090x+37.8	Y = -0.080x+1.85	Y = 27.8x+7.6	0.491	0.819	0.865

ATE, Acacia tannin extract, MG-TE, MG-TE, Acacia tannin extract encapsulated with maltodextrin+ gum Arabic; S-TE, Acacia tannin extract encapsulated with native starch. Q_0, Tannin to be released at zero time (mg); Q, amount of drug released at time t; t, time in hours. *n=3*

Effect of ATE encapsulated with starch or maltodextrin-gum Arabic on in vitro ruminal gas production and methane emission

Acacia tannin extract (ATE) treatment reduced total gas production up to 24 h incubation when compared with the control diet in both substrates ($p < 0.0001$). The inclusion of S-TE and MG-TE generally resulted in higher total gas production across the time intervals up to 24 h when compared with the control treatment in both *Eragrostis curvula* (EC) and total mixed ration (TMR) substrates. However, total gas production was lower in S-TE and MG-TE treatments than in incubations containing only the wall materials (Starch; GA/MD) ($p < 0.0001$). Acacia tannin extract tended to reduce methane emission in both EC and TMR substrates ($p < 0.0001$). The S-TE and MG-TE incubations did not reduce methane production when compared with the control or ATE treatment in both substrates ($p < 0.0001$). However, in the TMR incubations, methane values obtained in S-TE were lower compared to the starch only treatment ($p < 0.0001$). The intensity of methane produced when expressed as the ratio of methane volume to total gas volume showed that tannin inclusions did not affect methane concentration across the treatments in EC ($p = 0.38$) and TMR ($p = 0.13$) substrates. The rate of fermentation of the insoluble fraction ('c') showed that with starch and S-TE, 'c' was higher compared to the control, ATE and incubations with gum Arabic and maltodextrin in the Eragrostis hay substrate ($p < 0.0001$). However, no difference in fermentation kinetics across the treatments were observed in the TMR substrate ($p = 0.68$). Supplementation with ATE, S-TE or MG-TE did not affect rumen pH after 24 h incubation in both Eragrostis hay ($p = 0.15$) and TMR ($p = 0.11$) substrates. However, with Eragrostis hay as the substrate, ATE, S-TE and MG-TE reduced ammonia nitrogen concentration in rumen fluid after 24 h *in vitro* incubation ($P = 0.04$) while no differences were observed in the TMR substrate ($p = 0.11$). The unencapsulated-ATE were more effective in reducing total gas and methane production than samples containing S-TE and MG-TE.

Table 6.3 *In vitro* gas production and fermentation parameters due to the addition of Acacia tannin extract encapsulated with native starch or maltodextrin/gum arabic on *Eragrostis curvula* hay (EC) and total mixed ration (TMR) feeds

	Gas production (mL/g DM)				Methane (mL/g DM)				24 h Methane (%)	[2]Gas production kinetics		PH	NH_3-N (mM)
[1]Treatment	2 h	4 h	12 h	24 h	2 h	4 h	12 h	24 h		b	C		
Eragrostis Hay substrate													
Control (C)	12.0^{bc}	19.0^{cd}	27.5^{c}	50.2^{c}	1.06^{bc}	1.81^{cd}	2.77^{b}	5.4^{c}	10.6	125.8^{c}	0.02^{b}	6.93	11.5^{a}
C+ Starch	17.0^{b}	27.5^{bc}	75.4^{a}	167.7^{a}	1.76^{b}	3.04^{bc}	7.90^{a}	19.6^{a}	11.5	184.2^{b}	0.13^{a}	6.84	10.5^{ab}
C+ S-TE	14.8^{bc}	21.9^{c}	66.5^{ab}	162.5^{a}	1.49^{bc}	2.32^{fg}	7.26^{a}	18.8^{a}	11.3	191.5^{b}	0.13^{a}	6.85	9.6^{b}
C+ Maltodextrin-Gum Arab.	25.1^{a}	38.6^{a}	62.5^{ab}	99.6^{b}	2.85^{a}	4.09^{a}	6.51^{a}	10.7^{b}	10.5	225.5^{a}	0.02^{b}	6.72	10.5^{ab}
C+ MG-TE	23.6^{a}	35.7^{ab}	54.8^{b}	87.1^{b}	2.64^{a}	3.95^{a}	5.97^{a}	9.5^{b}	10.4	207.7^{ab}	0.02^{b}	6.85	10.0^{ab}
C+ ATE	9.9^{c}	14.1^{d}	18.8^{c}	36.8^{c}	0.90^{c}	1.32^{d}	1.86^{b}	3.5^{c}	10.7	100.7^{d}	0.01^{b}	6.91	10.1^{ab}
SEM	1.75	2.65	6.21	14.91	0.23	0.31	0.69	1.81	0.18	9.64	0.02	0.07	0.7
P value	0.005	0.002	0.001	<0.0001	0.003	0.001	0.002	<0.0001	0.38	0.0002	<0.0001	0.15	0.04
Total mixed ration (TMR) substrate													
Control (C)	24.1^{c}	42.1^{b}	89.0^{b}	154.2^{d}	2.82^{b}	4.30^{b}	9.41^{c}	17.0^{d}	11.2	341.2	0.06	6.60c	20.0
C+ Starch	25.5^{c}	45.9^{b}	129.4^{a}	256.1^{a}	2.88^{b}	4.85^{b}	14.3^{a}	31.0^{a}	12.1	336.5	0.06	6.34d	19.8
C+ S-TE	22.6^{c}	39.5^{b}	115.7^{a}	236.7^{b}	2.1^{b}	3.97^{b}	12.7^{ab}	28.7^{b}	12.0	288.3	0.05	6.68bc	18.4
C+ Maltodextrin-Gum Arabic	33.5^{ab}	59.2^{a}	124.2^{a}	193.6^{c}	3.47^{ab}	5.82^{ab}	13.0^{ab}	22.3^{c}	11.4	298.2	0.04	6.78ab	19.4
C+ MG-TE	38.6^{a}	63.2^{a}	123.4^{a}	184.4^{c}	4.66^{a}	7.12^{a}	135^{a}	21.4^{c}	11.4	250.3	0.05	6.72abc	18.6
C+ ATE	28.3^{bc}	45.8^{b}	90.1^{b}	146.7^{e}	3.13^{ab}	5.34^{ab}	10.7^{bc}	18.0^{d}	11.9	199.5	0.12	6.85a	18.8
SEM	1.92	2.96	5.49	12.1	0.28	0.37	0.61	1.59	0.12	26.8	0.01	0.08	0.8
p-value	0.007	0.004	0.001	<0.0001	0.07	0.03	0.007	<0.0001	0.13	0.53	0.68	0.11	0.11

[1]S-TE, Acacia tannin extract encapsulated with native starch; MG-TE, Acacia tannin extract encapsulated with maltodextrin+gum arabic; ATE, Acacia tannin extract. [2]b: gas production (gp) from the insoluble but slowly fermentable fraction of substrate (ml); c :the rate of gp from insoluble fraction per hour. SEM, standard error of mean. Means with different superscripts across same column are significantly different ($p < 0.05$).

6.4 Discussion

The variations in microparticle size as observed in this study are likely due to the influence of the size of core materials, the encapsulation method used and the molecular sizes of the wall material (Maa and Hsu, 1997; Tonon et al., 2009), which will ultimately influence the encapsulation efficiency of the microparticles (Kim and Park, 2004). The various chip/crumb-like shapes formed by MG-TE microparticles may be associated with the method of dehydration (freeze-drying) or the properties of the wall materials (Anandharamakrishnan et al., 2010; Ezhilarasi et al., 2013). The surface morphological characteristics of the MG-TE microparticles observed were similar to the description of microparticles reported earlier as flake-like structures, free of dents and shrinkage when gum-arabic/sucrose/gelatine was used as wall material in encapsulating limonene under freeze-drying conditions (Kaushik and Roos, 2007). A broken-glass shaped structure was similarly observed by Ezhilarasi et al. (2013) when gum-arabic was used to encapsulate garcinia fruit extract using freeze-drying. Agglomeration and stickiness of particles often noticed as a limitation when using maltodextrin as a wall material in previous studies (Ezhilarasi et al., 2013; Goula and Adamopoulos, 2008) was not observed in the current study. This may be an indication that the combination of gum-arabic with maltodextrin served as a more effective wall material for encapsulation of ATE. Loksuwan (2007) observed a smooth, round shape without dents in native starch encapsulated beta-carotene powders and this is in agreement with our SEM observations of S-TE microparticles. According to Zhang et al., (2000) materials with plasticizer properties enhance the formation of spherical microparticles with smooth surfaces during microencapsulation. The encapsulation process may have provided enough opportunity for the starch to interact with the tannin molecules. Pre-gelatinization of starch has also been found to improve its ability to encapsulate polyphenolic core materials (Loksuwan, 2007). Pitchaon et al. (2013) observed that a combination of maltodextrin and gum Arabic produced better encapsulation of phenolic antioxidants, with higher encapsulation efficiency.

The results of this study showed that beyond a 30:70 ratio of core to wall material, encapsulation efficiency decreased significantly for the MG-TE microparticles while for the S-TE microparticles, a significant decline occurred beyond a 15: 85 ratio. The interaction of the active ingredient (core material) and wall material seems to have a profound effect on encapsulation parameters, specifically the loading capacity and encapsulation efficiency. The proportion and nature of the core material in the total microparticles have been noted as a very

important factor influencing the efficiency of microencapsulation and the overall application of an encapsulated product. Previous research by Fernandes et al., (2014) with starch, maltodextrin, maltodextrin-gum Arabic and gum-Arabic reported encapsulation efficiency values that range from from 45.45 to 60.22% in encapsulating a lipophilic core material while Robert et al., (2012) reported values that range from 47 to 61% when starch or acetylated starch was used for encapsulation of gallic acid, a hydrophilic polyphenolic compound. However, very high encapsulation efficiency of 99.2% and an encapsulation yield of 89.71% were previously reported for maltodextrin-gum Arabic microparticles encapsulating grape seed extract (Zhang et al., 2007).

Core material concentration, as a factor, affected the encapsulation efficiency of the microparticles in this study just as Zhang et al., (2007) had reported earlier. The higher loading capacity and encapsulation efficiency obtained in the maltodextrin-gum arabic microparticles compared with microparticles encapsulated with native starch may be related to the structural differences of the wall materials, leading to a probable higher binding capacity of maltodextrin-gum Arabic combination over native starch. The result of this study showed that this wall material combination was effective for the tannin extract but not beyond 30% of the core material. Similarly, high encapsulation efficiency for maltodextrin-gum Arabic microparticles encapsulating phenolic antioxidants has been reported (Pitchaon et al., 2013). Encapsulation efficiency in maltodextrin and gum Arabic as encapsulating agents were higher than native starch with Acai powder core material, although the spray-drying method was applied in that study (Tonon et al., 2009). The properties of wall materials have been noted as an important factor that affects encapsulation efficiency (Fernandes et al., 2014; Jafari et al., 2008). The better entrapment of ATE in the maltodextrin-gum Arabic microparticles can be linked to the plasticity of gum arabic which is capable of forming a good film over the core material, and thus prevents the cracking of the matrix (Bertolini et al., 2001; Krishnan et al., 2005b). Encapsulation method also has significant effects on the extent and efficiency of encapsulation. When gum arabic was used as a wall material in preparation of spray dried microparticles, shrinking and denting of microparticles because of the evaporative dehydration process was observed. Spray-drying as an encapsulation method has been reported to result in the formation of spherical microparticles with concavities when maltodextrin was included as wall materials. This was attributed to the shrinkage of particles due to rapid moisture loss after cooling. In contrast, in that study, microparticles prepared by freeze-drying produced flake-like shapes

devoid of indentations and could be associated with the lack of forces to break up the frozen liquid into droplets (Papoutsis et al., 2018).

The initial rapid release of tannin in both S-TE and MG-TE may be attributed to the presence of surface (uncoated) tannin as well as the encapsulation properties which can be affected by wall material property, the interaction between wall material and the core material and method of encapsulation (Robert et al., 2012). This is an indication that these microparticles may be easily solubilised in the mouth or rumen of the ruminant animals. The solubility of starch in aqueous media and the tightly bound ATE particles to the wall materials and its gradual erosion may have influenced the second phase of ATE release. In the HCl buffer, a lower release of ATE was observed even after 8 h of dissolution of microparticles. Tannin dissociation from existing bonds has been known to depend on pH (Makkar, 2003a). During the gelatinization process, some amylose content of starch, which carries a functional group capable of attaching to tannins, is usually leached into solution (Barros et al., 2012). The interaction of amylose with tannins have been observed to slow starch retrogradation after gelatinization, and reduce its rate of *in vitro* degradation due to the formation of stronger hydrogen bonds (Andrade-montemayor et al., 2009; Wu et al., 2009). However, various modifications of starch such as high amylose starch, and other modified starch products like acetylated starch, have been found to further improve its binding ability. Similarly, it has been found that the protein binding activity of tannins can be affected by the presence of other polysaccharides such as pectin, gum arabic, carrageenan, xanthan, and gellan (Zhu, 2018). The ability of these polysaccharides to form hydrophobic pockets and encapsulate polyphenols have been observed to result from the formation of hydrogen bonds between the oxygen atom of the carbohydrates and the hydroxyl group of the tannin (de Freitas et al., 2003; Ozawa et al., 1987).

