Effect of microbial ecosystem from wild herbivores browsing tanniferous plants on goat rumen fibrolytic activity

By

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Declaration

I (**Nokwethemba Nqobile Philile Msimango**) declare that this dissertation is entirely my own work and has not been taken from the work of others except where I have appropriately acknowledged and referenced the original source. This dissertation has never been submitted for any degree for examination in any university.

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Signed on the _____ day of _____, 2016

Abstract

Ruminant have developed advanced microbial ecosystem for digesting fibrous feedstuffs over the past years. The efficiency of harnessing energy from these forages is still a major problem as less than 60% of forages consumed are still passed out as faeces. Many factors have been associated with the poor breakdown of these forages including; complexity of fibre in forage types, inefficient microbes, microbial population, low rumen pH and presence of anti-nutritional compounds (lignin and alkaloids, saponnins and tannin). Therefore, understanding microbial ecosystems' activities in ruminants is an essential step towards improving tanniferous browsers' utilisation especially in goats which are often supplemented in winter during forage shortages. The study was conducted to identify potential rumen microbial ecosystems browsing tanniferous forages that might have evolved in their ability to degrade plant fibre in the presence of tannin that can potentially be used to improve domestic goat browse utilisation.

Experiment 1 investigated the fibrolytic potential of microbial ecosystems giraffe, kudu, impala and consortia (A1 (giraffe + kudu, 1:1), A2 (giraffe + impala, 1:1), A3 (kudu + impala, 1:1), A4 (giraffe + kudu + impala, 1:1:1)). Crude protein enzyme extracts (CPZ) from fresh faecal samples were precipitated by 60% ammonium sulphate and assayed for exocellulase, endocellulase and hemicellulase by incubating with crystalline cellulose, carboxymethyl cellulose and xylan at 38℃ at pH of 5.5 for 1, 2, and 48 h, respectively. Enzyme specific activities were defined as μg of reducing sugar/mg CPZ. *In vitro* fermentation study was done by transferring 33 mL of fresh faecal inoculum into 67 mL of salivary buffer containing 1 g *Acacia sieberiana* and incubating for 72 h at 38℃. Apparent degradability (APD), true degradability (TD), neutral detergent fibre degradability (NDFdeg), acid detergent fibre degradability (ADFdeg), microbial yield (MY), metabolisable energy (ME) and total gas emitted (Gas) were measured. Cellulases, hemicellulase and *in vitro* degradability studies showed that microbial ecosystems from wild browsers (especially impala) and consortia possess a higher ($P<0.05$) potential to digest tanniferous forages with less enteric gas production than observed in goats, hence could be exploited as feed additives for improving digestibility and reducing enteric gas production in goats.

In experiment 2, the effect of tannins on the fibrolytic potential of microbial ecosystems from goats, wild giraffe, kudu, impala and consortia (A1, A2, A3 and A4) were monitored. The method was the same as in experiment 1 except for the addition of 10% tannic acid. A microbial ecosystem treated with 10% tannin showed higher ($P<0.05$) fibrolytic enzyme activities and

digestibility parameters compared to the control. The goat ecosystem showed some degree of adaptability to increased tannin but microbial ecosystems from wild browsers (impala and kudu) and consortia (A1, A2 and A3) showed a higher potential to digest tanniferous forages. The results demonstrated that wild herbivores especially impala and A1 consortia can tolerate minimal changes in tannin concentrations.

Experiment 3 assessed the effect of *in vitro* inoculation of goat microbial ecosystems with inocula from wild herbivores (impala, kudu and giraffe) on *in vitro* fermentation, gas production and cellulase activity in goats. The method was similar to that of experiment 2 but for goat microbial consortia (N1 (goat + impala, 1:1), N2 (goat + kudu, 1:1), N3 (goat + giraffe+ kudu, 1:1:1) and N4 (goat + giraffe + kudu + impala, 1:1:1:1)). Manipulation of the goat ecosystem with microbes from the wild generally increased ($P<0.05$) cellulase and hemicellulase activities as presented by their high hemicellulase, endocellulase and exocellulase activities. The highest ($P<0.05$) NDFdeg, ADFdeg and TD were observed in N3 while HEMdeg and CELLdeg were high in the N1ecosystem. Therefore, wild ruminant browsers may be harbouring important potential fibrolytic microbial population with relatively high tannin tolerance which can be used to improve the browses digestibility of goats.

The final experimental chapter monitored the effect of adding polyethylene glycol 4000 (PEG) to goat microbial consortia *in vitro* degradability of tanniferous feeds. The results showed that 5% PEG supplementation generally increased ($P<0.05$) hemicellulase, endocellulase and exocellulase activities in goats' consortia when compared to the control. Digestibility of fibre was lower for diets not treated with PEG. Apart from N3, PEG increased ($P<0.05$) dry matter degradability of *Acacia sieberiana* but not microbial yield. The improvement of enzyme activities and dry matter digestibility upon addition of PEG in goat consortia highlights the inhibitory effects still possessed by tannins even when inoculated with potential fibrolytic microbes from wild browsers. Therefore, microbial ecosystems from wild browsers can contribute potential fibrolytic microbes with relatively high tannin tolerance that can improve forage digestibility and reduce enteric gas production in goats.

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Table of contents

List of tables

Abbreviations

DM = Dry matter

APD = Apparent degradable fraction of feed

TD = Truly degradable fraction of feed

NDFdeg = Neutral detergent fibre degradability

ADFdeg = Acid detergent fibre degradability

HEMdeg = Hemicellulose degradability

CELLdeg = cellulose degradability

CTs = Condensed tannins

PEG = Polyethylene glycol

ME = Metabolisable energy

GP = Gas volume produced from fermentation

VFAs = Volatile fatty acids

ATP = Adenosine triphosphate

A1 = Giraffe + Kudu

A2 = Giraffe + Impala

A3 = Kudu + Impala

A4 = Giraffe + Kudu + Impala

N1 = Goat + Impala

N2 = Goat + Kudu

N3 = Goat + Giraffe + Kudu

N4 = Goat + Giraffe + Kudu +Impala

Chapter 1

General Introduction

1. Background

Animals can be classified according to what they eat. This classification includes; carnivorous, omnivorous or herbivorous animals. All these animals face different challenges to satisfy their nutrient requirements (Shipley, 1999b). Omnivores consume both plants and other organisms (animals) whereas carnivores consume only animal matter. The most interesting are herbivorous animals that can survive on fibrous feed (Natsir, 2012a). Mammalian herbivores can be divided into two major groups according to their gastro-intestinal anatomy and ability to digest feedstuff (Godoy-Vitorino et al., 2012). These two groups are hindgut fermenters (post-gastric fermenters) and foregut fermenters (pre-gastric fermenters) (Godoy-Vitorino et al., 2012; Van Soest, 1994). Hindgut-fermenting herbivores are described as animals that digest plant material more rapidly in their hindgut (colon) whereas foregut-fermenting animals can digest feed for longer periods in their rumen (Hume, 2002).

The feedstuff that animals feed on can be divided into roughages, trees and shrubs, conserved forages and fodder crops as well as crop residues (Ndikumana and Zziwa, 2013). The chemical composition of these forages is much more important to meet an animal's nutritional requirements. The chemical composition of forages usually consists of water, ether extracts (lipids and fats), crude fibre (cellulose and some lignin), nitrogen-free extracts (sugars, starch and some of the hemicellulose and lignin), crude protein (total nitrogen), ash (salt and mineral matter), ADF and NDF (Azim et al., 2011; Van Soest et al., 1991a). It is advantageous to determine the nutritional composition of feed especially the crude fibre, cell wall constituents and protein because they play a vital role in forage digestibility (Murphy and Colucci, 1999). Crude fibre content correlates positively with feed intake as well as the amount of energy animals obtain from a particular feed. Additionally, chemical constituents determine the nutritional quality of a feed.

Ruminant meet their nutritional requirements either by browsing or grazing on plant material (Niwińska, 2012). The most recognised domesticated and wild ruminants include cattle, sheep, goats, deer, antelopes and giraffes. These animals are typically classified based on their forage choice as either grazers or browsers (Redjadj et al., 2014). Browsers are usually those animals that rely on trees and shrubs for their nutrition whereas grazers base their diet on grass (Hummel et al., 2006). Furthermore, grasses are likely to have thinner cell walls than

browses and their cell wall content is highly digestible whereas browses have thicker cell walls consisting of slowly digestible plant fibres, such as cellulose and hemicellulose (Shipley, 1999a).

Giraffes (*Giraffa camelopardalis*), goats and impala are well-known browsing ruminants. Giraffes are the world's tallest ruminants usually found in South Africa browsing tall trees of the *Acacia* species. Their height helps them to browse on the upper leaves of the plant and therefore avoid competition with other animals. This is beneficial especially during the dry season when feed is scarce (Cameron and du Toit, 2007). Goats and impala belong to the small ruminant group and are active foragers that can utilise browse plants. These animals have also been reported to be efficient browsers which are highly selective feeders (Jakhesara et al., 2010). Goats and impala are classified as intermediate selective feeders or sometimes called mixed feeders while the giraffe is a browser (Dicko and Sikena, 1992; Raats, 1998). Moreover, diet selection and intake does not only depend on the available resources but also on the feeding behaviour of the animals (Dicko and Sikena, 1992).

Ruminants have four special stomach compartments (rumen, reticulum, omasum and abomasum) unlike non-ruminants (Parmar et al., 2015). Their compartments are designed in a way that they can digest different types of feed. The size of the chambers changes as the animal grows up. When forage is consumed by an animal, it is masticated, soaked with saliva and then swallowed. Saliva facilitates chewing and swallowing and above it all it contains potassium and sodium salts that aid in buffering the rumen (Parish et al., 2009). After salivation, the cud goes down into the fermentation vat (rumen) where it will be digested by the rumen microbial ecosystem (Umphrey and Staples, 1992). The rumen environment is complex and composed of microbes, gases and ruminal fluid (Niwińska, 2012). Therefore, the rumen microbial population is adopted to live at a constant temperature range between 36^0C to 40^0C and a pH of range of 6.8-7.0 (Tjakradidjaja, 2012).

Once the feed has been digested in the rumen, it flows to the reticulum where any unwanted particles (like stones) ingested with feed settle out. The fermented particles will go through to the third compartment (omasum) where nutrient absorption, especially volatile fatty acids, (supply animal with energy) takes place (Parmar et al., 2015) . After microbial fermentation, the partially fermented feed will then flow through to the abomasum where acid digestion takes place (Umphrey and Staples, 1992). Here the protein component of feed is digested by pepsin which is activated by hydrochloric acid from pepsinogen to pepsin. The partially digested

protein component and undigested portions flow through to the small intestine where protein digestion is completed prior to absorption (Nafikov and Beitz, 2007). Ruminal fermentation usually results in the degradation of plants' polysaccharides and proteins into useful products such as glucose, amino acids and volatile fatty acids (Niwińska, 2012). After absorption, these products are used by the host animal for their maintenance and production (Aluwong et al., 2013).

Microbial species that occupy the rumen include bacteria, ciliate protozoa and anaerobic fungi (Kamra, 2005). Bacteria is the largest population in the rumen followed by ciliate protozoa and fungi is the smallest (Tjakradidjaja, 2012). Both bacterial and fungal microorganisms serve a common function of degrading fibrous feed more efficiently (Sahu et al., 2004). Although these microbial ecosystems are said to be efficient in harvesting the energy required for production, the amount of browse or graze that are lost as faeces is still more than 50%. For browses many other factors influence their digestibility especially anti-nutrition compounds such as tannin and lignin. The ability of microbial ecosystems to ferment or degrade browses can be estimated using biological methods such as *in vitro* fermentation systems (Mould et al., 2005). This is because the system is less expensive and less time consuming (Niwińska, 2012). Therefore, there is a need to study more about the degradability of fibrous plant material by rumen microbial population for potential applications as feed additives.

1.1 Objectives

The broad objective of this study was to identify potential rumen microbial ecosystems that have evolved with their ability to degrade or digest browses in the presence of tannin and also to investigate the effect of potential microbial ecosystems with relatively high tannin-tolerance from wild herbivores on goat rumen fibrolytic activity and browse fermentation.

1.1.1 Unveil potential fibrolytic microbial ecosystems from giraffe, kudu, impala and consortia.

1.1.2 Monitor the effect of tannin on the fibrolytic potential of microbial ecosystems from goat, wild giraffe, kudu, impala and consortia.

1.1.3 Assess the effect of inocula from impala, kudu and giraffe on goat *in vitro* fermentation, cellulase and hemicellulase activities.

1.1.4 Determine the effect of polyethylene glycol (PEG 4000) on goat microbial consortia degradability of tanniferous feeds

1.2 Hypothesis

To test the above objectives, we hypothesized that:

- Fibrolytic potential of microbial ecosystems from wild herbivores (impala, giraffe, and kudu) will not differ from that of domesticated goat.
- Increased tannin percentages will decrease browse microbial fibrolytic activities.
- *In vitro* inoculation of goat microbial ecosystems with inoculum from impala, kudu and giraffe will not have an effect on rumen fermentation and fibrolytic activity.
- Inoculation of goat microbial ecosystem with inocula from wild herbivores treated with PEG could improve feed degradability by domestic goats.

Chapter 2

Literature Review

2.1 Rumen microflora

Ruminants have an advantage over non-ruminant because their rumen is well filled with a wide range of symbiotic microorganisms. These microorganism are responsible for the breakdown of indigestible roughages under anaerobic conditions (Gruninger et al., 2014). Rumen microbes require an open environment for effective fermentation patterns. The section will review the types of microbes found in the rumen and the factors which affect the rumen environment for efficient microbial growth and functioning. Rumen microbial populations consist of three main groups- bacteria, protozoa and fungi (Wang and McAllister, 2002). These rumen microbial ecosystems convert feed into short chain fatty acids and microbial biomass that serve the animals' main sources of energy and protein, respectively (Weimer, 1998). Table 2.1 shows the summary of the types, concentrations and function of rumen microorganisms.

Table 2.1 Summary of the types, concentration and function of rumen microorganisms.

Microorganism	Concentration	Function	Reference
Bacteria	10^{10}-10^{11} cells/ml	Ferment a variety of substrate and reproduce	(Shinkai et al., 2010)
Protozoa	10^{5}-10^{6} cells/ml	Ferment substrate and engulf bacteria and reproduce	(Kamra, 2005)
Fungi	10^{3}-10^{5} zoospores/ml	Source of cellulolytic enzymes	(Gruninger et al., 2014)

2.1.2 Bacteria

A number of bacterial species originate from the rumen and about 10^{10}-10^{11} bacteria per ml of rumen fluid have been estimated (Shinkai et al., 2010). Bacteria dominate rumen environments because of their large population density. These bacteria have generally been classified according to the substrates which they digest (cellulolytic, amalolytic and proteolytic bacteria). Bacteria that ferment cellulose are described as the most important among others. Furthermore cellulolytic and amylolytic bacteria require ammonia and branched chain fatty acids as growth factors (Zhang et al., 2013). Among rumen bacteria, Gram-negative *Fibrobacter succinogenes*, and two species of Gram-positive bacteria, *Ruminococcus albus* and *Ruminoccocus flavefaciens* have highest cellulolytic activity in the rumen (Wina et al., 2006).

However, the number of Gram-positive bacteria has been found to increase with an increase in high energy diets (Kamra, 2005). Table 2.2 shows the rumen bacteria with energy sources and fermentation end products.

Table 2.2 Rumen bacteria with energy sources and fermentation end products

Bacteria species	***Description***	***Typical energy source***	***Typical fermentation end products (excluding gas)***						***Alternative energy source***
			A	*P*	*B*	*L*	*S*	*F*	
Fibrobacter succinogenes	Gram-negative rods	Cellulose	+				+	+	Glucose (starch)
Ruminococcus flavefaciens	Gram-positive, Catalase-negative Streptococci with yellow colonies	Cellulose	+			+	+	+	Xylan
Ruminococcus albus	Gram-positive, singled or paired cocci, capsulated	Cellobiose	+					+	Xylan
Streptococcus bovis	Gram-positive, short changes of cocci, capsulated	Starch				+			Glucose
Prevotella ruminocola	Gram negative, oval or rod	Glucose	+				+	+	Xylan, starch
Megasphaera elsdenii	Gram-negative, large cocci, paired or in chains	Lactate	+	+	+				Glucose, glycerol
Lachnospira multipara	Gram-positive, curved or rod	Pectins	+			+			Glucose, fructose

A = Acetic acid, P = Propionic acid, B = Butyric acid, L = Lactic Acid, S = Succinic acid, F = Formic acid. Source: (McDonald, 2002)

2.1.2 Rumen protozoa and anaerobic fungi

Protozoa population in the rumen is about 10^5-10^6 of rumen content and is relatively influenced by feeding practices (Kamra, 2005). They are generally found in higher quantities when highly digestible feed (concentrate) is fed. They ingest bacteria as a source of protein. On the other hand, anaerobic fungi population is the smallest and contributes about 8 % of the

total microbial biomass (Varga and Kolver, 1997). They degrade polysaccharides (cellulose, xylan) but their contribution to fibre degradation might be low due to their small microbial population (Gruninger et al., 2014). All the above mentioned microbial population play a significant role in feed degradability in herbivores. Furthermore, a continual supply of substrate, and salivary buffering salts and the removal of end products and residues will result in a relatively stable rumen environment, thus promoting high microbial populations and increased biomass (Moran, 2005).

2.2 Importance of browse forage in ruminant nutrition

Livestock plays an important role in the lives of many people in developing countries. They provide a source of income, meat and milk which are considered first class proteins (Hassan et al., 2007). Nutrition is the most limiting factor in ruminant production in developing countries (Simbaya, 2002). Insufficiency of nutrients particularly protein is generally caused by a lack of protein rich forages (Azim et al., 2011). This is because ruminants survive on natural pastures and crop residues that are low in nutrients (Odenyo et al., 1999). Therefore improving feeding and maximizing the use of the available feed resources should be considered when researching the improvement of small ruminant production systems (Nampanzira et al., 2015).

Browse species have been recommended as a central source of energy and protein supplement especially during the dry season when forage is limited (Dynes and Schlink, 2002; Rubanza et al., 2003). These fodders have nutritional importance for ranging herbivores in extensive, communal systems (Aganga and Tshwenyane, 2003). In addition livestock, particularly goats, rely on browse species for their nutrition in dry seasons (Nampanzira et al., 2015). Therefore, studying the use of browse species as an alternative feed supplement is necessary to understand their positive and negative effects on ruminant nutrition.

Acacia species have played a significant role as browse species in feeding domestic animals especially during periods of shortages or drought in most developing countries (Abdalla et al., 2014). They have been documented as the main forage supplement when both quality and quantity of pasture is limited (Al-Soqeer, 2008). This is because they can remain green in the dry seasons due to their deep roots that allow them to extract water and minerals from the soil (Gebeyew et al., 2015). *Acacia species* have a high proportion of cell wall content but this depends on plant parts, stage of growth and season. Basha (2012) showed that the

chemical composition of browses had high fibre (NDF, ADF and ADL) fractions during the dry season compared to the early wet season. Tolera et al. (1997) also reported that DM increased with the stage of maturity while CP reduced with age of maturity. Therefore, maturity affects the crude fibre (CF) content of the plant. Basha (2012) again reported that crude protein (CP) is high during the wet season and lowest during the dry season. The cell wall of browse plants primarily consists of structural carbohydrates (cellulose, hemicellulose and lignin) and relatively high proteins compared to grasses (Horn et al., 2012). These compounds are the most important sources of energy for browsing ruminants as they aid in animals' growth and production (Fon, 2006).

2.3 Degradation of plant cell wall polysaccharides

In ruminant nutrition, plant cell wall polysaccharides represent the largest constituent of the diet and are important for meeting animals' energy requirements while maintaining rumen health. Plant cell walls are the most organic compound found in nature and they are composed of sugars (arabinose, xylose, mannose, galactose and glucose) and arranged as polysaccharides (Wang and McAllister, 2002). These polysaccharides are basically grouped as cellulose, hemicellulose and pectin (de Souza, 2013). Cellulose is the main component of all plant material and the most common carbohydrate on earth (Sukumaran et al., 2005). It is composed of two or more glucose molecules linked by β-1, 4 glycosidic bonds (Roman, 2005). Cellulose is said to be of two general forms in nature; crystalline cellulose and amorphous cellulose (Sukumaran et al., 2005). Crystalline cellulose creates the main cellulose segment while in low amounts is in an amorphous form (Palonen, 2004). These forms of cellulose are susceptible to enzymatic hydrolysis. Hemicelluloses are composed of different sugar units (de Souza, 2013). Xylan is the most common hemicellulose polymer of grass and woody plants and is composed of β-1, 4-linked xylose residues (Zahedifar, 1996). Rumen microbial ecosystems ferment dietary carbohydrates and produce energy in the form of adenosine triphosphate (ATP) which is beneficial to microbes and by-products that are important to the host animal (Fellner, 2009). The major fermentation end product includes volatile fatty acids (acetic, butyric and propionic acids), ammonia, carbon dioxide and methane (Van Soest, 1994). These end products, especially VFA, are the sources of nutrients to animals' gases (Fellner, 2009). Volatile fatty acids are absorbed into the blood stream through walls of the rumen (Van Soest, 1994), and they are the principal sources of energy to the host. Methane and CO_2 are often considered as waste since they are erucated into the atmosphere (Moran, 2005). Fermentation of feedstuffs depends on the quality and composition of feed (Moran, 2005). In

addition, plants also contain some other structural polymers such as waxes and proteins (Malherbe and Cloete, 2002).