The effect of ATE on *in vitro* ruminal gas production obtained in this study is consistent with previous studies involving the use of Acacia tannin extract in reducing total gas and methane production (Grainger et al., 2009; Hassen et al., 2016). However, encapsulating ATE with starch or gum arabic and maltodextrin triggered an increase in methane and gas production, rather than reducing methane *in vitro*. This can be attributed to the high concentration of the encapsulating materials (starch, maltodextrin, gum arabic) which are potentially fermentable and thus might have acted as substrates for rumen microbes. Santoso et al. (2007) reported significantly higher methane production in sheep consuming silage-based diets compared to those consuming hay based diets as a result of higher NDF digested. When rumen ammonia

nitrogen concentration is adequate, increase in fermentable carbohydrate results in greater microbial growth, and consequent increase in fermentation and gas production (Calabrò et al., 2012; Chottanom et al., 2014). The chemical nature and concentration of tannin as well as its concentration in the diet, and substrate type, may influence its biological activities such as its protein binding, antimethanogenic effect, rumen microbial function and subsequently methane production and nutrient digestibility (Beauchemin et al., 2007; Patra and Saxena, 2009).

Tannins are known to reduce total gas and methane production by a reduction in methanogenic activities, an overall reduction in fermentation or a combination of both (Jayanegara et al., 2012; McSweeney et al., 1999). The impact of the tannin inclusion in this study showed that tannin extracts in ATE, S-TE and MG-TE might have reduced organic matter fermentation rather than exert any specific effect on methanogenesis. This result is similar to previous studies (Hoehn et al., 2018) where condensed tannin extracts exert significant reduction on dry matter disappearance and gas production, as a consequence of reduced fibre degradation. Reduced fibre digestion may have negative consequences, especially when animals are consuming poor quality roughages such as EC and a compromise on digestibility may affect nutrient intake and performance (Jayanegara et al., 2012). The supplementation of ATE, S-TE and MG-TE did not affect the rate of fermentation in the TMR substrate although the addition of starch, as an encapsulating material, significantly increased fermentation rate in the EC substrate. Generally, the impact of tannins or tanniferous plants on substrate fermentation rate varies for various tannin sources (Pellikaan et al., 2011). While some researchers have reported a significant reduction in the rate of substrate fermentation (Huang et al., 2011), others have observed no such effect (Gunun et al., 2018). This may largely be due to the varying properties of the tannins or other diet characteristics. Tannin sources that reduce methane production but exhibited only minor impacts on gas production have better potential at being exploited as antimethanogenic supplements (Pellikaan et al., 2011).

Where the nutrient requirement of animals is adequately supplied, reduced ruminal crude protein degradation offers the potential bypass of dietary protein to the lower part of the digestive system. A shift in protein digestion to the hindgut may be advantageous to the animal and also, the reduced urinary N loss as against faecal N loss has potential environmental benefits (Goel and Makkar, 2012; Hristov et al., 2013). The presence of tannin extract is often associated with reduced protein degradability in the rumen, often resulting in lower concentration of ammonia nitrogen (Carulla et al., 2005). Although reduced nitrogen

degradation resulting in lower ammonia nitrogen is common with tannin supplementation (Beauchemin et al., 2007; Carulla et al., 2005) in the TMR diet, ammonia nitrogen concentration between S-TE, MG-TE and ATE was not different. This is an indication that encapsulation may not have affected the impact of tannin on protein degradation in the rumen after 24 h. In the EC, a substrate with lower crude protein content, S-TE resulted in reduced ammonia nitrogen concentration, a pattern that has been widely reported in previous studies (Beauchemin et al., 2007; Makkar, 2003a).

Therefore, the level of inclusion of these materials in encapsulating ATE posed limitations to its application in methane mitigation studies. This could be partly due to the low loading percentage of the tannin within the wall materials, the encapsulation process or the potential of the wall materials to serve as a source of fermentable energy for rumen microbes. Further studies are therefore needed to evaluate the effect of S-TE and MG-TE in other ruminant applications while other encapsulation techniques may be explored for tannin utilization when gas production and methane emission are of interest.

6.5 Conclusion

Starch and maltodextrin-gum Arabic were successfully used in encapsulating acacia tannin extract. The maltodextrin/gum-Arabic microparticles were smaller and more homogenous than those of native starch even at higher loading concentration. Microparticles produced using both wall materials exhibited the burst release profile under various pH conditions. In terms of methane production, encapsulated microparticles showed only a slight reduction in methane when compared with the samples containing only the wall materials but methane production was generally higher than in the unencapsulated-tannin extract. These encapsulated microparticles need to be tested in other ruminant applications and other encapsulation methods suitable for tannins used in enteric methane mitigation strategies need to be developed.

CHAPTER SEVEN

Preparation of tannin lipid-microparticles by solid-in-oil-in-water and melt dispersion methods: characterization and evaluation of their effect on ruminal gas production *in vitro*

Abstract

Tannin extracts have wide biological activity in ruminant nutrition. The possibility of masking their bitter taste and enhancing sustained release in the rumen can be achieved through encapsulation. The objectives of this study were to prepare an encapsulated Acacia tannin extract (ATE) suitable for ruminants using the solid-in-oil-in-water (S/O/W) method, and to evaluate the microparticles in terms of morphology, encapsulation efficiency and *in vitro* release under varying pH. Subsequently, the effect of the microparticles on rumen *in vitro* gas and methane production would be evaluated. Lipid microparticles were prepared using the double emulsion process with palm oil and lard, dichloromethane, and Tween80©/Span80© emulsifiers. The microparticles produced by S/O/W emulsion tended to be smaller (P= 0.06), and had greater encapsulation efficiency compared with those produced by the melt dispersion method. Scanning electron micrographs showed microparticles had stable cylindrical and spherical shapes, with mean size of 34± 10.2 µm. Maximum encapsulation efficiencies of 78.6% and 80.1% were obtained with lard and palm oil as lipid wall materials respectively, even under high core material loading percentage of 80%. Wall material type did not affect the characteristics of microparticles. In acetate buffer, only about 20% of tannin was released from the lipid-encapsulated microparticles into buffer media after 24 hours. In contrast, about 90% of the tannin had been released into solution before 8 hours in the crude extract. Lipid-encapsulated ATE reduced rumen gas and methane production *in vitro* (P <0.05) in both Eragrostis and total mixed ration (TMR) diet substrates, but the magnitude of reduction was lower than that obtained when unencapsulated ATE was the additive (10% vs 20% for total gas and 17% vs 24% for methane). Crude ATE and palm oil-encapsulated ATE reduced the concentration of methane in sampled gas (*P*= 0.054) when fermenting the TMR substrate, but this effect was not observed in the Eragrostis substrate. Ammonia nitrogen concentration was greater in encapsulated ATE compared with the crude ATE (P <0.001). These results show that the small-sized and more uniform lipid-encapsulated ATE microparticles with high encapsulation efficiency compared with microparticles prepared by melt dispersion. Encapsulation of ATE enhanced the sustained release of tannin in the rumen and the potential to improve gas production and reduce methane production.

Keywords: Acacia tannin extract, lipid microparticles, solid-in-oil-in water, encapsulation, rumen fermentation

7.1 Introduction

Tannin extracts as dietary additives are gaining recognition in ruminant production because of their many nutritional, and nutraceutical uses. Their many biological functions include prevention of bloat, aiding the bypass of dietary protein, control of gastrointestinal nematodes and rumen methane mitigation (Huang et al., 2010; Kamra et al., 2012; Naumann et al., 2014; Ribeiro et al., 2013). However, the limitations to the use of tannins include stability, astringency and bitter taste, and its strong affinity with dietary ruminal protein, which at high concentration may result in reduced voluntary dry matter intake and nutrient digestibility among other negative consequences (Dschaak et al., 2011). Recent studies have suggested that the microencapsulation technique may be able to mask or reduce the negative effects associated with bioactive compounds in the food or feed industry (Munin and Edwards-Lévy, 2011). Fang and Bhandari, (2010) noted that the administration of encapsulated extracts instead of the raw product could overcome drawbacks such as product instability and bitter taste while improving the bioavailability of the compounds at the required site. A tannin extract that is released gradually over a prolonged period in the rumen may improve tannin utilization in ruminant nutrition (Putnam and Garrett, 2005).

Microencapsulation has been described as a process by which small particles of a core material are surrounded by a homogenous or heterogeneous coating (wall material) and form capsules or beads using various applications (Borgogna et al., 2010). Active ingredients are encapsulated to achieve purposes such as masking unpleasant tastes or colours, extending shelf life, protecting against oxidative damage, controlled release or release at a targeted site (Anal and Singh, 2007; Nazzaro et al., 2012). Controlled-release systems are specialised encapsulation systems, which deliver the active ingredients over an extended period (Munin and Edwards-Lévy, 2011). These systems have been used in the administration of anthelmintics and antiparasitic drugs, antibiotics, vitamins, amino acids and other dietary additives for ruminants and humans (Anal and Singh, 2007). They have shown important benefits by reducing the frequency of administration and minimizing negative side effects such as acute toxicity, rumen pH fluctuation, or astringency (Mamvura et al., 2014).

However, the cost, availability and suitability of many of the food industry encapsulating materials for animal nutrition have been highlighted (Emanuele et al., 2006; Krishnan et al., 2005a). This could hamper their commercial application in ruminant systems. Therefore, there is a need to develop and evaluate cheap but effective wall materials for various livestock

applications. Multiple emulsion systems such as the S/O/W method offer innovative approaches to the administration of bioactive compounds such as anthocyanidin across the gastrointestinal passage because they are able to mask flavours or odours or control the release of ingredients during ingestion and digestion (Benichou et al., 2004; Dickinson, 2011). Membrane emulsification methods such as S/O/W could control particle size and enhance the stability of microparticles, unlike simpler techniques such as melt dispersion or solvent evaporation methods (Ho et al., 2017; Nakashima et al., 2000; Toorisaka et al., 2003).

In this study, palm oil and lard (fat rendered from a pig) were used as lipid wall materials to encapsulate Acacia (*Acacia mearnsii*) tannin extract (ATE) in S/O/W emulsion using Tween80 and Span80 as hydrophilic and lipophilic surfactants respectively. The lipid wall materials are economically viable, eco-friendly and can be hydrolysed and biohydrogenated in the rumen (Bainbridge and Kraft, 2016; Doreau and Chilliard, 1997; Veneman et al., 2015). Nutritionally, there is an increase in fatty acid bioavailability to the animal (Bainbridge and Kraft, 2016). Lard and palm oils have high melting points compared with many other vegetable oils and hence, microparticles prepared from them may be able to withstand disintegration caused by handling (Berger and Siew, 1982). Lard has been used extensively as a milk replacer for feeding calves and therefore, does not impose health risks on ruminant animals (Huuskonen et al., 2005).

The hypothesis of this study is that encapsulated-ATE will result in microparticles that exhibit slower *in vitro* release, compared with the crude extract, while retaining its characteristic properties such as reduction in methane gas. The S/O/W double emulsion method was adopted based on its controlled-release advantages and the microparticles were compared with microparticles prepared under melt dispersion, a simple method that has similarly been used for ruminant additives (Wood et al., 2009). The objectives of this study, therefore, were i) to prepare an encapsulated ATE using the S/O/W method with lard or palm oil as wall material, then compare it with the product of the melt dispersion method, ii) to evaluate the microparticles in terms of morphology, efficiency of encapsulation, and the *in vitro* release profile of the ATE under varying pH conditions, and iii) to evaluate the addition of microparticles from ii) in terms of their effect on *in vitro* gas and methane production, as compared with the crude extract.

7.2 Materials and Method

This study was carried out in accordance with the guidelines stipulated by the National Health Research Ethics Council of South Africa. The protocol was approved by the University of Pretoria Animal Ethics Committee (AEC) (approval number EC061-14).

Materials

Acacia mearnsii tannin extract (ATE) from UCL Company Pty (Ltd), South Africa, was used throughout this study. Span80 (HLB, 4.3), Tween80 (HLB, 15.0) and dichloromethane (99.9%, ACS HPLC grade) were procured from Sigma-Aldrich Inc. (USA); Filter bags used for *in vitro* was the F57 fibre filter bags purchased from ANKOM Technology (NY, USA). All reagents were of analytical grade in purity.

Microparticle Preparation

Acacia tannin extract was a commercial sample obtained from UCL Company Pty (Ltd) in Dalton, South Africa, and extracted from the bark of the black wattle (*Acacia mearnsii*) tree by steam distillation and then concentrated into powdered form. It has a molecular weight that range from 500 to 3000, with an average of 1250. It has a high amount of condensed tannin although it also contains other non-tannins (including low molecular weight polyphenols, salts, sugars, and organic acids). From laboratory analysis, the sample had total phenol, total tannin and condensed tannin concentrations of 65.8%, 58.5% (as tannic acid equivalent) and 30.5% (as leucocyanidin equivalent) according to the procedure of Makkar et al. (1993) and Porter et al. (1985). Dichloromethane, the solvent used in this preparation is the least toxic of the simple chlorohydrocarbons. The LD50 value is 1600 mg/kg via oral administration in rats, is reported to be non-toxic to aquatic life (Mizutani et al. 1988)and is commonly used in the food and pharmaceutical industries. The solvent was evaporated during microparticle preparation and therefore, its use would not likely create a toxicity risk to animals.