2.4 Protein degradation in rumen

Plant protein is the primary source of protein in an animal's diet and its requirements is usually expressed in terms of crude protein (CP) which is calculated from the nitrogen content of feed. Crude protein contributes energy to ruminants indirectly as it provides amino acids for rumen microbial cell growth and animal protein. Therefore, the more protein acquired from forage, the lesser the supplementation required. The ingested protein is broken down into amino acids by rumen microbial ecosystems through the secretion of proteolytic enzymes (Osuga et al., 2005). The efficiency of protein breakdown by rumen microbes is highly dependent on a balance of proteolysis which liberates peptides and amino acids that can be absorbed in the gastro-intestinal tract (Brooks, 2010). These metabolites are used by different bacteria to synthesize microbial proteins that are the main source of proteins in ruminants (Yisehak et al., 2014).

Dietary protein can be divided into ruminal degraded protein (RDP) and ruminal bypass protein (RBP) (Block, 2006). Ruminal degraded protein (RDP) is protein that is broken down in the rumen by rumen microorganisms whereas RBP escape the rumen to the small intestine for enzyme digestion and potential absorption (Bohnert et al., 2002). When RDP is more than required by ruminal microorganisms, the protein is converted into ammonia N, absorbed through the rumen walls, metabolised to urea in the liver and lost in the urine as uric acid (Bach et al., 2005). When ammonia supply in the rumen is deficient, the ammonia in blood then becomes a significant source of nitrogen for growth of rumen bacteria (Bohnert et al., 2002). Microbial proteins are synthesized in the rumen by rumen microbes. These microbes utilize ammonia, amino acids and peptides to synthesize microbial protein (Bach et al., 2005). However, the optimum utilization of proteins and carbohydrates by ruminants can be restricted due to plant defence mechanisms.

2.5 Limitations of browse forages as a source of feed in ruminants

Ruminants are well adapted to degrade plants' cell walls through their symbiotic relationship with rumen microbes. However, their rate of digestion is mainly influenced by coarseness of plant fibre. The nutritive value of feed can also be defined by the animal's ability to ingest and digest the feed. Therefore, factors that limit feed digestibility are negatively correlated with nutrient intake. Lignin is a major component of the cell wall that limits the

digestion of plant matter. Therefore, lignin concentration, composition, and structure all play a critical role in rumen digestion (Cesarino et al., 2012). Among plant cell walls, lignin has a major influence on feed digestibility. High levels of lignin limit microbial penetration through the plant cell, thus reducing nutrient intake (Sanon et al., 2008). Lignin concentration in browses increases with plant maturity (Cesarino et al., 2012) which is an important factor to consider during forage harvesting.

Plants have developed direct and indirect defence mechanisms to reduce herbivory (War et al., 2012). Direct defence mechanisms include structural barriers and toxic chemicals (Rasmann and Agrawal, 2009) whereas indirect barriers include volatiles. Structural barriers may include spinescenes, trichomes and sclerophyll while toxic chemicals include alkaloids, saponin and phenolic compounds (War et al., 2012). Alkaloids affect the mammalian nervous system and reduce appetite (Fürstenberg-Hägg et al., 2013) while phenolics serve as a defence compound. These phenolic compounds reduce palatability as well as nutrient digestibility in herbivores (Hanley et al., 2007).

2.5.1 Phenolic compounds

Phenolic compounds are the most abundant secondary metabolites of plants (Özeker, 1999). They are commonly found in dicot plants (Frutos et al., 2004) and they are generally involved in defence mechanisms against plants herbivory. Plant phenolic compounds are mostly synthesized through shikimic acid pathways (Özeker, 1999). The most common phenolic compounds on earth includes phenolic acids, flavonoids and tannins (Dai and Mumper, 2010). All these compounds protect plants against herbivory because they contain anti-nutritional factors such as strong odours (to deter browsing) and acute toxins which can result in the death of animals (Schardl, 2001). Among phenolic compounds, tannins have the most anti-nutritive effects on ruminant nutrition (Frutos et al., 2004). Therefore a closer look at tannins will assist in understanding their properties and management to herbivore advantage.

2.5.1.1 Tannins

The addition of concentrates in ruminant diets is aimed at increasing dietary energy, proteins, minerals and vitamins to improve forage utilization and animal performance (Olafadehan et al., 2014). However, in developing countries finance is a critical issue forcing them to use traditional plants as supplements which might not provide the necessary requirements of the animal (Iqbal et al., 2011). Browse plants are important local and cheap sources of forages for both domesticated livestock and game animals (Yisehak et al., 2014). Browse shrub and trees can provide a cheap and available crude protein and mineral

supplement for animals (Rubanza et al., 2006). However, their utilization can greatly be affected by the presence of anti-nutritional factors such as tannins (Aganga and Tshwenyane, 2003). Ozkose et al. (2011) defined tannins as water-soluble polyphenolic compounds that can be found in larger quantities in plant tissue. The name tannins was derived from the French word tan meaning the bark of a holm oak and other trees used for tanning (Frutos et al., 2004). In tanning, tannins are generally used for treating skin and hides making them more durable and less susceptible to decomposition (Bunglavan and Dutta, 2013; Waghorn, 2008).

Tannins are normally found in trees, shrubs and herbaceous legume plants. Tannins are mostly found in abundance in plant parts that are more likely to be eaten by herbivores e.g. new leaves and flowers (Frutos et al., 2004), hence protecting the plant from herbivory attack (Bunglavan and Dutta, 2013). They are usually found in higher concentration in *Acacia species* (McSweeney et al., 2001). Environmental and seasonal factors have an effect on the concentration of tannins in plants (Bunglavan and Dutta, 2013). Frutos et al. (2004) reported that tannin content in plants is influenced by environmental and seasonal factors such as high temperatures, water stress, extreme light intensities and poor soil quality. Waghorn (2008) also elucidates that tannin concentration increases as the plant matures. There are two types of tannins known as hydrolizable and condense tannins. Hydrolysable tannin is a molecule containing carbohydrate (D-glucose) as a central core (Ashok and Upadhyaya, 2012). They are generally composed of gallic acid or ellagic acids (Dai and Mumper, 2010). Hydrolysable tannins can be degraded by the microorganisms of the gastro-intestinal tract to a variable extent depending on the species of microbes. These microbes secrete a tannin degrading enzyme called tannase (Lal and Gardner, 2012). Tannase is an extracellular hydrolase capable of hydrolysing ester bonds between phenolic and saccharide molecules, thus liberating glucose and gallic acid as final end products (Kannan et al., 2011). Bacteria capable of degrading or tolerating tannins have been isolated from the alimentary tracts of several animals, for example; koalas (*Phascolarctos cinereus*), goats (*Capra hircus*) and horses (*Equus caballus*) as cited by Mosleh et al. (2014). According to Barman and Rai (2008), phloroglucinol, gallic acid, resorcinol and catechin were identified as degradation products of *Acacia* tannins in goat rumen fluid. On the other hand, condensed tannins are not degraded by rumen microbes but rather develop a tolerant mechanism towards this anti-nutritive compound and they are studied mainly because of their known anti-nutritional effects on both ruminants and monogastrics (Littlefield et al., 2011).

2.5.1.2 Condensed tannins and ruminants

In animal production, nutrition is one of the most important factors which influence productive performance (Lamy et al., 2011). Fodder trees and shrubs play a significant role in feeding domestic animals (Abdalla et al., 2014). However, their utilization is mostly restricted by the presence of dietary condensed tannins. Condensed tannins (CTs) are oligomers and polymers of flavonoid units, linked by carbon-carbon bonds which are more resistant to hydrolysis (McMahon et al., 2000). They are found on leaves of different browse and shrub legumes and their concentration differs with plant maturity (Min and Hart, 2003). Condensed tannins have both beneficial and harmful effects in ruminants (Lamy et al., 2011). Their effects are usually based on feed digestibility and animal production but the amount consumed is critical (Schofield et al., 2001). Condensed tannins exert their beneficial effects when consumed at moderate concentration due to their ability to prevent microbial degradation of dietary protein (Norton, 2000). They can also prevent bloat on grazing ruminants (Frutos et al., 2004). It has further been suggested as an alternative treatment for gastro-intestinal nematodes in small ruminants (Oliveira et al., 2011). This is because they inhibit the different developmental stages of *Haemonchus contortus* in sheep (Pathak et al., 2013). Min et al. (2005) reported that condensed tannin containing forages can reduce worm egg production and inhibit larval development. Athanasiadou et al. (2000) also showed that condensed tannins from Quebracho extract were responsible for the reduction of parasitic burden in infected sheep.

Ruminant agriculture contributes to greenhouse gas (GHG) emissions through methane (CH4) arising from enteric fermentation, and methane (CH_4) and nitrous oxide (N_2O) from animals waste (Grainger et al., 2009). Methane emission from enteric fermentation has been identified as an environmental concern based on its contribution to global warming. Apart from its association with environmental problems, it also represents a significant amount of energy loss from the animals (Jayanegara et al., 2015). However, CT can reduce enteric gas production with an increase in animal performance (Grainger et al., 2009). Although tanniferous feed prevent bloat, decrease methane and gastro-intestinal nematodes, their effect on ruminant production requires further investigation.

Ruminant production on forages is a function of intake, which is typically limited by rumen fill and fibre digestion (MacAdam and Villalba, 2015). This is probably due to the negative effects of tannins on ruminant nutrition. Condensed tannin exert their harmful effects when consumed at a higher concentration where they reduce voluntary feed intake as well as nutrient digestibility (particularly protein) (Lamy et al., 2011) and result in reduced weight

gain. Getachew et al. (2001) reported that high levels of condensed tannin in the *Acacia species* depress digestibility. Selection and intake of forages depends not only on the availability of feed but also on the feeding behaviour of animals. Animal feed selection is highly dependent on the palatability of the feed (Raats, 1998). However, condensed tannins are usually associated with a decrease in palatability and consequently discourage browsing (Lamy et al., 2011). Condensed tannins have been reported to reduce voluntary feed intake (Frutos et al., 2004). They have a bitter taste which reduces the palatability of forages to animals and, therefore, results in a reduced intake of feed and dry matter digestibility.

The bitter taste is often caused by the binding of salivary protein and taste bud proteins with the phenolic compounds of tannins. Thus, a reduction in animal weight gain is imminent with a decrease in palatability and intake (Norton, 2000). Souri et al. (2015) reported that the low intake of tannin rich feed is attributed to their astringent taste. Dludla (2010) revealed that goats treated with low condensed tannin concentration gained more body weight compared to those treated with high condensed tannin. Frutos et al. (2004) also reported that the consumption of plant species with high CT contents (generally > 50 g kg-1 of dry matter, DM) significantly reduces voluntary feed intake, while medium or low consumption (< 50 g kg-1 DM) seems not to affect it.

2.5.1.3 Effect of tannins on dietary proteins and plant cell wall polysaccharides

In terms of nutrient digestibility, condensed tannins have been linked with their strong affinity to bind proteins and other polymers such as cellulose and hemicellulose (McSweeney et al., 2001) inhibiting their availability, function or digestibility. Tannins bind with dietary proteins, salivary proteins, endogenous enzymes and gut microbes (Bohnert et al., 2002). The ability of condensed tannin to form complexes with dietary protein depends on the characteristics of both protein and tannin (Marais, 2012). High concentrations of tannin reduce ruminal protein degradation. When forming complexes with proteins, condensed tannins increase post ruminal nitrogen flow (Frutos et al., 2004), thus reducing nitrogen availability in rumen microflora since enzymatic hydrolysis of protein has been inhibited (McSweeney et al., 2001). Krebs et al. (2007) revealed that high faecal nitrogen indicates strong condensed tannin activity in dietary nitrogen (N) being excreted in the faeces as a result of tannin-protein complexes. Most *in vitro* studies indicate that crude protein digestibility CPD) decreased as the CT level increased. This suggests that CT may have a high affinity to bind with proteins other than those of organic components in the diet, particularly fibre fractions (Jayanegara and Palupi, 2011). Barman and Rai (2008) reported that the digestibility of dry matter, organic

matter and crude protein decreases with an increasing amount of tannins in feed but crude protein was greatly affected over other constituents. This was also confirmed by Jayanegara and Palupi (2011) when *in vitro* nutrient digestibility decreased with increasing levels of tannins. Condensed tannins form complexes with carbohydrates usually with a lower affinity than for protein (McMahon et al., 2000). Yisehak et al. (2014) reported that the lower dry matter digestibility by bulls fed on condensed tannin (CT) rich diet might be due to higher CT content in diets. Hervás et al. (2000) found that tannic acid reduced ruminal degradation of a soya bean meal.

Yisehak et al. (2014) reported that tannins in tree leaves are present in significant amounts in the neutral detergent fibre (NDF) and acid detergent fibre (ADF) fraction. Binding tannins to carbohydrates can also decrease ruminal gas production. It has been reported that a low correlation coefficient between gas production and dry matter disappearance may be due to the interference of tannins in leaves (Kamalak et al., 2005). Furthermore, tannin-complexing with enzymes which are also protein in nature exerts a huge negative impact on rumen fibre fermentation (McSweeney et al., 2001). Microbial inhibition by tannins may reduce the potential ability of enzymes to degrade lignocellulose hence reducing fibre utilisation by ruminants (McSweeney et al., 2001). To continue to utilise plants as food resources, herbivores that depend on plants must develop resistance and tolerance to tannins (Schardl, 2001). Tolerance may arise from adaptability and a symbiotic relationship with microbes inhabiting their rumen.

2.6 Ruminant adaptability to tannins

2.6.1 Salivary proteins

Most ruminants contain tannin-rich binding salivary proteins to counter the effect of tannin rich feeds. Among ruminants, such proteins have been demonstrated in the saliva of several browsers and intermediate feeders (Yisehak et al., 2011). These saliva are said to be either proline-rich or histidine-rich (Yisehak et al., 2012). Table 2.3 shows the Anti-nutritional effects of tannins in shrub and tree forages

Table 2.3 Anti-nutritional effects of tannins in shrub and tree forages

Fodder Tree/Shrub	Predominant Tannin	Animal	Nutritional Effect
Acacia aneura	CT	Sheep	Reduction in N digestibility decreased wool yield and growth, decreased S absorption
A. cyanophylla	CT	Sheep	Reduced feed intake, negative N digestibility, loss in weight
***A. nilotica* (pods)**	CT	Sheep	Low growth rate, reduced N and NDF digestibility
***A. sieberiana* (pods)**	HT	Sheep	Low growth rate, reduced N and NDF digestibility
Albizia chinensis	CT	Goat	Reduced *in sacco* N digestibility
Leucaena leucocephala	CT	Poultry	Poor N retention, low apparent metabolisable energy value
Manihot esculenta	CT	*In vitro*	Inhibits digestibility
Prosopis cineraria	CT	Sheep	Reduction in feed intake protein, digestibility, decreased wool yield & growth, decreased iron absorption
Robinia pseudoacacia	CT	Rat	Reduced protein digestibility
		Rabbit	Reduced feed intake & growth, cecotrophy increased protein digestibility
Terminalia oblongata	HT	Sheep	Reduction in feed intake, toxicity but no effect upon digestibility
Ziziphus nummularia	CT	Sheep	Reduction in feed intake protein and DM digestibility; decreased wool yield and weight loss

CT: Condensed tannins; HT: Hydrolysable tannins

Source: (Kumar, 2003)

2.6.2 Microbial degradation of plant cell wall polysaccharide

Utilisation of poor quality feed can be improved by the microbial population inhibiting the rumen (Paul et al., 2004). The presence of these microbes in the rumen can be influenced by the composition of feed and pH. Most nutrition researchers believed that the symbiotic relationship between microbes and animals can shape the world (Karasov and Carey, 2010) by improving fibre utilisation of different forages. Rumen microbial ecosystems are considered to

be more important because they are capable of producing enzymes that degrade plant fibre (Paul et al., 2004). Rumen micro-organisms work together to degrade ingested feed and most often produce useful end products that may increase the productivity of ruminants (Fon, 2012). Mammalian herbivores do not secrete cellulolytic or hemi-cellulolytic enzymes but rather depend on the synergetic relationship with rumen microbes in order to degrade fibrous feed (Gruninger et al., 2014). These microbes secrete carbohydrate-active enzymes (cellulases and hemicellulase) which assist in rumen fermentation (de Souza, 2013). These microbial species have been found to differ between cow and calf in terms of species, population and fibrolytic potential (Canbolat et al., 2005). McSweeney et al. (2001) also elucidated the variation of microbial species and their fibrolytic potential within animal species grazing or browsing in the same field.

2.6.1.1 Fibrolytic enzymes in the rumen

Enzymes are defined as compounds that assist chemical reactions by increasing the rate of reaction at which they occur (Seiboth et al., 2011). Enzymes are protein in nature but not all proteins are enzymes. This means that enzymes are proteins found in all living things. They are the biological catalyst that catalyses all metabolic processes within a cell. The compounds that enzymes act upon are known as substrates (Seiboth et al., 2011). The substrate can bind to a specific place in the enzyme called the active site (Matthews, 1993), and after binding the substrate is changed to a product while the enzyme remains unchanged. Therefore, Microbial ecosystems (bacteria, fungi and protozoa) are considered to be important because they produce the enzymes that degrade plant fibre (Paul et al., 2004). Efficient degradation of cellulose requires a combination of different cellulase enzymes (Toth, 2014).

Cellulose is the most abundant component of plant cell walls. Ruminal cellulolytic microorganisms play a central role in the nutrition of ruminant animals fed forages (Kumar and Sirohi, 2013). The degradation of cellulose in the rumen is made possible by the production of fibrolytic enzymes. Bacteria *Fibrobacter succinogenes* and *Ruminococcus flavafaciens* were found as the predominant bacteria in digesting fibre (Shinkai et al., 2010). Cellulose degrading enzymes are traditionally divided into two classes which include endoglucanases and cellobiohydrolases (exocellulase). These enzymes act together on cellulosic substrate where they hydrolyse the B-1, 4 cellulose linkages (Wilson, 2011), and produce their end product. Endocellulase can hydrolyse the internal bonds of amorphous cellulose whereas cellobiohydrolases act on the terminal ends produced by endogluconases (Lee et al., 2002).

Both enzymes can degrade amorphous cellulose but exocellulases (most important enzyme) are the only enzyme that efficiently degrades crystalline cellulose (Pérez et al., 2002).

In addition, both enzymes (cellobiohydrolases and endoglucanases) degrade cellulose to cellobiose which is then subjected to hydrolysis by another enzyme called cellobiase. It hydrolyses cellobiose to soluble sugars (glucose) as the end product. Paul et al. (2004) indicated that fungal isolates have the highest stimulating effect on apparent degradability, true degradability and neutral detergent fibre digestibility. These enzymes are often assayed *in vitro* specific to their substrates. Carboxymethyl cellulose (CMC) is a soluble form that is an excellent substrate for endocellulase, whereas exocellulase can be measured using crystalline cellulose as a substrate (Zhang and Zhang, 2013).

The biodegradation of hemicellulose requires xylanase enzymes. This is because xylan is the primary carbohydrate found on hemicellulose (Polizeli et al., 2005). Xylanase enzymes hydrolyse the B-1, 4 linkages of xylan (Collins et al., 2005). These enzymes act on the amorphous structure of xylan to liberate soluble sugar called xylose. Enzymes are assayed by measuring the rate at which they degrade specific substrates and the total reducing sugars produced (Collins et al., 2005). The end products (reducing sugars) of enzyme substrate reaction are mostly assayed using the Dinitrosalicylic acid (DNS) method (Miller, 1959). However, enzyme specific activities can be affected by different factors including pH, temperature and substrate concentration (Scopes, 2002). Cao and Tan (2002) reported that cellulase enzymes have an optimum range of pH 6.5-8 and temperature range of 39ºC. Since enzymes are proteins they can also be affected by the presence of tannins in substrate ingested by an animal. Therefore, this study is evaluating the ability of these enzymes to degrade fibrous feed in the presence of tannins without losing their fibrolytic potential.