Solid-in-oil-in-water method: The double-step procedure used in the preparation of multiple phase emulsions as described by Castellanos et al. (2001) was used for encapsulation of ATE by S/O/W method. The primary solid-in-oil (S/O) phase was prepared by suspending ATE powder in 30 mL lipid solution (50 mg/mL) of dichloromethane (DCM) containing Span80© as a surfactant and homogenized at 20,000 rpm for 120 seconds (PRO400DS, Pro Scientific Inc., Oxford CT 06478 USA). The resulting S/O suspension was added to an aqueous phase of distilled water containing 0.1% (w/v) Tween80© and homogenized for 180 seconds at 20,000

rpm to produce a secondary S/O/W emulsion. Varying concentrations of the ATE and external aqueous phase was compared, to determine the optimum conditions for encapsulation. The resultant emulsion was placed on a magnetic stirrer and rotated at 800 rpm for three hours to allow for evaporation of the water-immiscible organic solvent (DCM). The microparticles that were formed were collected by filtration through a 60 µm glass filter crucible, washed with approximately 100 mL distilled water, freeze-dried and stored at 4°C for analysis. Preliminary tests were conducted to establish the best amount of Tween80© and Span80© combination that would give good emulsion stability. The parameters that were kept constant during the preparation of the S/O/W microparticles included concentration of surfactants (0.5% w/v Span80© in DCM and 0.1% w/v Tween80© in water), homogenization conditions (S/O phase: 20,000 rpm, 120 s and O/W phase: 20,000 rpm, 180 s) and solvent evaporation conditions (800 rpm, 3 h). The schematic chart of the encapsulation procedure is shown in Figure 7.1. The various preparation concentrations of the external aqueous phase and tannin extract are designated L-1 to L-4 in lard-encapsulated microparticles and P-1 to P-4 in palm oil-encapsulated microparticles. Each preparation was repeated in three replicates for evaluation.

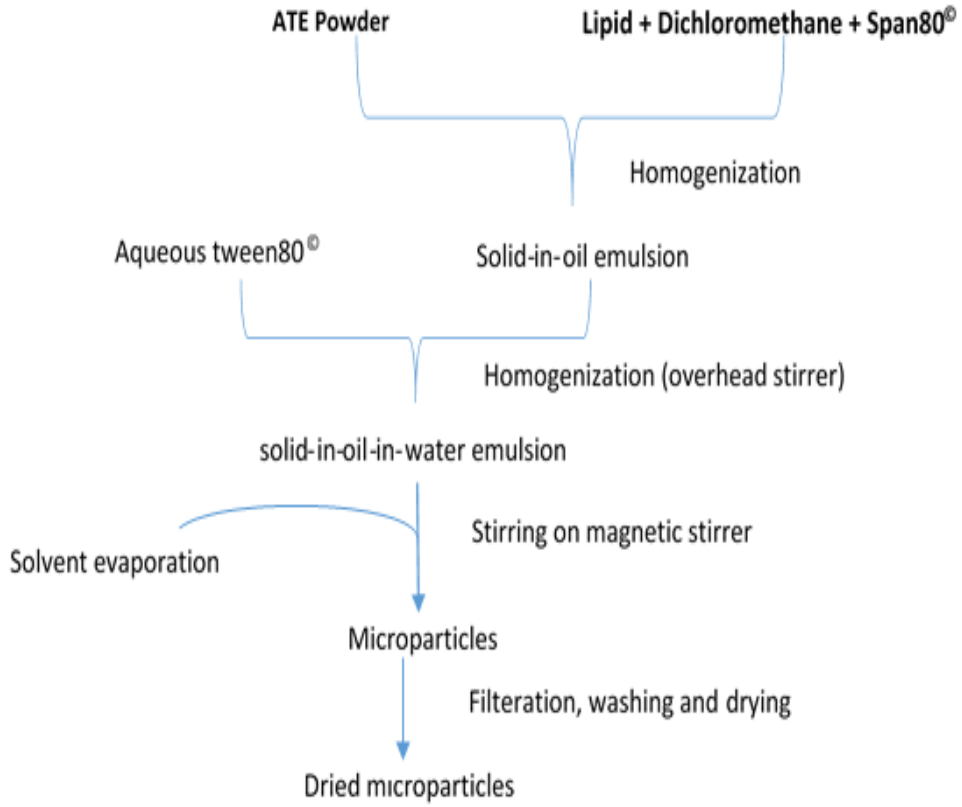


Figure 7.1 Schematic diagram showing the preparation of S/O/W microparticles

Melt dispersion method: Preparation of the microparticles using the melt dispersion method was done according to the procedures of Richardson (2004) and Wallace et al (2006) with minor modifications. Briefly, 2 g palm oil or lard was added to a beaker and melted under gentle heat and continuous stirring using a magnetic stirrer until the liquid was clear. Acacia tannin extract powder (8 g) was placed in an IKA A10 electric mill and the melted lipid sprayed over the powder, and then the mixture was blended for two minutes. Thereafter, a spatula was used to scrape any powder that had stuck to the sides and lid of the mill, and the blending was repeated several times until the powder was thoroughly coated with the oil. Then, 120 mg of Span80© was added and the blending was repeated several times. Coated samples were passed through a six-mesh screen to break up agglomerates and then stored in an airtight container at 4°C. Three separate samples were prepared for evaluation. The melt dispersion method is considered simple and has been similarly used in ruminants (Richardson, 2004; Wallace et al., 2006). It was therefore used to compare microparticles prepared by the S/O/W method.

Microparticle morphology and size determination

Morphology of microparticles and particle size distribution were evaluated using scanning electron microscopy. The encapsulated powder was coated with carbon before sputtering with gold under an argon atmosphere (Emitech K550X, Ashford, UK), followed by viewing under a JSM-840 microscope (JEOL, Tokyo, Japan). The size of the microparticles were determined by comparing the scanning electron microscopy images with those of a scale bar of the same magnification. At least 50 microparticles of each treatment were measured.

Determination of tannin yield and encapsulation efficiency of microparticles

The actual loaded tannin (LA) of microparticles was determined using a procedure described by Castellanos et al., (2001) with slight modifications. Briefly, 100 mg of microparticles were dispersed in 20 mL DCM, and the lipid wall material dissolved by sonication for 10 min. The solid tannin was pelleted by centrifugation for 10 minutes at 2500 rpm. The supernatant was discarded and the pellet was dissolved in 20 mL 70% aq. acetone. The tannin concentration in the resulting clear solution was determined from its absorbance spectrophotometrically to estimate actual loaded tannin. The encapsulation efficiency of the microcapsules enclosing ATE was estimated as follows:

$$Ee\ (\%)\ =\ (\frac{L_A}{L_T})X\ 100$$

where LA is the actual loaded tannin and LT is the theoretical loaded tannin (%, w/w) in the lipid microparticles. The theoretical loaded tannin was the actual weight of tannin added during preparation of the microparticles.

In vitro release of tannin from microparticles

Many *in vitro* release studies use diffusion cells or dialysis tubes for the evaluation of the release of bioactive compounds from solid microparticles (Reithmeier et al., 2001; Venkateswarlu and Manjunath, 2004). In this study, F57 ANKOM filter bags with a porosity of 25 µm were used. These filter bags are designed for rumen fermentation simulations. The *in vitro* release of tannin extract from the encapsulation matrix in the gut of ruminant animals was simulated using product solubility in various pH media following the procedure of Rossi et al. (2003). Elution media used were: acetate buffer (pH 5.4), phosphate buffer (pH 6.8) and HCl buffer (pH 2.2). Microparticles (100 mg) were weighed into fibre filter bags (F57; ANKOM), suspended in 50 mL of elution media and then vortexed at 50 rpm at 39°C. Aliquots of 2 mL were removed at 30 min, and at 1, 2, 4, 8, and 24 hours after incubation. The initial volume was maintained by the addition of fresh buffer media. Aliquots were frozen immediately for subsequent analysis. The release of tannin was monitored by spectrophotometric evaluation of the samples as described above. The obtained release data for acetate buffer were applied in zero order, first order and Higuchi square root equations to find the best prediction of the release of tannin extract in the rumen, where tannin release is of nutritional interest (Chime et al., 2013).

In vitro gas production

The *in vitro* gas production procedure detailed by Menke et al., (1979) was used to evaluate the effect of lipid-encapsulated ATE on gas and methane production and on rumen fluid parameters. During the *in vitro* procedure, a semi-automated gas pressure transducer and digital tracker were used to record gas pressure at time intervals and converted to gas volume while gas samples were analysed for methane concentration using gas chromatography (8610C BTU Gas Analyser GC System, SRI Instruments, Germany). Rumen fluid was obtained from three Merino rams feeding on Lucerne hay *ad libitum*, mixed with buffer under continuous CO_2 flushing, and used as inoculum (40 mL/bottle). Detailed procedures are described in Adejoro and Hassen, (2017). *Eragrostis curvula* hay (CP, 55 g/kg; NDF, 784 g/kg; ADF, 492 g/kg) and a TMR sheep diet (CP, 180 g/kg; NDF, 301 g/kg; ADF, 214 g/kg) were used separately as substrates (400 mg DM). For each substrate, treatments include i) diet only, ii) diet plus crude

ATE iii) diet plus lard-encapsulated ATE iv) diet plus palm oil-encapsulated ATE v) diet plus palm oil only vi) diet plus lard only. To each treatment containing ATE or encapsulated ATE, an equivalent of 30 mg ATE was added, which corresponds to 2.63% CT (leucocyanidin equivalent). Lard only and palm oil only treatments were added in amounts that were equivalent to those of the wall materials present in the lipid-encapsulated ATE to account for gas produced because of the wall material inclusion. Rumen fluid only incubation was included in each run to account for fermentation arising from the rumen fluid. The volume from this incubation was subtracted from gas volume for each time point. Four replicate bottles were incubated for each treatment with three repeated incubation runs. Gas production and methane concentration were measured at 2, 4, 8, 12 and 24 hours after incubation and cumulated to get the cumulative gas production at the time points. The rate and extent of gas production was determined by fitting the gas production data into the non-linear equation $y = a + b\ (1 - e^{-ct})$ of Ørskov and McDonald (1979) where y= gas production at time t, a= gas production from the soluble fraction (ml g^{-1} DM), *b*= gas production from the insoluble but slowly fermentable fraction (mL g^{-1} DM), and c= rate of fermentation of fraction 'b' (mL h-1). Rumen fluid pH after 24 hours of incubation was measured using a pH meter (Mettler Toledo 230 pH meter) while ammonia-nitrogen concentration was analysed as described by (Broderick and Kang, 1980).

Statistical analysis

For the *in vitro* gas production, within each substrate, individual bottles for each treatment in each incubation run served as analytical replicates while each repeat incubation run served as a statistical replicate. Gas volume was plotted against incubation time using the non-linear equation to predict fermentation kinetics variables (Ørskov and McDonald, 1979). Data on microparticle characteristics, gas production and fermentation parameters were expressed as least square means and were analysed using the PROC MIXED Procedure of SAS 9.4 (SAS Inst. Inc., Cary, NC). The model statement included $Yhijk = \mu + Sh + Ri + Tj + Qk + + ShTj + eij$ where, Y_{hijk}= mean of individual observation, μ= overall mean, S_h= effect of substrate, R_i= effect of incubation run, T_j = effect of treatment/additives, Q_k= effect of run within treatment, S_hT_k= substrate-treatment interaction effect, and e_{hijk}= residual error. Incubation run and run within treatment were set as random effects, whereas substrate, treatment and substrate-treatment interaction were fixed effects. Mean separation was done using Tukey's test.

7.3 Results

Characterization of microparticles

Figure 7.2 shows the scanning electron micrograph images of ETE^{L} and ETE^{P} microparticles obtained under optimal conditions. These microparticles had a mean diameter of approximately 34 µm and the encapsulation efficiency (EE) of approximately 80% (L-1; P-1) (Table 7.1). Encapsulation efficiency, yield, and particle size in the S/O/W emulsion process with palm oil (ETE^{P}) and lard (ETE^{L}) compared with microparticles prepared by the melt dispersion method showed that core material concentration and volume of the external aqueous phase affected microparticle properties. Microparticles prepared by the melt dispersion technique resulted in a high yield of encapsulated tannin (95%) but low EE (46%) compared with microparticles prepared by the S/O/W emulsion method ($P< 0.0001$). The melt dispersion method produced microparticles that were more spherical in shape but were larger compared with the cylindrical microparticles obtained from the S/O/W emulsion process ($P= 0.06$). Based on EE and mean particle diameter, the lipid-encapsulated ATE microparticles from L-1 and P-1 were subsequently used in the *in vitro* release and *in vitro* gas production tests.