2.7 Use of chemical binding agents to reduce the adverse effects of condensed tannins

Trees and shrubs are important fodder sources for both livestock and wild ungulates in tropical and dry environments. These browses are known to have a high concentration of condensed tannins. These dietary CTs affect the growth and body weight gain of animals as well as digestion and absorption of proteins, carbohydrates, lipids and minerals (Yisehak et al., 2014). Thus addition of a tannin-complexing agent, polyvinylpyrrolidone (PVP) and polyethylene glycol (PEG) to tannin-rich diets may improve the feeding value of browse foliage (Silanikove et al., 1996). Besharati and Taghizadeh (2011) illustrated that the addition of PEG and PVP inactivated effects of tannins and increased gas production, metabolisable energy, organic

matter digestibility and VFA in grape yield by products. Tolera et al. (1997) also reported *in vitro* digestibility depression with tannin but increased rumen fermentation and gas production with PEG supplementation. An improvement on nutrient digestibility and nitrogen retention after PEG addition was also observed by Mlambo et al. (2003). However Nsahlai et al. (2011) reported a reduction in microbial yield after PEG treatment. Therefore, addition of PEG and PVP could overcome adverse effects of tannins on nutrient availability. (Getachew et al., 2000) also confirmed that the addition of PEG increases ammonia-nitrogen concentration and net production of short chain fatty acids. Therefore, PEG application in tanniferous feed could be beneficial to animals by increasing their rate of fermentation. Furthermore, PEG with molecular masses (2000-3500) are the most tested chemicals on the improvement rumen digestibility of tannin rich feed (Getachew et al., 2000). Polyethylene glycol (PEG) is also reported to increase the activity of enzymes thus indicating that tannins depress the digestibility of feed in the rumen when not treated with PEG.

2.8 *In vitro* evaluation of feed for ruminants

2.8.1 *In vitro* digestibility of feed

Microbial effect on fermentation can be estimated using *in vitro* digestibility (Getachew et al., 2004). *In vitro* digestibility is an anaerobic fermentation method performed in the laboratory to simulate digestion in the rumen. It has been used for years because of its convenience and accuracy (Mould et al., 2005). *In vitro* digestibility has a high degree of correlation to *in vivo* digestibility although the technique is time consuming (Rymer et al., 2005). The *in vitro* technique is used to predict digestibility of ruminant feedstuffs since it mimics the rumen environment (Rymer et al., 2005). This system functions in the presence of rumen fluid as a source of microbial inoculum under anaerobic conditions (Mould et al., 2005). Rumen fluid can be collected from fistulated animals, slaughtered animals or through the oesophagus (Salanitro et al., 1977). The use of herbivore faeces has been recommended as a potential alternative source of microbial inoculum for *in vitro* digestibility (Rymer et al., 2005).

2.9 Conclusion

The review has shown that ruminant production decreases due to forage shortages especially in winter. Energy and protein were found to be the most limiting nutrient for both domestic and wild ruminants. Energy deficiencies were not entirely due to lack of forages but cellulose complexity, inability of rumen microbes to digest such complex forages since more that 50% is still wasted as faeces and the presence of anti-nutritive compounds especially in browse

species. Therefore, monitoring small ruminant microbial ecosystems in fibrous diets and browses can be useful for the maintenance of their population. However, direct *in vivo* measurement methods in the rural communities are difficult, scarce and expensive. Therefore, faecal sampling has been proposed as reported by Verheyden et al. (2011). Dietary condensed tannins cause a negative influence on animal performance and overall digestion. This is also a major problem in domesticated systems (especially in goat production) where animals browsed tanniferous feeds in winter. This tended to decrease dry matter intake, ruminal protein and dry matter digestion, ammonia concentrations and ruminal VFA concentration. Therefore looking for alternative microbes from wild browsers that might have evolved with their fibrolytic potential and tannin tolerance might be a solution to the high waste percentage (50%) observed in goats.

Chapter 3

Monitoring the fibrolytic potential of microbiomes from domestic and wild ruminants browsing tanniferous forages

Abstract

Although the rumen microbiome has been reported to synthesize a rich source of symbiotic enzymes (exocellulase, endocellulase, hemicellulase and cellobiase), the digestion of tropical C_4 grasses and browses by ruminants is still limited. Therefore, this study aimed to unveil potential fibrolytic microbial ecosystems from giraffe, kudu, impala and consortia (A1 (giraffe + kudu), A2 (giraffe + impala), A3 (kudu + impala), and A4 (giraffe + kudu + impala)) browsing tanniferous plants, which can be used to improve forage utilization in domesticated goat. Crude protein enzyme extracts (CPZ) from fresh faecal samples were precipitated by 60% ammonium sulphate and assayed for exocellulase, endocellulase and hemicellulase by incubating with crystalline cellulose, carboxymethyl cellulose and xylan at 38℃ with optimum pH of 5.5 to 6.5 for 1, 2, and 48 h, respectively. Enzyme specific activities were defined as μg of reducing sugar/mg CPZ. *In vitro* fermentation study was done by transferring 33 mL of fresh faecal inoculum into 67 mL of salivary buffer containing 1 g *Acacia sieberiana* and incubating for 72 h at 38℃. Apparent degradability (APD), true degradability (TD), neutral detergent fibre degradability (NDFdeg), acid detergent fibre degradability (ADFdeg), microbial yield (MY), metabolisable energy (ME) and total gas emitted (Gas) were measured. Exocellulase activities were higher (P <0.05) in all wild animals and consortia than in goat except for A4. Differences in hemicellulase activities (P <0.05) were observed among goat and wild animals and consortia, while endocellulase activity was generally higher (P <0.05) in goat than that of the rest of the systems. Apart from A3, TD, NDFdeg and ADFdeg were higher (P <0.05) in all microbial ecosystems from wild animals and consortia than those in goat. Apparent degradability, MY and ME also varied (P <0.05) among these systems. Giraffe, Kudu and A3 produced lower (P <0.05) gas than the goat system. This study showed that microbial ecosystems from wild browsers (especially impala) and consortia possess a higher potential to digest tanniferous forage with less enteric gas production compared to domesticated goat. Therefore, these microbiomes could be exploited as microbial feed additives for improving digestibility and reducing enteric gas production in domesticated goat.

Key words: Cellulase, Hemicellulase, Fermentation, Tanniferous forage, Gas production

3.1 Introduction

Ruminants derive a large quantity of nutritional requirements (energy) from the breakdown of plant cell wall polysaccharides (Natsir, 2012b). The primary constituent of plant cell walls is cellulose and it is also known to be the most abundant polysaccharide in nature (Bielecki et al., 2005). Often, these complex polysaccharides are bound to lignin, tannin and pectin rendering it inaccessible for digestion. Ruminants are considered as cellulose degrading animals but these complex polysaccharides are degraded by the symbiotic rumen microorganisms which they harbour (Wilson, 2008). These microorganisms include bacteria, protozoa and fungi (Salem et al., 2015; Santra and Karim, 2003). They inhabit mostly the rumen, caecum and sometimes the colon of some mammalian herbivores where they ferment forages into volatile fatty acids, methane and carbon dioxide. These microbes produce a collection of highly active plant cell-wall degrading enzymes such as cellulase, hemicellulase and cellobiase (Dashtban et al., 2010; Koike et al., 2003; Ozkose et al., 2011a).

Cellulases hydrolyse the β -1, 4 linkages into cellulose molecules and are very different from the majority as they degrade insoluble substrate (Wilson, 2011). They are produced as a multiple component enzyme system consisting of three enzymes which include endoglucanases, exoglucanases and β-glucosidases (Horn et al., 2012). During cellulose degradation, enzymes secreted by microbes diffuse through rumen liquor to substrate liberating free glucose molecules (Wilson, 2011). However, some enzyme complexes consisting of endogluconases, exogluconases and β-glucosidase may also be attached to the substrates and act symbiotically while liberating free glucose molecules (Wang and McAllister, 2002).

Endoglucanases are thought to be non-active against crystalline cellulose but they hydrolyse amorphous cellulose and soluble substrates such as carboxymethyl cellulose (Sona and Mukundan, 2004). This enzyme randomly cut β-glycosidic bonds of cellulose chains and yielded new end products (glucose and cellobiose) that are beneficial for both microorganisms and host animal (Ozkose et al., 2011a). Exoglucanases are also described as cellobiohydrolases, and play a significant role in hydrolysing crystalline cellulose to either oligosaccharides, cellobiose or glucose (Horn et al., 2012). The third enzyme β-glucosidase converts cellobiose (the main product of the endo- and exoglucanases mixture) to glucose but its activity on insoluble cellulose is insignificant (Linton and Greenaway, 2004).

Hemicellulose are considered as secondary factors affecting fibre hydrolysis (de Souza, 2013). Xylan is the most common hemicellulose component of grass and wood that contains up to 45% of the polysaccharide constituent of ruminant feed (Malherbe and Cloete, 2002). Xylan structure is composed of β-1, 4-linked xylose residues (Dunne, 2010). Effective degradation of xylan in the rumen also involves a mutual relationship of rumen microbial population (Wang and McAllister, 2002). These microorganisms have the capability to produce highly active fibrolytic enzymes (xylanase) that degrade xylan to xylose (Wang and McAllister, 2002). Xylanase catalyse β-linkages of xylan to produce xylose as energy substrates for rumen microbes releasing by-products that can be used by ruminants.

The ability of tannin complexing with cellulose, forage proteins and even microbial protein (enzymes and microbes' cell wall proteins) has been a major concerned as it decreases forage degradability and increases production cost. This is even more prominent in browsers as browses are relatively higher in tannin concentrations. These imply that the microbes are faced with a daunting task of first annihilating the effect of tannin before hydrolysing the fibre. Because of the huge diversity of tannin structures, their effects on rumen microbes will also vary widely (Gemeda and Hassen, 2015). If a rumen microbiome is as diverse as that of tannin, it implies that microbes will have to adapt and evolve accordingly with tannin type and concentrations in browses in a browsing niche. However, consuming tannin herbivores are also evolving in their ability to negate the effects of tannin by secreting tannin binding protein in saliva to decrease its effect on rumen microbial protein.