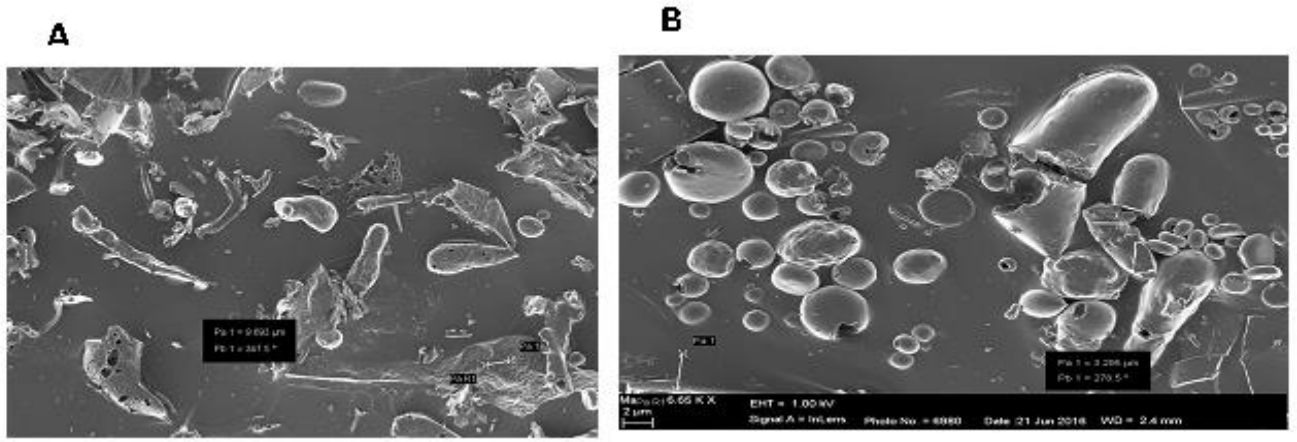


Figure 7.2 Scanning electron microscopy images of freeze-dried ATE-Lipid microparticles prepared by solid-in-oil-in-water encapsulation method using (a) lard and (b) palm oil

Table 7.1 Encapsulation efficiency and particle size of Acacia tannin extract-lipid microparticles prepared using melt dispersion and solid-in-oil-water encapsulation techniques

			Yield (%)	Encapsulation efficiency (%)	Mean particle diameter (µm)
[A] Melt dispersion			94.5[a]	46.0[c]	58.0[a]
[B] Solid-in-oil-in-water					
Batch	ATE conc. (g)	External aqueous phase (mL)	Yield (%)	Encapsulation efficiency (%)	Mean particle diameter (µm)
L1	8.5	300	57.3[d]	78.6[a]	33.9[bc]
L2	9.0	300	65.7[c]	75.1[ab]	39.5[bc]
L3	8.5	500	71.4[bc]	68.6[b]	44.7[abc]
L4	9.0	500	73.2[b]	74.6[ab]	48.6[ab]
P1	8.5	300	63.1[cd]	80.1[a]	26.8[c]
P2	9.0	300	65.8[c]	77.8[a]	32.7[bc]
P3	8.5	500	72.5[b]	74.3[ab]	30.4[bc]
P4	9.0	500	74.5[b]	68.8[b]	36.5[bc]
SEM			2.03	2.05	2.55
P-value			<0.0001	<0.0001	0.057

[1]L, batches 1-4 of S/O/W microparticles prepared using lard as wall material; P, batches 1-4 of S/O/W microparticles prepared using palm oil as wall material. Mean values with different superscript within the same column are significantly different ($P < 0.05$). Mean values are calculated from a minimum of three repeat batches

In vitro release behaviour of ATE-encapsulating lipid microparticles

Figure 7.3 shows the percentage of ATE released in different dissolution media from the lipid microparticles with lard (ETE^L) or palm oil (ETE^P) as wall material. The unencapsulated ATE produced a burst release in all dissolution media with 65% release within 2 hours and about 90% release before 8 hours of incubation. A slow release pattern however, was obtained for the lipid-encapsulated ATE products. After 24 hours in the dissolution media, 20%, 34% and 25% of the extract were released from the ETE^L microparticles in acetate, phosphate and HCl media respectively while 19%, 30% and 22% were released from the ETE^P microparticles in the same buffer media, respectively. The release of ATE from both lipid matrixes in acetate buffer followed the Higuchi equations better than the zero-order and first-order equations (Table 7.2).

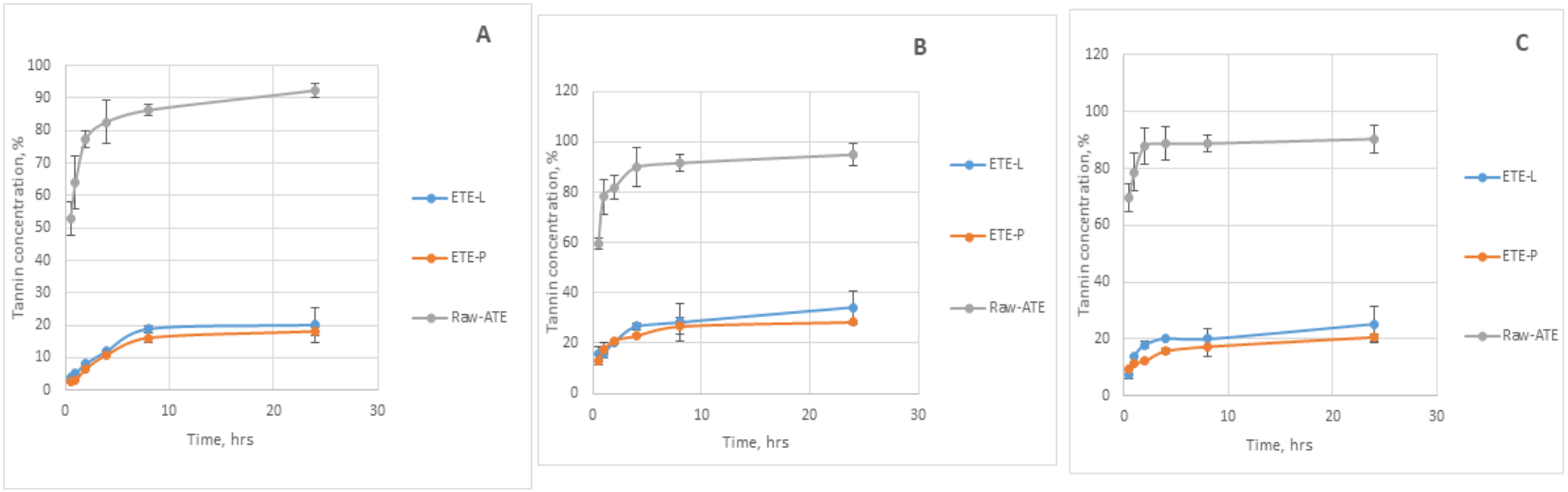


Figure 7.3 Release profiles of Acacia tannin extract (TE) and tannin extract microparticles encapsulated with lard (ETE^{L}) and palm oil (ETE^{P}) prepared by solid-in-oil-in-water encapsulation method in (A) acetate (0.1M, pH 5.50, (B) phosphate buffer (0. I M, pH 7.4) and (C) HCl buffer (0.1M, pH 2.2)

Table 7.2 *In vitro* release kinetic parameters in acetate buffer media (pH, 5.6), of Acacia tannin extract (ATE), or encapsulated-ATE prepared with lard (ETE^L) and palm oil (ETE^P) using the solid-in-water-oil method (*n*= 3)

	Zero Order, Q vs. t	First Order, ln (Q0-Q) vs. t	Higuchi, Q vs $\sqrt{t}$	R^2		
				Zero Order	First order	Higuchi
ETE^L	Y= 0.7988X+5.8823	Y= -0.0079X+1.9854	Y= 6.2174X-0.4151	0.6814	0.9177	0.9854
ETE^P	Y= 0.7504X+4.6174	Y= -0.0072X+1.9899	Y= 5.6666X-1.0398	0.7042	0.9292	0.979
ATE	Y= 2.2159X+53.925	Y= -0.0658X+1.6814	Y= 21.702X+29.357	0.3537	0.7366	0.7082

Q0, tannin to be released at zero time (mg); Q, amount of tannin released at time t: time in hours

In vitro fermentation, total gas production and methane emission of Eragrostis hay and a total mixed ration as influenced by lipid-encapsulated Acacia tannin extract

Table 7.3 shows *in vitro* gas production over a 24 hour period as affected by incubation of the *E. curvula* (EC) and TMR substrates with the ATE-encapsulated lipid microparticles. There was a strong interaction effect between substrate type and treatment for the cumulative gas and methane production at each of the incubation times ($P< 0.05$) except methane production at 2 hours. Generally, regardless of the inclusion of additives, gas and methane volumes were higher in the TMR substrate compared with the EC substrate across the incubation times. When both substrates were incubated with lipid wall materials only (lard or palm oil), there was no difference ($P <0.0001$) in gas production at 24 hours, when compared with the diet only (control). At 24 hours, incubation with crude ATE reduced the total gas production (mL/g DM) by 19.5% and 18.8% for EC and TMR substrates, respectively when compared with the control. The ETE^L and ETE^P incubations reduced ($P< 0.0001$) 24 hour total gas production when expressed in mL g^{-1} DM by 6.8% and 7.2% respectively in the EC substrate and by 13.4% and 13.2% respectively in the TMR substrate when compared with the control (diet only). The inclusion of ATE, ETE^L and ETE^P reduced cumulative methane production at 24 hours in both EC and TMR substrates ($P< 0.001$). The inclusion of the crude ATE reduced methane production by 23.8% in both EC and TMR substrates when compared with the control. In a similar trend, ETE^L and ETE^P reduced 24 hour methane production by 16.3% and 12.4% respectively in the EC substrate, and 16.7% and 21.4% respectively in the TMR substrates. Both palm oil only and lard only treatments had no effect on methane production after 24 hours incubation in the EC substrate but lard only treatment reduced 24 hour methane production in the TMR substrate when compared with the control. Generally, the oil type did not influence the properties of microparticles as reflected by the lack of significant differences observed between ETE^L and ETE^P in total gas or methane production. Substrate type and the inclusion of tannin additives did not have any significant interaction effect on methane concentration after 24 hour of *in vitro* incubation ($P> 0.05$). However, the inclusion of ATE, ETE^L, and ETE^P incubations only tended to reduce methane concentration in both EC and TMR substrates ($P= 0.09$). Similarly, methane concentration as a result of the inclusion of additives, tends to be lower in the EC substrate compared with the TMR substrate ($P= 0.06$).

There was a strong interaction effect of substrate type and treatment on the fermentation kinetics ($P< 0.05$). When compared with the control diet, the inclusion of ETE^P and ETE^L resulted in higher gas production from the rapidly fermentable portion ('a' fraction) of the TMR

substrate. Similarly, ETE^P reduced the 'a' fraction of gas production from the EC substrate, when compared with the control, while ETE^L did not have any effect ($P < 0.05$). The volume of gas production from the slowly fermentable portion of substrates ('b' fraction) showed that in the EC substrate, ETE^L and ETE^P inclusion increased the 'b' fraction when compared with the control while ATE inclusion did not elicit any effect. In contrast, no difference in 'b' fraction was observed across the treatments in the TMR substrate. On the rate of gas production in the TMR substrates, the effect of the encapsulated extract showed that ETE^L and ETE^P were intermediate between the highest rate observed in the control diet and the lowest rate observed in the crude ATE treatment. In contrast, no difference was observed in the rate of gas production when ETE^L and ETE^P incubations were compared with the crude ATE or with the control treatment in the EC substrate. The inclusion of extracts did not affect rumen fluid pH after 24 hours incubation ($P > 0.05$), but rumen pH was lower in the TMR substrate compared to the EC substrate ($P < .0001$). The addition of crude ATE, ETE^L and ETE^P reduced ammonia nitrogen concentration of rumen fluid after 24 hour incubation in the TMR substrate but no such effect was observed in the EC substrate. However, the crude ATE reduced rumen ammonia concentration more than that recorded by ETE^L when supplemented in the TMR substrates ($P < 0.0001$).

Table 7.3 Influence of Acacia tannin extract and lipid-encapsulated Acacia tannin extract (ETE^L, ETE^P) on *in vitro* gas production, methane, and fermentation parameters of *Eragrostis curvula* hay (EC) and total mixed ration (TMR) feeds

	Total gas (ml/g DM)				Methane (ml/g DM)					[3]Gas production constants				
[1]Treatment	2 h	4 h	12 h	24 h	2 h	4 h	12 h	24 h	[2]Methane, %	a	b	c	pH	NH_3-N
EC														
Control	11.3	18.2	27.7	47.1^{a}	1.04	1.72^{a}	2.61	4.92a	10.7	10.3^{a}	149.8^{b}	0.011	6.94	11.9
Lard	10.7	17.2	28.2	50.1^{a}	0.90	1.50^{ab}	2.50	4.81^{a}	9.61	8.38^{a}	155.3^{ab}	0.012	6.92	11.7
ETE^L	10.1	15.6	24.5	43.9^{ab}	0.86	1.47^{ab}	2.33	4.12^{ab}	9.79	7.29^{ab}	203.3^{a}	0.009	6.76	9.5
Palm oil	8.90	16.0	26.8	47.2^{ab}	0.81	1.49^{ab}	2.49	4.65^{a}	9.86	6.17^{b}	231.3^{a}	0.010	6.89	12.0
ETE^P	9.13	15.7	25.3	43.7^{ab}	0.80	1.45^{ab}	2.37	4.31^{ab}	9.86	6.90^{b}	200.9^{ab}	0.009	6.92	9.0
ATE	6.50	13.0	21.1	37.9^{b}	0.58	1.20^{b}	1.93	3.75^{b}	9.91	5.42^{b}	176.3^{ab}	0.008	6.87	9.0
TMR														
Control	29.2^{a}	50.9^{a}	97.6^{a}	157.1^{a}	1.97	3.79^{a}	8.29^{a}	16.8^{a}	10.7	-42.7^{a}	189.6	0.178^{a}	6.66	22.0^{a}
Lard	27.1^{a}	49.0^{a}	92.2^{a}	152.2^{a}	1.89	3.78^{a}	8.10^{a}	15.7^{b}	10.3	-41.3^{a}	186.2	0.176^{a}	6.67	21.7^{a}
ETE^L	20.9^{b}	39.3^{b}	79.0^{b}	136.1^{b}	1.70	3.30^{ab}	7.26^{b}	14.0^{c}	10.3	-32.5^{b}	173.7	0.163^{b}	6.65	16.8^{b}
Palm oil	27.2^{a}	49.1^{a}	93.3^{a}	155.7^{b}	2.38	4.24^{a}	8.67^{a}	16.1^{ab}	10.4	-39.6^{a}	187.9	0.177^{a}	6.59	21.6^{a}
ETE^P	20.8^{b}	41.0^{b}	78.4^{b}	136.4^{b}	1.63	2.43^{b}	6.09^{b}	13.2^{c}	9.7	-33.0^{b}	172.9	0.162^{b}	6.59	16.4^{bc}
ATE	19.9^{b}	37.8^{b}	75.1^{b}	127.5^{b}	1.55	2.89^{b}	6.64^{b}	12.8^{c}	10.0	-34.6^{b}	191.3	0.148^{c}	6.78	15.5^{c}
SEM	1.36	2.52	5.23	8.55	0.11	0.19	0.45	0.90	0.09	4.71	4.92	0.02	0.03	0.99
[4]*P*-value: S	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.059	<.0001	0.718	<.0001	<.0001	<.0001
[4]*P*-value: T	<.0001	<.0001	<.0001	<.0001	0.148	0.003	<.0001	<.0001	0.086	0.001	0.016	<.0001	0.197	<.0001
[4]*P*-value: S*T	0.004	0.001	<.0001	0.001	0.426	0.027	0.001	<.0001	0.625	<.0001	0.004	0.0001	0.088	<.0001

[1]ETE^L, lard-encapsulated Acacia tannin extract; ETE^P, palm oil-encapsulated Acacia tannin extract; ATE, Acacia tannin extract. SEM, standard error of mean. [2]Methane, '% of methane in the gas sample. [3]a, gas production from soluble fraction (ml g^{-1} DM); b, gas production from slowly fermentable fraction (mL g^{-1} DM); c, rate of fermentation of fraction 'b'(mL h^{-1}). [1]24 h methane, mL methane per 100 mL total gas. [4]*P*-values: S, effect of substrate, T, effect of treatment/additives, S*T, effect of substrate and treatment interaction. Mean values with different superscript within the same column are significantly different ($P < 0.05$).

7.4 Discussion

Important considerations in the encapsulation of bioactive products for oral administration are the particle size and EE. The larger particle sizes of microparticles have been related to the higher viscosity of lipid melt compared to lipid solution applicable in the S/O/W emulsion process (Reithmeier et al., 2001). For the S/O/W emulsion process, as the ATE/lipid ratio was increased by increasing the initial weight of ATE dissolved in DCM, there was a decrease in EE and ATE yield percentage (Table 7.1). As ATE concentration increased at constant volume of the external aqueous phase, mean particle diameter tends to increase. Scanning electron microscopy revealed that spherical particles were produced for all ATE/lipid ratios with indentations on the surface. The generally high concentrations of ATE used in this experiment may be responsible for the indentations, as was observed in a previous report (Jeffery et al., 1991). High concentration of ATE may have resulted in leaching of ATE into the surrounding external aqueous phase causing the indentation of microparticles surfaces. However, for all ATE concentrations, EE was high, ranging from 62 to 80%. The lower ATE: lipid ratio in batch L-1 may have ensured that a lower amount of core material was dispersed across the phase boundary thus resulting in higher EE. Another factor that may be responsible for the generally high EE is the nature and amount of surfactant used in both S/O and oil-in water (O/W) phases. Preliminary trials were conducted to establish the amount of Tween80© and Span80© that would give good emulsion stability. The concentration of the emulsifiers was subsequently kept constant at the levels determined most appropriate. It has been noted that surfactant concentration had a significant impact on the yield and EE of microparticles by contributing to the stabilization of the double emulsion. According to Jiao and Burgess (2003), droplet size and stability of emulsions depend on the concentration of both lipophilic and hydrophilic emulsifiers. An increase in the volume of the external aqueous phase also resulted in an increase in EE and particle size (Table 7.1). The concentration of the external aqueous phase (1% Span80) was kept constant for all batches.

The increase in microparticle size associated with an increase in the volume of the external aqueous phase can be attributed to a reduction in agitation intensity that occurred with larger volumes during the preparative process, which would result in the formation of larger microparticles. A reduction in mixing efficiency may occur due to an increase in the volume of the external aqueous phase. The present result is consistent with a previous trial (Castellanos et al., 2001) in which a decrease in homogenization intensity, because of the increased volume

of external aqueous phase, resulted in reduced microparticle size and EE. However, in the current study, the yield of microparticles increased as the volume of the external aqueous phase increased.

This study envisaged preparing a tannin product that would mask the bitter taste of the tannin and would not be easily dissolved under salivary conditions of the mouth, but would dissociate in the rumen. While the *in vitro* release protocol is an indication of solubility of the ATE-encapsulating lipid microparticles under the various pH media, it does not fully describe their dissolution under rumen conditions. For example, rumen microbes would expectedly hydrolyse the wall materials to free fatty acid (Bainbridge and Kraft, 2016; Veneman et al., 2015); thus exposing the core material to rumen fermentation. The *in vitro* release results indicate that the ATE-encapsulated lipid microparticles would not easily be solubilised in saliva during ensilivation, mastication and swallowing processes that depend on product solubility. An encapsulated fumaric acid preparation had similarly been found to exhibit a sustained release pattern of the core material in rumen fluid (Wood et al, 2009).

Several researchers (Huang et al., 2011; Jayanegara et al., 2012; Patra, 2010) have reviewed the effect of tanniferous plants or tannin extracts on methanogenesis. This study aimed to develop and evaluate an encapsulated tannin extract for rumen methane mitigation in ruminant animal production. An encapsulated ATE can be useful in decreasing rumen methane production, bypassing protein from the rumen, among other benefits without the astringency properties affecting feed intake. With a sustained release system, enteric methane reduction may be enhanced even more effectively. The use of the lipid-encapsulated tannin improved 24 hour gas production slightly compared with the use of the crude ATE. Gas production is an indication of dry matter fermentation, particularly the carbohydrate components of the feed (Carro et al., 1994; Menke et al., 1979). This showed that the reduction in gas production often associated with inclusion of tannin was slightly curbed as a result of the encapsulation of tannin, which makes them less available per time. However, the emission intensity, as judged in terms of mL of methane produced per 100 mL of total gas showed that only slight reduction in methane intensity could be associated with the inclusion of the tannin additives, either ATE, ETE^{L} or ETE^{P}. The effect of condensed tannins observed in the current study appeared to be associated more with reduced fermentation of dry matter rather than antimethanogenesis. The anti-methanogenic activities of condensed tannins have been related to a combination of direct

toxicity on methanogenic archaea, reduced fibre degradation or reduced OM digestibility (Patra, 2010).

Substrate type is known to influence microbial population and tannins have been noted to exhibit varying inhibitory effects on various rumen microbes (Hassanat and Benchaar, 2013). In this study, substrate type only tended to influence the antimethanogenic activity of the tannin extracts as seen from the methane concentrations. In a high crude protein diet like the TMR, it appeared that antimethanogenesis became less obvious compared to a lower crude protein diet like the Eragrostis hay substrate. This interaction between diet type and methane production had been reported previously by (Calabrò et al., 2012). A gradual release of tannin from the microparticles upon dissociation of the wall materials may be responsible for higher gas production recorded in the ETE^{L} and ETE^{P} treatments when compared with the crude ATE treatment. Inclusion of lard or palm oil only did not have any effect on gas production.

The results of reduced gas production associated with tannin reported in this study, agrees with those of various researchers on the effect of condensed tannin on *in vitro* gas production observed for plants with high CT content (Calabrò et al., 2012) or incubations with tannin extracts (Hassanat and Benchaar, 2013; Huang et al., 2011). Lipid-encapsulated tannin (ETE^{P} and ETE^{L}) resulted in higher gas production compared with the crude ATE treatment despite containing equivalent amounts of the active ingredient. This may be related to reduction in the initial solubilization of substrate and tannin extract in the mixed rumen fluid, or the reduction in the rate of attachment between the mixed microbial population and the incubation ingredients as can be seen by the slightly higher 'a' fraction recorded in the ETE^{L} compared to ATE in the Eragrostis hay substrate. It is not clear the reasons for the negative 'a' value observed in the TMR substrate and not in the Eragrostis hay. Negative values of 'a' fraction of substrates have been reported in previous trials (Chanthakhoun and Wanapat, 2012; Khazaal et al., 1995) when using gas production mathematical models. In a number of studies, this has been attributed to a deviation from the exponential course of fermentation, delayed fermentation due to microbial colonization or a lag phase after the soluble part of the substrate has been consumed (Blummel and Becker, 1997).

Although the rate of Eragrostis hay fermentation was not different in ETE^{L} and ETE^{P} compared with the ATE treatment, fermentation rate was significantly higher in the TMR substrate up to 24 hours of incubation as a result of the encapsulated ATE inclusion, rather than the crude ATE. For the degradation parameters, "b" represents components that are progressively but

slowly fermented, while ‘c’ represents the rate of degradation of ‘b’. Tannin extracts have been reported to slow the rate of dry matter digestion *in vitro* (Huang et al., 2011), but with encapsulation, a higher degradation rate can be achieved. The effects of condensed tannin on *in vitro* fermentation and nutrient digestibility depends largely on the formation of complexes with proteins, to a lesser extent on complexes with fibre fibre, as well as their effect on the mixed microbial population (Hassanat and Benchaar, 2013). In the TMR substrate, the higher ammonia concentration observed in the lipid-encapsulated ATE treatments when compared with the crude ATE treatment, is an indication that encapsulation of ATE can positively influence the activity of rumen fermentation depending on the diet characteristics. The encapsulation of tannin did show some promise in improving gas production compared to the application of the crude ATE, while still exhibiting typical tannin activities such as antimethanogenesis.

7.5 Conclusion

The present study showed that lard or palm oil could be used to encapsulate a tannin extract. The lipid-encapsulated ATE microparticles prepared using the S/O/W process exhibited good morphological characteristics, had high encapsulation efficiencies, and displayed sustained release of the tannin extract over time. The lipid-encapsulated microparticles also reduced total gas and methane production *in vitro* and therefore, could be utilized to modulate rumen fermentation positively.

CHAPTER EIGHT

Effect of lipid-encapsulated tannin on feed intake, nutrient digestibility, nitrogen balance and methane emission in sheep

Abstract

Condensed tannins are an important phytochemical which is increasingly being used in ruminant animals for a wide range of biological activities. However, some of its limitations such as astringency and reduced feed digestibility remains a critical challenge for its utilization as a dietary additive in ruminant nutrition. The potential use of encapsulated tannin extract instead of the crude extract may help to overcome some of these challenges. In this study, four rumen cannulated Merino wethers were used in a 4 × 4 Latin square design to determine the effect of encapsulating *Acacia mearnsii* tannin extract on feed intake, nutrient digestibility, nitrogen balance, and methane emission in sheep. The animals were fed a total mixed ration with one of the following four treatments: TMR only (control), TMR+ crude Acacia Tannin Extract (ATE), TMR+ lipid-encapsulated-ATE + TMR+SilvPro, which is a commercial tannin-based additive added as positive control. In each cycle, the experiment lasted for 26 days of which 14 days were used for adaptation followed by 7 days of data collection on total feed, orts, faeces and urine. An additional 5 days were used for *in vivo* methane emission measurement using an open-circuit respiratory chamber. Generally, nutrient intake for the supplemented animals was not different compared with the control animals ($p > 0.05$). Although inclusion of tannin reduced CP digestibility, encapsulation of ATE resulted in a relatively higher crude protein digestibility when compared with the crude ATE extract. However, compared to the control, the encapsulated ATE and Silvpro did not reduce DM, OM and NDF digestibility while the crude ATE resulted in a significantly lower DM, OM and NDF digestibility values compared to the control. Compared to the control treatment, total N-excretion was not different between the treatments ($p > 0.05$). However, ATE and encapsulated ATE treatment significantly increased the daily faecal-N excretion, but the urinary-N excretion did not differ across the treatment ($p > 0.05$). The total volatile fatty acid (TVFA) concentration, as well as acetate proportion of TVFA, was not affected by the treatments ($p > 0.05$). However, the Acetate: propionate ratio appears to be reduced in animals receiving the crude ATE and Silvpro extracts, but not in animals that received the encapsulated ATE ($p = 0.080$). Methane production expressed in L/kg $BW^{0.75}$/day was reduced by 20%, 33% and 26% in animals receiving SilvPro, encapsulated ATE and crude ATE, respectively. Both crude ATE and encapsulated ATE tend to reduce methane emission in L/kg $BW^{0.75}$ per day, in the experimental animals ($p = 0.065$). The reduction in methane output per unit of dry matter, per unit of NDF intake and per unit of dry matter digested for sheep receiving the encapsulated ATE treatment, suggests the potential use of encapsulated ATE to improve the utilization of tannin in ruminant diets by enabling the sustained release of the tannin to the rumen microbial population.

Keywords: Encapsulated Acacia tannin extract, Methane emission, Nitrogen balance, Nutrient digestibility

8.1 Introduction

The controversies on the use of antibiotic growth promoters in many countries have elicited greater interest in the use of plant secondary metabolites as a natural alternative in improving livestock productivity and reducing their impact on the environment (Cieslak et al., 2012). Some of the most common environmental impacts of ruminant production include the emission of methane from enteric fermentation (Hassen et al., 2016; Steinfeld et al., 2006), as well as methane, nitrous oxide, phosphorus and nitrogen from animal manure (Montes et al., 2013a; Pelletier et al., 2010). Condensed tannins (CTs) are water-soluble polyphenolic compounds of high molecular weight and wide biological activity. They have shown potential for wide application in animal production for modulating rumen fermentation (Gemeda and Hassen, 2015; Hoehn et al., 2018; Hristov et al., 2013). Past research has revealed that condensed tannins are able to bind with protein in plants, saliva, tissue, enzymes, and microbes (Reed, 1995), thereby reducing rumen protein degradability and increased intestinal absorption of amino acids (Dentinho et al., 2014). Other applications of CTs include management of bloat and intestinal parasites (Naumann et al., 2014) in ruminant production systems as well as a reduction in enteric methane emission (Carulla et al., 2005; Gemeda and Hassen, 2015; Hassen et al., 2016).