Enteric gas production (methane) has been shown to decrease or vary with tannin type and concentration in plant species (Gemeda and Hassen, 2015). There are also suggestions that some rumen microbes may have a higher ability of synthesizing tannin binding proteins. Therefore, the aim of this research was to investigate the potential of fibrolytic microbial ecosystems from wild browsers (giraffe, kudu, impala or their consortia (A1 (giraffe + kudu), A2 (giraffe + impala), A3 (kudu + impala), A4 (giraffe + kudu + impala), which can potentially be used to improve browse digestibility in domesticated goat. To achieve this objective, we hypothesized that fibrolytic potential of microbial ecosystems from wild herbivores (impala, giraffe, and kudu) will not differ from that of domesticated goat.

3.2 Materials and method

3.2.1 Faecal collection and inoculum preparation

Faecal inoculum was preferred in this study because it has been previously investigated as an alternative inoculum for rumen fluid and secondly rumen cannulated wild animals and domestic goat were not available and would be very expensive to cannulate and manage (Osuga et al., 2005). Faecal samples were collected fresh (within 5 min) after defecation from giraffe, kudu and impala browsing the thorn veldt at Tala Game Reserve (KwaZulu-Natal Province, South Africa) and a goat from KwaMthethwa village, Empangeni browsing the communal fields in winter. Faeces were transferred in a pre-warmed thermo flask that had been flushed with CO_2 (Getachew et al., 2004; Posada et al., 2012) and taken to the laboratory of the Department of Agriculture, University of Zululand. Inocula were prepared by mixing a 60g faecal sample with 250ml of warm salivary buffer solution (2L of warm Solution A ($NaHCO_4$ 19.60g, Na_2HPO_4 7.40g, KCl 1.14g and MgCl.6H2O 0.26g)) containing 2 mL of Solution B (5.3g CaCl. $2H_2O$ in 100 ml distilled water)) that was pre-warmed at 39℃ prior use before squeezing through four layers of cheese cloth to get filtrate (inoculum). Microbial consortia were prepared prior use as follows; A1 (giraffe + kudu, 1:1), A2 (giraffe + impala, 1:1), A3 (kudu + impala, 1:1) and A4 (giraffe + kudu + impala, 1:1:1).

3.2.2 Crude enzyme extraction

Protein extraction for enzyme analysis was done as described by Byrne et al. (1975) with quantities and volumes slightly modified by Fon et al. (2014b). Prior crude protein enzyme (CPZ) extraction, 200 ml of inoculum was treated with 1,500 µL of phyenylmethylsulfonyl fluoride (PMSF) (0.1 mM PMSF 1: 100 faecal fluid) to inhibit proteases from lysing enzymes of interest (Owolabi et al., 1988). Faecal fluid (100 ml) from each inoculum was used for enzyme extraction after cell disruption by sonication to release proteins. After sonication, the samples were centrifuged at 10 000 $\times g$ for 15min at 4℃ until a clear supernatant was obtained. Ammonium sulphate (60%) was dissolved in the supernatant before centrifuging at 7500 $\times g$ for 15min at 4℃ to precipitate proteins. After centrifugation, the supernatant was discarded and the remaining precipitate was dissolved in 7mL storage buffer (20 mM sodium acetate, 0.02% NaN_3 and 0.1 mM EDTA at pH 5.01) before dialyzing.

3.2.3 Dialysing and concentration of crude enzyme protein

After crude enzyme extraction, the samples were dialysed as described by Fon et al. (2014b) and Wright and Upadhyaya (1998). Dialysis membrane was cut into desired volume (7mL) and soaked into distilled water for a few minutes (to soften membrane for easy access) and filled with enzyme solution (7mL). The membrane was then immersed in a 2L homogenization buffer (50mM sodium acetate, 0.02% NaN_3 and 0.1 mM EDTA at pH 5.02) of two changes in for 24 h to completely remove the salt (NH_4SO_4). The dialysed solution was precipitated with polyethylene glycol 20 000 (PEG 20000). The 7mL membrane was completely covered with granules of PEG 20000 and constantly monitored for water loss until 2mLwhen it was stopped. The 2mL sample was then pipetted into 1mL of storage buffer and stored in the fridge for protein quantification and enzyme assays.

3.2.4 Crude protein enzyme quantification

Bradford dye binding assay (Bradford, 1976) was used to determine protein concentrations. Known concentrations were prepared with bovine serum albumin (ABS) (0.5 to 40µg BSA/500ul reaction buffer). A known concentration of BSA solution (500 µL) was pipetted into 1 mL eppendorf tube and then 500µL of Bradford reagent was added into each eppendorf and mixed by vortexing before placing in the dark for 5min. The absorbance at 595nm was measured after 5min in 1mL cuvette against reagent blank. The responses of the standard were used to plot a standard curve. The unknown protein samples were prepared in the same manner as the standard and their protein concentrations were determined from a standard plot of known concentrations. Each BSA concentration and experimental sample was replicated 5 times.

3.2.5 Enzyme assays

3.2.5.1 Endocellulase activity

Endocellulase activity was assayed following the method described by Fon et al. (2014a). Endocellulase was measured by pipetting 0.5 mLof 0.5% (m/v) in the reaction buffer (20 mM sodium acetate, 0.02% (m/v) NaN_3 and 0.1 mM EDTA at pH 5.0) into 0.5 ml of crude protein solution obtained from the faecal inoculum and incubated for 2 h at 38°C to determine endocellulase activity. The enzyme reaction was stopped by boiling at 100°C, and 400 µL of sample solution was analysed for reducing sugars using the Dinitrosalicylic (DNS) method. Each enzyme assay was replicated three times with three pseudo repeat for each run. Specific enzyme activity was measured in µg glucose/µg crude protein.

3.2.5.2 Exocellulase activity

Exocellulase activity was assayed as described by Fon et al. (2014a). Crystalline cellulase was measured by pipetting 0.5 ml of 0.5% (m/v) crystalline cellulose in the reaction buffer (20 mM sodium acetate, 0.02% (m/v) NaN_3 and 0.1 mM EDTA at pH 5.0) into 0.5 ml of crude protein solution obtained from the faecal inoculum and incubated for 72 h at 38°C to determine exocellulase activity. The enzyme reaction was stopped by boiling at 100°C, and 400 µL of sample solution was analysed for reducing sugars using the DNS method. Each enzyme assay was replicated three times with three pseudo repeats for each run. Specific enzyme activity was measured in µg glucose/µg crude protein.

3.2.5.3 Hemicellulase activity

Hemicellulase activity was assayed as described by Fon et al. (2014a). Hemicellulase was measured by pipetting 0.6 ml of 0.1% (m/v) xylan solution in the reaction buffer (20 mM sodium acetate, 0.02% (m/v) NaN_3 and 0.1 mM EDTA at pH 5.0) into 0.4 ml of crude protein solution and incubated at 38ºC for 2 h to determine hemicellulase activity. The enzyme reaction was stopped by boiling at 100°C, and 400 µL of sample solution was analysed for reducing sugars using the DNS method. Each enzyme assay was replicated three times with three pseudo repeats for each run. Specific enzyme activity was measured in µg xylose/µg crude protein.

3.2.6 Determination of reducing sugars by Dinitrosalicylic Method

The concentration of reducing sugar (glucose or xylose) released in the reaction was determined using either glucose or xylose standards as described by the Dinitrosalicylic (DNS) method (Miller, 1959). Glucose or xylose solution containing different sugar concentrations (1 to 150 µg and 5 to 40 µg, respectively) in a reaction buffer (20 mM sodium acetate, 0.02% (m/v) NaN_3 and 0.1 mM EDTA at pH 5.0) were used to prepare standard solutions. Dinitrosalicylic (DNS) reagent 600 µL was pipetted into 400 µL of standard solution, incubated in boiling water for 5 min and cooled on ice along with the blank. Absorbance versus the concentration of the standards were plotted to construct xylose and glucose standard curves. The same procedure was followed to determine enzyme specific activities which are the amount of reducing sugar (glucose or xylose) released from reaction. Dinitrosalicylic reagent (600 µL) was pipetted into 400µl of enzyme reaction solution and absorbance at 540 nm. The amount of reducing sugar released was calculated from the linear equation generated from glucose or xylose plots. The concentration of reducing sugar released was measured either as µg glucose/mg crude protein or µg xylose/mg crude protein.

3.2.7 Chemical analysis of *Acacia sieberiana*

Acacia sieberiana was used for *in vitro* degradability study. Dry matter (DM), crude protein (CP), neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL) and condensed tannin (CT) were analysed for *Acacia sieberiana*. Nitrogen (N) concentration was measured by the Kjeldahl method described by Basha (2012) using nitrogen analyser system. The chemical components (NDF, ADF and ADL), cellulose and hemicellulose were determined according to Van Soest et al. (1991b) principles using ANKOM fibre method. The acid-butanol proanthocyanidins assay was used to determine CT (Makkar and Goodchild, 1995).

3.2.8 *In vitro* degradability and gas production

Gas production was measured using the computerised pressure transducer system as described by Nsahlai et al. (2011). Approximately 1g of ground (pass through 1mm sieve) *Acacia sieberiana* dry matter was transferred into 250 ml Duran bottles containing 67ml of salivary buffer. Faecal inoculum (33 ml) was then added into the bottle while flushing with CO_2 to maintain anaerobiosis. The bottles were tightly closed with stoppers and placed in an incubator for 72hrs at 39°C with blanks lacking substrate only. After incubation, the contents of each incubation was centrifuged at 8000 x g for 15 min at 4ºC and dried for 72 hrs at 60°C. Apparent degradability (APD), true degradability (TD), neutral and acid detergent fibre degradability (NDFdeg and ADFdeg) and hemicellulose degradability (HEMdeg) were measured. Metabolisable energy (ME) was estimated from gas production and microbial yield following the method described by (Afshar et al., 2011). Metabolisable energy was calculated as follows:

ME (MJ/kg DM) = 2.20 + 0.136 + GP + 0.057 X MY

GP = Net gas production (ml/g)

MY = Microbial yield (g/kg DM)

3.2.9 Statistical analysis

A statistical Analysis System (SAS 9.3, 2013) was used to determine gas production from the regression equation described by Campos et al. (2004). The treatment effects for APD, TD, NDFdeg, ADFdeg, HEMdeg, microbial yield, and gas parameters obtained from the regression equation were evaluated using linear analysis of Variance (ANOVA).