Condensed tannins exhibit their anti-methanogenic activity directly by inhibiting the growth of methanogens through the tanning action of their functional proteins (Field et al., 1989; Field and Lettinga, 1987), resulting in bacteriostatic and bactericidal effects (Tavendale et al., 2005). The indirect methanogenic activity of condensed tannin is mainly achieved through its defaunating action on the methanogen-associated protozoal population (Animut et al., 2008a, 2008b; Bhatta et al., 2009; Patra et al., 2006). However, moderate to high concentrations of dietary CT, which proved consistently effective in methane reduction, may also significantly depress dry matter intake and nutrient digestibility (Aerts et al., 1999) depending on the source, molecular weight and biological activity of the tannin. The compromise on intake and nutrient digestibility, if not carefully balanced, may impose a serious challenge on production performance. The astringency of tannins were particularly responsible for the negative responses in feed intake observed in ruminants (Lascano et al., 2003; Waghorn, 1990).

Thus, encapsulation of condensed tannin extract has the potential of masking this astringency, by restricting the biological activity of CT in the mouth and regulating its release and effect in the rumen and the post-rumen environment. According to Mamvura et al. (2014), the

productivity of bioactive compounds may be enhanced when they are fully available at the precise location where they are required such as in the rumen or intestine. It is hypothesised that encapsulated-ATE will confine the effect of tannin to the rumen, resulting in improved antimethanogenic effect without an associated depression in feed intake. The aim of this study, therefore, is to determine if encapsulating *Acacia mearnsii* tannin extract in a lipid matrix will favourably mask the bitter taste of tannin and possibly slow down its release in the rumen. By this, the anticipated utilization of encapsulated CT extract for the purpose of methane reduction can be achieved without the associated negative effects on voluntary dry matter intake, nutrient digestibility and N-balance in South Africa Mutton Merino wethers.

8.2 Materials and Methods

Animal management protocols were carried out in accordance with the University of Pretoria animal ethics committee (AEC) guidelines as stipulated in the approval number EC061-14.

Micro-encapsulation of Acacia mearnsii tannin extract

Acacia mearnsii tannin extract was supplied by UCL Company (Pty) Ltd. Dalton, South Africa. The water-soluble powdered product was analysed for its total phenol, total tannin and condensed tannin concentration following the procedure described by Makkar (2003b). The product contained 0.651 g/g total phenol, 0.58 g/g total tannin (as tannic acid equivalent) and 0.35 g/g condensed tannin (as leucocyanidin equivalent). Encapsulated ATE was prepared by the double-phase emulsion micro-encapsulation technique using palm oil as the lipid wall material (Adejoro et al, 2018; Chapter 7). The stability of the encapsulated tannin extract and its release rate was validated using *in vitro* culture technique at various pH buffer media, simulating the different segments of the ruminant gastrointestinal tract (Adejoro et al, 2018; Chapter 7).

Animals, diets, and experimental design

Four Merino wethers fitted with rumen cannulas (body weight, 75.8 ± 5.4 kg) were assigned to one of the four experimental treatments using 4 × 4 Latin square design. The animals received the treatments in four different periods of 26 d each (i.e., 14 d of adaptation, 7 d of digestibility data collection and 5 d of methane emission measurement). At the start and end of each period, animals were weighed and the average was used in calculating the average body weight of each individual animal. The four treatments were control (C), crude acacia tannin extract (ATE; 42 g/kg), lipid-encapsulated acacia tannin extract (encapsulated-ATE; 52.5 g/kg) and SilvPro (10 g/kg) as a positive control. The encapsulated tannin extract contains about 80%

Acacia tannin extract while SilvPro, under the market name SilvPro$^{(R)}$, is a commercial additive marketed across the European Union by Silvateam S.p.a (San Michele Mondovi, CN-Italy) as a vegetable tannin-based product. Although the active ingredients were not disclosed, according to the manufacturer's guidlines the powdered product is able to minimise protein digestion in the rumen, regulate bloat and inhibit enteric methane formation, andthus it was included as a positive control for comparisive purposes.

Apparent digestibility and N-balance measurment

Experimental animals were fed a total mixed ration diet *ad libitum*, and this was provided daily, in two instalments (0700, 1600 h). The TMR consists of approximately 50% roughage from Eragrostis hay and Lucerne meal, and this was mixed with concentrate and vitamin-mineral premix before feeding, to meet the maintenance nutrient requirement of the animals (Table 8.1). Clean water was also provided *ad libitum* and its consumption was monitored daily. During each study period, tannin extract was added to the respective daily TMR portion, and hand-mixed thoroughly inside a wide plastic container, before offering it to the animals. Feed refusal was weighed once daily and recorded before the next feed was offered, and samples of refusal and feed offered were collected daily and stored at -20°C. Samples of diet and refusals were pooled across days, within each period, freeze-dried and ground to pass through a 1-mm screen. Faeces were collected in faecal bags and urine was collected in metal trays with a funnel that emptied into a plastic bucket, which contained 50 mL of sulphuric acid (10% v/v) to prevent N-volatilization. Total faecal excretion and urine output were recorded daily. A sub-sample (10%) of urine was taken for urine-N analysis and stored at -20°C. At the end of each period, faeces and urine were pooled across days, for each animal, and subsequently stored at -20 °C until chemical analysis. At the end of each period, rumen fluid was sampled and pH was immediately determined, thereafter the samples were taken for the analysis of VFAs by preserving the rumen fluid samples in 25% orthophosphoric acid (5:1 v/v) and for ammonia N by preserving samples in 0.5 M sulphuric acid (6:1 v/v).

Open circuit respiratory chamber

The open circuit respiratory chambers are made of steel frames and UV-resistant clear flexible polyvinyl chloride (PVC) sheet on every side. The lower section of one side of each chamber has an air space to allow air inflow while at the top opposite side, an opening was made to channel air through a pipe connected to an exhaust fan to force air out of the chamber in one direction through the creation of negative air pressure. These fans have a speed control that

allows regulation of airspeed. Air exiting each chamber was channelled through a pipe containing two 90-degree bends prior to being vented to the outside of the building via PVC pipes. Each chamber was pre-calibrated via a methane recovery test by releasing a known concentration and amount of methane gas into the chambers and sampling methane concentration of outflowing air as well as the airflow through the exit ducts. This calibration of chambers was done at the start and at the end of each cycle of the methane emission measurement.

Methane emission measurement

While the animals were inside the open circuit respiratory chamber, feed supply was kept constant, and refusals were weighed daily. The Four animals, representing each treatment, were rotated every 24 h across the 4 different chambers to avoid any confounding effect that can be associated with the chambers. The animals were adapted to the chambers for one day and this was followed by 4 consecutive days of data collection. Animals were fed their ration in one daily portion, water was provided *ad lib* and the chamber was cleaned within 1.5 h while air sampling occurred for the remaining 22.5 h. Airflow within each chamber was monitored via a fixed hot wire anemometer probe, fitted with automatic data-loggers, and airspeed was recorded every 5 s from which mean airflow through the pipe was estimated. This reading was corrected with a manual vane anemometer measurement taken daily. A sub-sample of ambient air entering and leaving each chamber was continually withdrawn from halfway along a straight section of the PVC pipe using an 8-channel peristaltic pump (Masterflex 77292-50 L/S, Cole-Palmer Instrument company, The USA.). This was collected continuously via tubings into big deflatable Teflon bags fitted with a 3-way corkscrew for approximately 22.5 h period. Approximately 10 subsamples of air were withdrawn from each chamber's collection bag and ambient bag using gas tight syringes, and analysed by gas chromatography, (SRI 8610C Gas Chromatograph {GC} BTU Gas Analyser GC System) to determine the concentration of methane in each chamber. Net methane concentration (emission of each animal) was calculated as the difference in methane concentration of air coming out of each chamber minus ambient air methane concentration. Total daily methane emissions were therefore calculated by multiplying the total air volume extracted from each chamber in a day with the net methane concentration for each chamber. The recovery percentage determined for each chamber was used as a correction factor to adjust the daily methane volume produced by the animal in each chamber.

Chemical analyses

Samples of feed offered, refusals and faeces were analysed following the standard procedure described in AOAC, (2000) for dry matter (DM; ID 934.01), total ash (ID 942.05) and crude protein (ID 968.06). Neutral detergent fibre (NDF) was determined following the procedure described by Van Soest et al., (1991), using the ANKOM filter bag technique with the addition of heat-stable alpha amylase and sodium sulphite. The ADF was also analysed (non-sequential) using the ANKOM Technology Corp. (Fairport, NY.) fibre analysis procedure. Both NDF and ADF are expressed exclusive of residual ash. Rumen fluid pH was analysed using a pH meter (model PB-10/c, Sartorius, Germany) while its ammonia nitrogen concentration was analysed spectrophotometrically as described by Broderick and Kang, (1980) using a Specord 200 Analytik Jena UV-Spectrophotometer (Konrad-Zuse-Strasse, Germany). The concentration of volatile fatty acids in rumen fluid samples were determined using a gas chromatography machine (Shimadzu GC-2010 Tracera; Shimadzu corp., Kyoto, Japan) fitted with a 30m Inert Cap Pure Wax column (df = 0.25µm, I.D. = 0.25 mm).

Statistical analyses

Apparent nutrient digestibility, nitrogen balance and methane production were analysed as a 4 × 4 Latin square design, using PROC MIXED procedure of SAS, (SAS Institute, Inc., Cary, NC) with the following model:

$$Y_{ijk} = \mu + Pi + A_j + T_k + e_{ijk,}$$

Where Y_{ijkl} is the observation from animal (independent variable); μ is the overall mean; P_i is the effect of period (i= 1, 2, 3, 4), A_j is the effect of animal (j= 1, 2, 3, 4), T_k is the effect of treatment/diet (k= 1, 2, 3, 4) and e_{ijk} is the residual error effect. Each animal was considered as random effect while period and treatment/diet as fixed effects. Means were compared using Tukey's test and results reported as least square means and standard error of the means. Differences among treatment means, with $P < 0.05$ were accepted as significant.

8.3 Results

The ingredients and chemical composition of the experimental diet are presented in Table 8.1. The TMR has a roughage: concentrate ratio of 50:50 and CP, 162 g/kg DM; NDF, 373 g/kg DM and ADF, 210 g/kg DM. The addition of ATE, encapsulated-ATE or SilvPro as an additive

Table 8.1 Ingredient and chemical composition of experimental diet

Ingredients[1]	Inclusion, g/kg
Eragrostis curvula hay	300
Alfalfa hay	200
Hominy chop	140
Wheat bran	90
Maize germ meal	190
Urea	10
Molasses	60
Mineral and vitamin premix[2]	5
Calcium carbonate	5
Analysed composition	
Dry matter, (g/kg)	908.2
Organic matter, g/kg DM	922.3
CP, g/kg DM	162.1
NDF, g/kg DM	373.7
ADF, g/kg DM	210.5

[1]All ingredients was provided as a total mixed ration. [2]providing per kg: Zn, 25 g; Mn, 15 g; Se, 0.5 g; Co, 0.3 g, mg, 30 g, Fe, 15 g, Na, 500 mg and Cl, 280 mg; vit A, 2500 IU; vit D, 400mg; vit E, 3.5IU.

did not affect dry matter intake of the animals ($p= 0.57$), with their consumption ranging from 1341-1479 g/kg DM per day (Table 8.2). Similarly, dietary inclusion of tannin additives did not affect dry matter intake when expressed in g/kg $BW^{0.75}$/d, compared to the control treatment ($p= 0.53$). Similarly, dietary treatments did not show any effect on daily OM, CP, NDF and ADF intake of animal across the experimental treatments.

Treatment effect showed that tannin additives generally reduced DM and OM digestibility in the animals ($p< 0.05$). While ATE significantly reduced DM, OM, NDF and ADF digestibility, the inclusion of encapsulated-ATE and SilvPro did not seem to reduce DM, OM, NDF and ADF digestibility of the feed compared to the control treatment. However, dietary inclusion of both ATE and encapsulated ATE reduced CP digestibility when compared with the control treatment ($p< 0.05$). Encapsulation of ATE tended to improved NDF and ADF digestibility in animals consuming the Encapsulated ATE compared to the animals that received the crude ATE.

Table 8.2. Feed intake and apparent nutrient digestibility in Merino wethers fed total mixed ration containing crude or lipid-encapsulated acacia tannin extract (ATE) and SilvPro.

Parameter[1]	Control	SilvPro	ATE	Encapsulated ATE	[2]SEM	p-value
DMI, g/head/d	1479	1386	1341	1359	71.6	0.57
DMI, g/kg $BW^{0.75}$/d	57.3	53.8	51.9	52.7	2.61	0.53
OM, g/head/d	1358	1287	1228	1279	2.46	0.49
OM, g/kg $BW^{0.75}$/d	52.6	49.9	47.6	49.6	2.42	0.27
CP, g/head/d	244.0	207.7	203.3	195.2	11.0	0.19
NDF, g/head/d	542.5	538.1	517.3	547.0	27.9	0.55
ADF, g/head/d	306.9	303.8	283.8	307.3	15.4	0.50
Nutrient digestibility						
DM	63.2[a]	64.3[a]	50.4[b]	59.1[a]	1.52	0.01
Organic matter	64.2[a]	65.6[a]	51.2[b]	60.8[a]	1.53	0.01
Crude protein	77.1[a]	73.3[a]	61.2[c]	68.3[b]	1.30	0.005
NDF	38.6[a]	45.0[a]	24.0[b]	43.0[a]	2.53	0.01
ADF	37.7[a]	43.8[a]	21.8[b]	34.6[ab]	2.99	0.006

[1]DMI, dry matter intake; OM, organic matter; CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre. [2]SEM, standard error of means.

Dietary inclusion of SilvPro, ATE and encapsulated ATE did not affect overall N-intake, g/head/day ($p > 0.05$) when compared with the control treatments (Table 8.3). Similarly, total N-excretion, in g/head/day and g/kg $BW^{0.75}$/day was not different across the treatments ($p > 0.05$). However, the faecal-N excretion, when expressed in terms of g/kg N-intake increased in animals that received the crude ATE, and encapsulated-ATE but not in animals that received SilvPro additive, when compared with animals on the control diet ($p < 0.05$). Inclusion of additives did not affect urinary-N excretion ($p > 0.05$) and N-retention ($p > 0.05$) when compared with the control treatment.

Table 8.3. Nitrogen intake, excretion and retention in Merino wethers fed total mixed ration containing crude or lipid-encapsulated acacia tannin extract (ATE) and SilvPro

Parameter	Control	SilvPro	ATE	Encapsulated ATE	[1]SEM	*P*-value
N-intake, g/head/d	39.0	33.2	32.5	31.2	1.76	0.186
N-intake, g/kg $BW^{0.75}$/d	1.51	1.29	1.26	1.21	0.07	0.111
N-excretion, g/head/d	28.8	24.0	22.9	22.8	1.90	0.475
N-excretion, g/kg $BW^{0.75}$/d	1.12	0.93	0.89	0.88	0.08	0.387
Faecal-N proportion, g/kg N-intake	229.5^{c}	267.0^{c}	387.9^{a}	317.1^{b}	13.0	0.005
Urinary-N proportion, g/kg N-intake	511.5	454.5	314.2	412.7	40.7	0.217
Retained-N proportion, g/kg N-intake	259.0	278.6	297.9	270.2	41.7	0.979

[1]SEM, standard error of means. Means with different superscript across a row are significantly different ($P< 0.05$).

The rumen fermentation parameters of animals that received SilvPro, ATE or encapsulated-ATE is shown in Table 8.4. Rumen pH in the experimental lambs was not affected by the inclusion of the various additives. Similarly, rumen ammonia-nitrogen concentration was not different across the treatments ($p> 0.05$) although SilvPro, ATE, and encapsulated-ATE supplemented animals produced 11%, 30% and 26% lower rumen ammonia-nitrogen concentration when compared with the control treatment respectively. The total volatile fatty acid (VFA), acetate, butyrate, iso-butyrate, iso-valerate and valerate concentrations were not affected by the treatments ($p> 0.05$). However, the inclusion of additives tended to increase the propionate concentration ($p< 0.071$) in the animals. While encapsulated-ATE resulted in only a slight increase in propionate concentration, SilvPro and ATE inclusion resulted in significant increase in propionate concentration. Similarly, acetate to propionate ratio seems to reduce due to the inclusion of the additives, when compared with the control treatment ($p= 0.080$). In this case, while encapsulated-ATE recorded a slight decrease, there was a significant decrease in both SilvPro and ATE treatment when compared with the control treatment.

Table 8.4. Ruminal fermentation characteristics in Merino wethers fed total mixed ration containing crude extracts or lipid-encapsulated acacia tannin extract (ATE) and Silvpro

Parameter	Control	SilverPro	ATE	Encapsulated ATE	[1]SEM	*P*-value
pH	6.53	6.56	6.66	6.56	0.04	0.369
NH_3-N, mg/dL	19.4	17.3	13.5	14.3	2.17	0.104
Total volatile fatty acid (VFA), mmol/L	96.4	104.8	83.6	91.3	7.51	0.325
VFA molar proportion, mol/100mol						
Acetate	57.2	56.0	54.5	55.8	2.20	0.58
Propionate	19.4	24.9	27.9	21.2	1.43	0.071
Butyrate	19.6	15.3	13.0	18.5	2.30	0.618
Isobutyrate	1.10	1.03	0.83	1.13	0.13	0.335
Isovalerate	1.60	1.40	2.01	1.74	0.31	0.446
Valerate	1.42	1.76	1.98	1.92	0.13	0.244
Acetate: propionate ratio	2.98	2.32	1.99	2.69	0.19	0.080

[1]SEM, standard error of means. Means with different superscript across a row are significantly different ($P< 0.05$).

The *in vivo* methane emission by experimental animals as influenced by the addition of SilvPro, ATE and encapsulated-ATE is shown in Table 8.5. Compared to the control treatment, inclusion of both crude ATE and encapsulated-ATE as an additive tended to reduce enteric methane output when expressed in terms of L of CH_4/head/day ($p= 0.067$), L of CH_4/kg $BW^{0.75}$/d ($p= 0.067$), g of CH_4/kg DM intake ($p< 0.05$) and g of CH_4/kg NDF digested ($p< 0.05$). Animals that received SilvPro additive recorded reduced methane emission when expressed in terms of L of CH_4/kg $BW^{0.75}$/day ($p<0.05$). When methane production is expressed in terms of g/kg DM digested, there was no difference across the treatments.

Table 8.5. Methane emissions in Merino wethers fed a total mixed ration containing crude extract or lipid-encapsulated Acacia tannin

Parameter	Control	SilverPro	ATE	Encapsulated ATE	SEM[1]	P-value
L/head/d	79.4[a]	63.7[ab]	53.6[b]	58.6[b]	4.73	0.067
L/kg $BW^{0.75}$/d	3.06[a]	2.46[b]	2.08[b]	2.28[b]	0.17	0.065
g/kg DM intake	39.5[a]	34.6[ab]	27.5[b]	31.2[b]	2.07	0.025
g/kg NDF intake	105.6[a]	87.6[ab]	71.1[b]	80.0[b]	5.32	0.020
g/kg DM digested	62.0	52.0	56.1	52.5	5.52	0.348

[1]SEM, standard error of means. Means with different superscript across a row are significantly different ($P < 0.05$).

8.4 Discussion

The inclusion of SilvPro, encapsulated-ATE and ATE did not reduce DM and OM intake when compared with the control diet. Reduced DM and OM intake associated with tannin supplementation has been reported in some studies as a limitation (Bhatta et al., 2002; Fernández et al., 2012) while others have reported that tannin supplementation did not affect dry matter and nutrient intake (Animut et al., 2008b; Beauchemin et al., 2007).

The influence of tannin on dry matter intake may vary widely and depend on a number of factors such as concentration of tannin present, animal species and their age, biological characteristics of the tannin compound, prolonged adaptation of animals to tannin, diet characteristics, etc. (Beauchemin et al., 2007; Dschaak et al., 2011; Lascano et al., 2003; McNabb et al., 1996). Although the quantity of ATE inclusion in this trial is comparable to the one used by Carulla et al. (2005), the butanol-HCL method used in evaluating the tannin concentration for this study is reported in terms of Leucocyanidin equivalent (Makkar, 2003b). Nevertheless, the astringency associated with CT consumption may have waned after the adaptation period as previously observed (Landau et al., 2000b). The Silvpro, ATE and encapsulated-ATE did not affect CP intake when compared with the control treatment. Both Beauchemin et al. (2007) and Carulla et al. (2005) also observed that Acacia tannin inclusion did not significantly affect CP-intake. However, Animut et al. (2008b) observed increased N-intake with increasing levels of *Kobe lespedeza* tannin inclusion in goats. This phenomenon of increased n-intake may be due to the ability of animals to select nutrients based on their need

(Hutchings et al., 2003) but the magnitude and underlying principles are not clear. In this trial, animals were offered the respective diets *ad libitum* with an allowance for about 10% orts.

The reduced digestibility of DM, CP, OM, NDF and ADF recorded in the crude ATE and encapsulated ATE is related to the ability of tannin to bind to protein and fibre, thereby reducing their digestibility in the rumen (Carulla et al., 2005; Dentinho et al., 2014; Grainger et al., 2009). According to Dentinho et al. (2014), tannins reduce DM and CP digestibility by reducing the solubilization and degradation of CP in the rumen. In their study, the decrease observed in rumen degradability of CP was mainly associated with a significant reduction in initial solubilization (decrease in "a" value of digestion kinetics) (Dentinho et al., 2014). In particular, the degradation rate (c) was not affected by the inclusion of tannins. A significantly higher CP and ADF digestibility in the lipid-encapsulated ATE treatment, compared with the ATE treatment is an indication that more protein may have been digested or more protein was available for microbial digestion as a result of the encapsulation due to a slow release of the condensed tannin from the encapsulated ATE in the rumen. Encapsulation may have ostensibly made tannin release slower in the rumen prior to the establishment of new linkages with protein and other nutrients in the feed. This mechanism for slow release of tannin may offer a huge potential in the administration of tannins and other phytochemicals in animal nutrition. The DM, OM, NDF and ADF digestibility of the encapsulated ATE was comparable to the SilvPro and control treatments. Condensed tannins have been noted to reduce the digestibility of NDF and ADF (McSweeney et al., 2001a). The negative impact on the activity of cellulolytic bacteria in the rumen may have been reduced by the encapsulation of ATE, as evidenced in the higher DM, OM and NDF digestibility compared with the ATE treatment. Cellulose and hemicellulose are the major components of NDF. The high concentration of tannin that remains free after binding with protein, readily complexes with lignocellulose, and is thus capable of depressing NDF and ADF digestibility (Barry and Manley, 1986). In tanniferous forages, it has been reported that the ratio of soluble to insoluble tannins will affect its binding ability to proteins and fibre. If the ratio of soluble to insoluble tannin is high, there is a shift to tannin-protein-complex formation and if the ratio is low, there is a shift to tannin-fibre complexes (Giner-Chaves, 1996). The preferential binding characteristics of each tannin with dietary proteins, and subsequently with endogenous protein in the lower parts of the digestive system (McSweeney et al., 2001b) may, therefore, determine its potential application to achieve digestible bypass protein through tannin supplementation.

Higher faecal N-loss in ATE, encapsulated-ATE and SilvPro is consistent with previous results reported on the effect of tannin on N-loss in animals (Animut et al., 2008b, 2008a). When tannin is included in the diet, it is common to observe the environmentally advantageous shift in nitrogen loss form urine-N to faecal-N. In this study, the shift was not observed. Urinary-N losses were associated with very large error term and therefore the lack of significance may be attributed to experimental error. The confounding effect associated with the experimental design and in rotating experimental animals across the treatments may have been responsible for this. Values for N-retention and total-N loss from the animals on tannin treatments were not different when compared with the control treatment. Faecal-N is a more environmentally stable source of N-loss to the environment compared to urine-N (Śliwiński et al., 2004). While faecal-N is in organic form, urine-N is largely in the form of urea-N that can be quickly hydrolysed to ammonia and nitrified to nitrate (Eckard et al., 2010). Ammonia is volatile while nitrate can leach into groundwater as a pollutant, or can be converted to nitrous oxide which is a highly potent greenhouse gas (Steinfeld et al., 2006). In contrast, the CT-protein complex in faeces will improve the quality of manure as a fertiliser by increasing the nitrogen content of the manure, as well as slowly releasing the nitrogen into the environment due to the slow dissociation of CT-protein complex in the soil. This, in turn, reduces volatilization of ammonia and improves the utilization by plants because nitrogen is slowly released into the soil. Thus it will indirectly reduce the environmental impact of livestock production over a long-term period (Eckard et al., 2010).

Rumen pH and TVFA was not affected by the inclusion of SilvPro, ATE or encapsulated-ATE in the diets of the animals and this result is consistent with some reports on the effect of inclusion of tannin extract in sheep and cattle diet on TVFA (Calabrò et al., 2012; Carulla et al., 2005). This may be an indication of the fermentability of the diets, in which SilvPro, ATE or encapsulated-ATE did not reduce VFA production. Rumen pH often relates to the production of different VFA by fibrolytic or amylolytic microbes in the rumen. There was a slight tendency for reduction of rumen ammonia concentration in animals receiving SilvPro, ATE or encapsulated-ATE, however, these levels were not significantly different from the control treatment. Significant reduction in rumen ammonia concentration was observed in some previous trials (Beauchemin et al., 2009; Cieslak et al., 2012). Ammonia is the end product of the deamination of amino acid and non-protein nitrogen (urea). The high amount of NPN in the diet of the animals may have ensured a high concentration of rumen ammonia, sufficient to meet the need of rumen microbes despite the reduced CP digestibility. The

threshold of rumen ammonia-nitrogen concentration required for optimum microbial function is 5- 25 mg/dL NH_3-N (Pinho et al., 2018; Satter and Slyter, 1974). According to Calabrò et al., (2012) when ammonia nitrogen is high in the presence of sufficient carbohydrate, more amino acids are utilised in building microbial biomass, resulting in increased fermentation and total volatile VFA. Therefore, the impact of tannin on rumen ammonia may depend not only on the CP content of the feed but also on the amount of NPN present.

While Carulla et al., (2005) and Beauchemin et al., (2007) observed reduced A/P ratio in rumen fluid with supplementation of Acacia tannin and Quebracho tannin respectively, Puchala et al., (2005) did not observe any change in A/P ratio with supplementation of tannin from *Lespedeza cuneata*. Tannin source or concentration may have played a profound effect in these differences. According to Makkar et al., (1995) generally the reduction in A/P ratio could be due to either significant reduction in acetate proportion or marginal increase in propionate proportion. This is usually by the activities of microbes inhibiting acetate-forming bacteria or by inhibiting the production of their preferred substrates. In the current study, the slight reduction in A/P ratio noted was associated with an increase in propionate proportion for the crude ATE or encapsulated ATE treatments but acetate proportion was unaffected as was observed by Castro-Montoya et al. (2011). Compared to the crude ATE extract, A/P ratio was higher in the encapsulated ATE supplemented animals as a result of higher propionate proportion. We are not sure how this relates to the nature of tannin, encapsulation, or the release of tannin extract into the mixed microbial system. A sustained release tannin system may have preferentially reduced the activities of methanogens, allowing more hydrogen to go via propiogenesis, rather than a reduction in overall organic matter fermentation.