3.3 Results

3.3.1 Chemical composition of *Acacia sieberiana*

The chemical compositions of *Acacia sieberiana* were successfully determined and are shown in Table 3.1.

Table 3.1 Chemical composition of *Acacia sieberiana*

Chemical composition	***Acacia sieberiana*, g/kg**
Dry matter	947±57.7
Crude protein	127±15.3
Neutral detergent fibre	658±26.9
Acid detergent fibre	515±28.8
Acid detergent lignin	393±38.9
Condensed tannin	68.7±4.3
Cellulose	122±54.9
Hemicellulose	142±38.9

3.3.2 Enzyme assays

Table 3.2 Hemicellulase, endocellulase and exocellulase specific activities of crude protein extracts

Animal	**Enzyme specific activities**		
	Hemicellulase ,µ xylose/mg	**Endocellulase ,µg glucose/mg**	**Exocellulase ,µg glucose/mg**
Goat	29.98^{c}	99.93^{a}	65.87^{d}
Giraffe	30.05^{c}	95.63^{c}	72.69^{bc}
Kudu	29.77^{d}	95.83^{c}	72.91^{bc}
Impala	30.88^{a}	93.10^{d}	80.23^{a}
A1	30.37^{b}	95.18^{c}	76.03^{ab}
A2	30.11^{c}	96.03^{c}	70.34^{bc}
A3	29.67^{d}	96.88^{b}	70.97^{bc}
A4	31.06^{a}	95.67^{c}	61.80^{d}
SEM	0.03	0.12	0.85
***P*-value**	0.05	0.05	0.05

A1 = giraffe + kudu, A2 = giraffe + impala, A3 = kudu + impala, A4 = giraffe + kudu + impala, SEM= standard error of the means, ^{a,b,c,d}Numbers in a column with different superscript letters are significantly different ($P < 0.05$).

Enzyme specific activities are shown in Table 3.2 and the results showed that enzyme activities varied (P <0.05) among microbial ecosystems. According to the results, exocellulase activities were higher (P <0.05) in all wild animals and consortia than those in goat except for A4. Differences in hemicellulase activities (P <0.05) were observed between goat and wild animals or consortia. Hemicellulase activity was highest in A4 followed by impala, A1, A2 and giraffe. Endocellulase activity was generally higher (P <0.05) in goat than that in the rest of the microbial ecosystems.

3.3.3 *In vitro* degradability.

Table 3.3 *In vitro* degradability of *Acacia sieberiana* from both domestic and wild herbivores and their consortia

Animal	APD, g/kg DM	TD, g/kg DM	NDFdeg, g/kg DM	ADFdeg, g/kg DM	HEMdeg ,%	MY, g/kg DM	ME, MJ/kg DM	Gas, mL/g DM
Goat	256.00^{b}	453.35^{c}	278.96^{c}	193.44^{b}	64.85^{bc}	197.35^{e}	26.40^{ab}	70.43^{a}
Giraffe	152.30^{cd}	483.29^{ab}	318.52^{ab}	240.36^{a}	65.59^{bc}	330.99^{b}	21.46^{b}	14.18^{c}
Kudu	243.65^{b}	499.14^{ab}	339.35^{ab}	249.39^{a}	72.83^{a}	255.49^{cd}	20.27^{b}	21.01^{c}
Impala	383.30^{a}	527.24^{a}	376.42^{a}	298.02^{a}	71.52^{a}	143.94^{f}	22.29^{b}	51.76^{ab}
A1	116.60^{d}	523.72^{a}	371.78^{ab}	302.67^{a}	67.04^{abc}	407.129^{a}	30.27^{a}	65.97^{ab}
A2	242.85^{b}	485.61^{ab}	321.52^{ab}	255.44^{a}	60.70^{bc}	242.76^{d}	24.00^{ab}	53.06^{ab}
A3	127.00^{d}	435.30^{c}	255.15^{ab}	178.6^{b}	58.59^{c}	308.30^{b}	23.61^{ab}	42.30^{b}
A4	201.45^{cb}	496.00^{b}	335.22^{ab}	259.42^{a}	66.27^{abc}	294.55^{cb}	26.70^{ab}	61.85^{ab}
SEM	8.38	5.41	6.90	6.90	17.70	7.22	9.44	2.96
***P*-value**	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05

A1 = giraffe + kudu, A2 = giraffe + impala, A3 = kudu + impala, A4 = giraffe + kudu + impala, SEM= standard error of the means, APD = apparent degradability, TD = true degradability, NDFdeg = neutral detergent fibre degradability, ADFdeg = acid detergent fibre degradability, HEMdeg = hemicellulose degradability (%), MY = Microbial yield, ME = Metabolizable energy, ^{a,b,c,d}Numbers in a column with different superscript letters are significantly different (P <0.05).

The results obtained from *in vitro* degradability of *Acacia sieberiana* showed that APD, TD, NDFdeg, ADFdeg, HEMdeg, MY, ME and total gas production varied (P<0.05) among the different microbial ecosystems (Table 3.3). Apparent degradability in impala was the highest among all the systems. True degradability and NDFdeg were highest in impala and A1,

moderate in A2, A4, giraffe and kudu and lowest in goat and A3. For HEMdeg, impala and kudu showed the highest activity. Interestingly, MY was the lowest in impala, followed by goat while the rest were relatively moderate with the highest observed in A1. The ME was the highest in impala while the rest of the microbial ecosystems seem to have similar quantities of ME. Gas production from goat was the highest ($P<0.05$), followed by A1, A4 and A2 while the rest of the microbial ecosystems showed relatively lower values compared to goat.

3.4 Discussion

Most microbes in the rumen can utilize the monomeric units of cellulose or hemicelluloses or their by-products after fermentation, but only a few of them can produce enzymes that have the potential to degrade these complex polysaccharides (Ozkose et al., 2011a). That is why to efficiently digest cellulose requires a combination of symbiotic hydrolytic enzymes (Pérez et al., 2002). Despite the complexity of these polysaccharides, tannin and lignin complexing with these polysaccharides render them inaccessible and difficult to degrade (Mlambo and Mapiye, 2015; Ndagurwa and Dube, 2013) especially in browse species with high levels. Many studies have demonstrated that tannins plays a huge part in influencing cellulose digestibility either by binding to cellulose, cellulases, rumen microbes or to rumen symbiotic enzymes (Horner et al., 1988; Ximenes et al., 2010).

Most goats in communal systems as well as in commercial farms suffer nutrient deficiency (often seen in poor body condition scores and weight loss) especially in winter (dry season), yet some of them browse the thorn veldt or are supplemented with browses. Often, more than 50% of forages browsed are eliminated through faeces. Therefore, this study looked at microbes from impala, goat and kudu which are browsing the thorn veldt at Tala Game Reserve hoping to find microbes that have not only evolved in their fibrolytic potential but also with the ability to resist and manage variable tannin conditions with minimal effect on fibrolytic enzymes.

The results obtained from crude enzyme extracts showed that domesticated goats microbial ecosystems can digest or ferment amorphous or soluble polysaccharides (endocellulase activity) as good as wild ruminants and their consortia. However, this was not true for exocellulase activities which are responsible for complex fibre degradation as it varied. The impala showed the highest potential in hydrolysing cellulose which was 20% greater than that of goat. This high activity was associated with microbes that have evolved with their fibrolytic potential influenced by diet type browsed in the fields (Pitta et al., 2014) as impala

was observed browsing *Lantana camara* during winter. *Lantana camara* at that time of the year appeared to be high in fibre, tannin and lignin as demonstrated by its dark blue-green colour. Both giraffe and kudu had a 10% increase in hemicellulose degradability compared with that of goat. Their activities were also influenced by their browsing habit. However, they were seen browsing leaves from tall acacia plants with a relatively lower content of cellulose, tannin and fibre favoured by their height. Although the statistical analysis of hemicellulase activity showed that it varied among the different microbial ecosystems, the difference was really small. The hemicellulolytic activity of impala and A1 microbial ecosystems were the highest at 3 and 1.2%, respectively, different from that of goat. The impala seemed to show greater efficiency in digesting both cellulose and hemicellulose which was a bit higher than observed in a previous study by Fon et al. (2014a). A similar study by Nagpal et al. (2011) also demonstrated a higher fibrolytic activity from wild deer browsing as well.

The observed chemical composition of *Acacia sieberiana* leaves used in this study was similar to that previously reported by Nsahlai et al. (2011) on *Acacia sieberiana.* For *in vitro* degradability studies, impala, giraffe, kudu and consortia showed greater potential in fermenting *Acacia sieberiana* than goat except for A3. Microbial ecosystems in impala and A1 showed the highest percentage difference in TD (16.3 and 15.2%), NDFdeg (34.9 and 33.2%) and ADFdeg (54.0 and 56.4%, respectively) when compared with goat. This result was consistent with the higher exocellulase activity observed from crude enzyme extracts for impala and A1. Therefore, we have reason to believe that impala may be harbouring microbes that might have evolved with their ability to ferment forages in the presence of tannins. Although TD (6.6 and 10.2%), NDFdeg (14.1 and 21.6%) and ADFdeg (24.2 and 28.9%, respectively) for giraffe and kudu were higher than those of goat, their consortia (A1) were even greater for TD (15.5%), NDFdeg (33.2%) and ADFdeg (56.4%).

The increase in A1 degradability was associated with a positive symbiotic microbial activity. Interestingly, impala with the highest ability to ferment *Acacia sieberiana* produced the least microbial yield as one of its products of fermentation (Rymer et al., 2005). It was suggested that the highest degradability values observed for impala were associated with microbial efficiency rather than population. It was logical to assume that high carbohydrate and protein degradation will provide both energy (ATP) and nitrogen for microbial growth (Pathak, 2008) but this was not the case. It was also very interesting to find out that goat produced more gas than microbial ecosystems from wild animals and consortia. This confirms

the adaptability of goat microbial ecosystems in digesting soluble polysaccharides (with high gas as a characteristic by-product) than high fibre forages.

High enteric gas production is not cost effective to farmers (energy loss and metabolic disorders like bloat) as well as to environmentalists where it is a major contributor to global warming especially in the form of methane. Therefore, giraffe, impala, kudu and consortia did not only harbour microbes with relatively higher fibrolytic potential but also had the potential to decrease the amount of energy lost as enteric gas (Singh et al., 2010). Other studies have shown that microbes respond differently to forages and different types of tannins (Gemeda and Hassen, 2015), therefore we cannot absolutely conclude that impala can replicate its efficiency in digesting forages with different types of phenolic compounds without investing its effect in the presence of different types of tannins. The hypothesis was rejected as microbial ecosystems from giraffe, impala, kudu and consortia showed a higher ability in digesting *Acacia sieberiana* than the domesticated goat microbial ecosystem.