The ATE and encapsulated-ATE reduced methane production expressed in g/kg DM intake by 30% and 21.0% respectively. This result confirms the anti-methanogenic properties of acacia tannin extract also found by some previous researchers (Carulla et al., 2005; Grainger et al., 2009). Usually, methane reduction may be dependent on dose, mode of administration and biological activity of the active compound, therefore results from each trial needs to be examined carefully and comparisons are made cautiously. In a previous trial involving *Acacia mearnsii* extract, dairy cows were drenched twice daily with 163 and 244g CT d^{-1} (Grainger et al., 2009) while in a related study, Carulla et al. (2005) added *Acacia mearnsii* extract to diets of Sheep at 25g CT/kg feed DM. While Grainger et al. (2009) recorded 14 and 29% reduction in methane (g d^{-1}); 10 and 22% methane reduction when expressed in terms of DMI (g/kg

DMI), Carulla et al. (2005) reported a 13% reduction in methane (g/kg DMI). In this study, compared to animals that received crude ATE only, animals on lipid-encapsulated ATE produced slightly less methane (56.1 vs 52.5 g/kg DM digested). This is an indication that the likely slow release mechanism from encapsulated-ATE may have ensured a better utilization of the tannin extract in mitigating the activity of methanogens rather than a reduction in dry matter digestion. A meta-analysis study by Jayanegara and Palupi (2010) concluded that tannin concentration has a negative correlation to methane production. The anti-methanogenic properties of tannins relate to a combination of direct toxicity on the methanogenic archaea, reduced fibre degradation or reduced OM digestibility (Beauchemin et al., 2007; Patra and Saxena, 2011). Reduced fibre digestion often result in reduced acetate and butyrate production. In the current study, TVFA, acetate, and butyrate concentrations were unaffected by tannin additives, and less methane was produced per unit of DM and NDF intake, an indication that the extracts may have exerted a direct antimethanogenic effect in the rumen.

While the quantity of extracts added in the crude ATE and lipid-encapsulated ATE treatments were decided based on laboratory condensed tannin (CT) analysis according to the butanol-HCL-Iron method (Makkar, 2003b), these values may not fully describe the characteristics of the plant extract, especially its methane reducing potential or protein binding characteristics. According to Jayanegara et al. (2009), CT values, unlike the tannin bioassay, are a poor predictors of the methane reducing potential (MRP) of tannin-containing plants/extracts. Similarly, the relationship between CT values and the protein binding affinity as well as anti-methanogenic properties of tannin sources have not been fully elucidated (Waghorn, 2008). The potential presence of hydrolysable tannins and other non-tannin phenolics in the crude ATE and encapsulated-ATE used in our study may also partly exacerbate the detrimental effects of CT (Grainger et al., 2009). The molecular weight of CTs have also been shown to relate very strongly to its protein binding ability and methane reducing ability (Kariuki and Norton, 2008; Osborne and McNeill, 2001) irrespective of the concentration. Condensed tannins have a varying extent of the inhibitory effect on rumen fibrolytic bacteria species (McAllister et al., 2005) with tannins of low molecular weight showing greater inhibitory effect (Patra and Saxena, 2011). The manufacturer of the ATE used in this study quoted a molecular weight range of 500- 3000 and mean molecular weight of 1250. The distribution of tannin molecules within this range is not clear.

The commercial use of extracts such as lipid-encapsulated ATE could have importance in mitigation of GHG from agriculture. However, farmer acceptance for such product would largely depend on the benefits for sustainable animal production being able to exceed the cost of inclusion of such products as a dietary additive, factoring that the lipid wall material for encapsulation will constitute added ingredient to the final feed. The process of encapsulation seems to enhance the utilization of ATE as evidenced by higher improvement in OM and NDF digestibility, a slight improvement in feed intake and other nutrient digestibility with a significant reduction in methane production. According to Waghorn (2008), while CT protects proteins from excessive ruminal degradation and reduces enteric methane production, they can also inhibit the absorption of the protected protein. A tannin product such as encapsulated ATE may be useful in meeting this delicate balance.

8.5 Conclusion

The use of encapsulated-ATE as a feed additive is justified by the significant reduction in methane production with a slight improvement in the digestibility of NDF and CP recorded when compared with the use of crude ATE. Overall, both ATE and encapsulated-ATE significantly reduced methane emission in the experimental animals.

CHAPTER NINE

General conclusion, recommendation and critical evaluation

Conclusions and recommendations

In much of the tropical and sub-tropical ruminant production systems of Africa, livestock depends mainly on forage, crop-residues and other low quality feeds. The low nitrogen content and low digestibility of these feeds remain a major challenge. In such systems, supplementing animal diets with a NPN source could improve rumen fermentation of poor quality forages especially when dietary crude protein is the most limiting nutrient. In the context of reducing the environmental footprints of livestock production from such systems, the use of nitrogen-based supplement and other feed additives capable of improving the utilization of available resources, while also reducing anthropogenic greenhouse gas emission becomes a climate-smart approach. Such technologies will have a huge potential to improve the competitiveness of the system on a global scale.

The first part of the study, which focused on the supplementation or treatment of Eragrostis hay with calcium nitrate instead of urea, sought to evaluate the incorporation of calcium nitrate as a rumen hydrogen acceptor into the existing production practices in order to improve forage utilization while reducing the enteric methane production from such a system. The result showed that both urea and calcium nitrate improved the digestibility of Eragrostis hay. Although urea gave a superior improvement in digestibility, nitrate significantly reduced methane production either when supplemented or used in the ammoniation process. The effect of inclusion of nitrate in the diet of sheep instead of urea in the subsequent experiments, apart from reducing enteric methane emission, it has resulted in improved feed utilization efficiency.

The study conducted on the combined use of tannin extracts with various NPN sources sought to evaluate the effect of the tannin-nitrogen interaction occurring in the rumen under a high amount of non-protein nitrogen. The result revealed that animals under nitrate diet had superior growth performance compared to those on urea, while tannin inclusion neither improved growth performance nor significantly reduced enteric methane emission in the lambs. Although a significant reduction in crude protein digestibility was recorded due to the inclusion of *Acacia mearnsii* tannin extract, the proportion of faecal nitrogen excretion was increased without affecting the overall nitrogen retention values of the experimental animals. No clinical or sub-clinical signs of morbidity were noted due to the inclusion of the NPN sources or tannin as part of the TMR diet. No intoxication symptoms were recorded as judged in the haematology or

biochemical parameters of blood samples evaluated after the growth period. This implies that *Acacia mearnsii* tannin extract or calcium nitrate at the current dose can be fed safely to the growing lambs, without any serious negative consequences.

One of the likely negative effects of the administration of crude tannin extract to ruminants is the astringency properties among others. The study conducted on the preparation and evaluation of an encapsulated Acacia tannin extract product was mainly aimed at developing a product that is able to mask the effect of tannin in the mouth while also guaranteeing sustained release in the rumen. After testing a number of encapsulating materials, and conducting a series of *in vitro* experiments, the solid-in-oil-in-water double phase emulsion method was identified for developing an encapsulated Acacia tannin extract product. This extract exhibited good morphological characteristics and high encapsulation efficiency even under a high loading percentage of up to 80%. Lipid-encapsulated ATE prepared under optimum conditions was further evaluated in subsequent studies using an *in vivo* experiment. The lipid-encapsulated extract reduced methane production *in vivo,* and its efficacy was comparable to the commercial product SilvPro. The findings of the *in vivo* study showed that the administration of tannin extract could be improved using an encapsulated product such as the lipid-encapsulated Acacia tannin extract tested in this study. This product resulted in considerable reduction in methane emission per NDF intake, in mature Merino rams compared to the crude extract.

While the Acacia tannin extract did not seem to have any prolonged effect in reducing methane emission during the growth trial in the Merino lambs, both the crude extract and the lipid-encapsulated tannin extract significantly reduced methane emission in mature Merino rams during the *in vivo* digestibility trial. Although it is problematic to give a proper explanation for the observed discrepancy in terms of enteric methane response between the two experiments, duration of tannin feeding might have confounded the result perhaps due to gradual adaptation of the methanogens to a long-term feeding of tannin extract. This hypothesis needs to be tested in the future. The supplementation of low protein diets with calcium nitrate as an NPN source should be promoted as a climate-smart strategy for guaranteed sustainable agricultural production.

In addition, the encapsulated tannin extract needs to be refined further and evaluated for other applications of tannin in ruminant animals such as its effectiveness to enhance bypass protein in order to protect the degradation of high-quality protein in the rumen. Despite the low response of the tannin extract used in this study to reduce enteric methane especially after

prolonged feeding, the potential of encapsulating tannin for ruminant animals have been established. Other tannin sources with higher potency should be tested using the encapsulation method described in this study, for both enteric methane as well as other ruminant applications.

Critical evaluation of research

It is important to consider some important issues that were not included in this study but may have a profound impact on the result already presented, or relevant to other researchers interested in using some of the methodologies used in this study to conduct similar research in the future.

- For the *in vitro* ruminal gas production trials, a 48 hour end-point was used to monitor gas production and methane emission associated with the test diet and the additives. While this may be acceptable for certain diet types, the 48 hour duration has shown to be insufficient to predict the pattern of fermentation and gas production in a tropical grass species like the Eragrostis hay. The Orskov and McDonald equation would therefore be better able to predict the fermentation characteristics after an asymptote point has been reached and this would occur only after 96-120 hours of *in vitro* fermentation in most tropical grasses. This is therefore recommended as a better approach.
- There are significant limitations to the quality of result obtainable with the end-point measurement of rumen fluid samples as was done for the *in vitro* ruminal gas production as well as in the animal experiments where rumen fluid were sampled at the abattoir. There is a documented hourly wide variation in rumen fluid parameters as a result of feeding, retention times and the type of additive offered to the animals. A better approach may be to include rumen fistulated animals in the experimental set up for the purpose of repeated sampling of the rumen.
- The biological value of tannins is known to depend on the tannin source among other factors. Different tannin sources with the same concentration of tannin, as determined by the photometric methods used in this study may have a significantly different biological activity such as protein binding potential, hence the comparison of tannin content values must be treated with caution.
- Tannic acid was used as a calibration standard during the quantification of total phenol and total tannin content of the extract while condensed tannin content was determined by the butanol-HCl method and expressed as leucocyanidin equivalent. Expression of CT as leucocyanidin equivalent has limitations. It has been widely suggested that for each tannin

source, a separate standard with similar molecular structure is required to generate calibration curves during the photometric analysis. This is best derived by elution of pure samples from the same tannin product. With the availability of required equipment, this approach will provide a more accurate result of the total phenol, total tannin and condensed tannin content of any extract.

- The analysis of methane using the SRI GC relies on the standardization of the equipment and the generation of calibration curves to plot the concentration of the unknown gas samples. Variation in results could be associated with the operational conditions of the GC and calibration gas used as standards. This variation may be significant from batch to batch. However, although the individual trials of this study were carried out at different times, comparisons were made across treatments for each trial and repeat runs were carried out within a short time from each other under similar operating conditions. This, therefore, would not have any profound effect on the ranking of the experimental treatments reported in these trials.
- The inclusion level of the non-protein nitrogen sources, as well as the crude protein content of the feed used in the growth experiment for lambs, may be considered to be on the higher side. The magnitude of dietary effects of the tannin or NPN sources on parameters measured such as methane reduction may become more or less obvious at varying levels of dietary CP. It may, therefore, also be important to evaluate the inclusion level of calcium nitrate as a replacement for urea at lower levels of CP.
- the lack of adequate isonitrogenous precision is significantly noted in the crude protein values of the urea-based TMR and nitrate-based TMR ration. This was due to the purchase of calcium nitrate through a third party company, which was not analysed prior to feed formulation. Relying on the nitrogen content of the calcium nitrate as provided by the manufacturer, resulted in deviation from actual nitrogen content of the final feed. Future trials with calcium nitrate should be preceded with a preliminary analysis of nitrate source.
- The *in vivo* measurement of methane emission inside the open circuit respiratory chamber relied on the generation of negative air pressure inside the chamber in order to ensure that the flow of air out of the chambers is in one direction only. The chambers were designed and maintained to be sufficiently airtight, to minimise air loss from the system. However, the magnitude of errors that can be associated with chamber air mixing and air ducting (from chambers to collecting bags) were not quantified. Therefore, comparison of values reported in the current study with that obtained from other research centres may need to be

considered with caution. Nevertheless, the periodic recovery tests, serving as correction factors, and the rotation of the experimental animals/treatments across each of the chambers ensured that the results obtained can be safely compared across treatments with a high degree of certainty.

- From this study, only a slight reduction in enteric methane production was associated with the Acacia tannin extract after prolonged administration. However, the magnitude of the effect of dietary tannin in reducing the overall animal derived greenhouse gas emission may be higher when an increase in nitrogen excretion in faeces as against urine is evaluated in terms of net greenhouse gas emission at a farm scale, by taking into account the enteric methane and nitrous oxide production from manure. Although, in much of Africa, manure is packed in solid form under various composting methods, whereas in many temperate countries of Europe and America, manure is commonly stored in the form of a slurry. Manure may not produce significant methane emission when in the solid form, unlike slurry. The binding of tannin to faecal protein ensures that there is a prolonged dissociation of the protein-tannin complex in the faeces, whereas urine is a more volatile source of nitrogen loss. Nitrous oxide emission from manure has a global warming potential that is about fourteen times that of methane. Thus, the effect of supplementation of nitrate and/or encapsulated or acacia tannin extract as it is, needs to be modelled at a farm scale, in order to quantify the net gain that can be achieved in terms of production as well as greenhouse gas reduction that is associated with the use of these additives in the feeding system of ruminant animals.

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