3.5 Conclusion

The results from exocellulase activity and *in vitro* degradability of *Acacia sieberiana* showed that giraffe, impala, kudu and consortia can utilise browse forages better than the domesticated goat. Microbial ecosystems in impala and A1 showed the highest potential in utilising *Acacia sieberiana* as forage and could be used as a potential inoculum to improve the digestibility of tanniferous forages. The wild microbial ecosystems and their consortia produced less enteric gases than the domesticated goat. Therefore, an improved understanding of fibre degradability at a slightly high concentration of tannins by microbial ecosystems from both wild and domestic ruminants will provide an easy process to select species with a greater performance on tannin-rich browses.

Chapter 4

Effect of tannin on the fibrolytic potential of microbial ecosystems from domestic and wild browsers and consortia

Abstract

Trees and shrubs are important sources of forage (protein supplement when both quality and quantity of pastures are limited) for animals particularly during the long dry seasons of West Africa or winter in Southern Africa. However, their consumption is restricted by the presence, variation and complexity of phenolic compounds called tannins. Therefore, browsing different microbial ecosystems in search of potential microbes that have evolved in their ability to digest fibre and tannin tolerance can be important to improve browse digestion by domestic browsers. This study monitored the effect of tannin on the fibrolytic potential of microbial ecosystems from domestic goat, wild giraffe, kudu, impala and consortia (A1 (giraffe + kudu, 1:1), A2 (giraffe + impala, 1:1), A3 (kudu + impala, 1:1), and A4 (giraffe + kudu + impala, 1:1:1)). Fresh faecal samples were collected and 50g was mixed with a homogenisation buffer (50ml) for crude protein extraction. Crude protein enzyme extracts (CPZ) were precipitated with 60% ammonium sulphate and assayed for exocellulase, endocellulase and hemicellulase by incubating with crystalline cellulose, carboxymethyl cellulose and xylan at 38°C with an optimum pH of 5.5 to 6.5 for 1, 2, and 48 h, respectively. Each reaction mixture contained 100 µL of 10% tannin acid while the control had no tannin. Enzyme specific activities were defined as µg of reducing sugar/mg CPZ. An *in vitro* fermentation study was done by transferring 33 mL of fresh faecal inoculum into 67 mL of salivary buffer containing 1 g *Acacia sieberiana* and 10% tannin (substrate 6.2% was made up to 10% by adding 50 µL containing 3.8 mg tannic acid) before incubating for 72 h at 38°C. The control incubations had no tannic acid. Apparent degradability (APD), true degradability (TD), neutral detergent fibre degradability (NDFdeg), acid detergent fibre degradability (ADFdeg), hemicecullase degradability (HEMdeg), microbial yield (MY), metabolisable energy (ME) and total gas emitted (GAS) were measured. Endocellulase and hemicellulase activity were generally higher ($P < 0.05$) when incubated with tannin than no tannin with the highest activity observed in impala. Exocellulase activity increased ($P < 0.05$) in all systems when incubated with tannin but for goat that decreased. Impala and A1, showed the highest ($P < 0.05$) exocellulase activity. Apparent degradability, TD, NDFdeg and ADFdeg varied ($P < 0.05$) between microbial ecosystems

incubated with tannin and no tannin as well as among the tannin treatment groups. True degradability increased ($P<0.05$) in all systems when incubated with tannin than no tannin. Impala and A1 showed the highest TD, NDFdeg and ADFdeg. Apart from goat and A2, HEMdeg tended to increase upon incubation with tannin. Metabolisable energy decreased with tannin incubation. The highest decrease in GAS was observed in goat followed by A1, A4 and A3. However, GAS increased in impala, kudu, and giraffe. The wild herbivores especially IM and A1 consortia possess a higher fibrolytic potential under high tannin concentrations. It was also noticed that tannins play a major role in reducing enteric GAS production especially in microbial consortia which is a major environmental concern for global warming. Therefore, investigating these microbial ecosystems effect on domestic goat both *in vitro* and *in vivo* may improve browse utilisation and decrease enteric gas production.

Keywords: Tannin, metabolisable energy, enteric gas production, condensed tannin

4.1 Introduction

The world food crisis highlights the need for alternative fodder resources especially among rural farmers who have been experiencing a continuous decline in livestock productivity due to low forage availability (Yisehak et al., 2014). Therefore the use of local resources such as fodder trees and shrubs for animal nutrition is key especially during the long dry season in West Africa or long dry and cold winters in Southern Africa (Tshabalala et al., 2013). These browses are often preferred as protein supplements because they maintain their nutrients into the long and dry winter periods which is not very common with most pasture vegetation (limited quality and quantity) (Ng'ambi et al., 2011). However, these plants contain a significant amount of naturally occurring anti-nutritional factors known as plants' secondary metabolites. These plants' secondary metabolites are also known as tannins (Kamra, 2005; Ng'ambi et al., 2011) and are generally classified into two broad groups known as condensed and hydrolysable tannins (Gordon et al., 2002).

Anti-nutritional factors are defined as those constituents produced in natural feedstuffs by the normal metabolism of plants which affect animal feed breakdown and utilisation (Kumar, 1992). Tannins are generally produced as a defensive mechanism in forage trees, shrubs and legumes to deter insects and animals from its consumption (McSweeney et al., 2001). Condensed tannins reduce the nutritive value of browses and tree foliage (Salem et al., 2007). Similar effects have also been observed with tannic acids (Hervás et al., 2000) but are

said to be poisonous in ruminants and non-ruminants when excess amounts are present in feed (Ozkose et al., 2011b). The anti-nutritive effect of condensed tannins has been associated with reduced voluntary feed intake and nutrient digestibility (Frutos et al., 2004; Mlambo et al., 2008). Condensed tannins' adverse effects have also been observed in animals browsing legumes such as *Acacia* species as a significant part of their diet (Njida and Ikhimoya, 2012). *Acacia* species reduce feed digestibility and this has been strongly associated with condensed tannins (Barman and Rai, 2008).

Condensed tannin is harmful to most herbivores, but some animals (mostly ruminants) have developed a defence mechanism such as secreting a high amount of salivary proline-rich proteins to neutralise tannin effect in their gastro-intestinal tract (Ng'ambi et al., 2011; Ozkose et al., 2011b). Rumen microbes have been shown to secrete a thick glycocalyx as a tolerance mechanism to a relatively high amount of tannins. This tolerance also depends on the type of tannin as well as concentration which is likely to become toxic if too high. Condensed tannin can also influence the digestive process by complexing with secreted feed proteins, endogenous proteins, enzymes (proteolytic enzymes, cellulolytic enzymes and other rumen enzymes) and carbohydrates such hemicelluloses and celluloses (McSweeney et al., 2001; Nelson et al., 1995).

Many mechanisms have been suggested on how these secondary compounds affect forage intake, digestibility and absorption (Ng'ambi et al., 2011; Paul et al., 2004) but all these will strongly depend on the type of microbes and their tolerance as well as the type of tannin present. Therefore the study aims to determine the effect of tannin on the fibrolytic activity of microbes from giraffe, llama impala and consortia (A1 (giraffe + kudu), A2 (giraffe + impala), A3 (kudu + impala), and A4 (giraffe + kudu + impala)) that can be used to improve domestic goats' digestibility. It was hypothesised that increased tannin percentages will decrease browse microbial fibrolytic activities.

4.2 Materials and Method

4.2.1 Faecal collection and inoculum preparation

Faecal inoculum was preferred in this study because it has been previously investigated as an alternative inoculum for rumen fluid (Osuga et al., 2005). Faecal samples were collected fresh (within 5 min) after defecation from three ruminants; giraffe, kudu and impala browsing the thorn veldt at Tala Game Reserve (KwaZulu-Natal Province, South Africa) and a goat from

KwaMthethwa village, Empangeni browsing the communal fields winter (May to August). Faeces were transferred in a pre-warmed thermo flask that had been flushed with CO_2 (Getachew et al., 2004; Posada et al., 2012) and taken to the laboratory of the Department of Agriculture, University of Zululand. Inocula were prepared by mixing a 60 g faecal sample with 250 mL of warm salivary buffer solution (2 L of warm Solution A ($NaHCO_4$ 19.60 g, Na_2HPO_4 7.40 g, KCl 1.14 g and $MgCl{\cdot}6H_2O$ 0.26 g)) containing 2 mL of Solution B (5.3 g $CaCl{\cdot}2H_2O$ in 100 mL distilled water)) that was pre-warmed at 39℃ prior to use before being squeezed through four layers of cheese cloth to get a filtrate (inoculum). Microbial consortia were prepared prior to use as follows; A1 (giraffe + kudu, 1:1), A2 (giraffe + impala, 1:1), A3 (kudu + impala, 1:1) and A4 (giraffe + kudu + impala, 1:1:1).

4.2.2 Crude enzyme extraction, dialysis, concentration and quantification

Crude protein enzyme extraction, dialysis and concentration as well as enzyme quantification, was carried out as described in chapter 3 (section 3.2.2, 3.2.3 and 3.2.4 respectively) for goat, kudu, impala and giraffe.

4.2.3 Enzyme assays

4.2.3.1 Endocellulase activity

Endocellulase activity was assayed following the method described by (Fon et al., 2014a) except for the addition of tannic acid. Endocellulase was measured by pipetting 0.5 ml of 0.5% (m/v) containing 100 µL of 10 % tannic acid in the reaction buffer (20 mM sodium acetate, 0.02% (m/v) NaN_3 and 0.1 mM EDTA at pH 5.0) into 0.5 ml of crude protein solution obtained from the faecal inoculum and incubated for 2 h at 38℃ to determine endocellulase activity. The enzyme reaction was stopped by boiling at 100°C, and 400 µL of sample solution was analysed for reducing sugars using dinitrosalicylic (DNS) method. Specific enzyme activity was measured in µg glucose/µg crude protein.

4.2.3.2 Exocellulase activity

Exocellulase activity was assayed as described by (Fon et al., 2014a) except for the addition of tannic acid. Crystalline cellulase was measured by pipetting 0.5 ml of 0.5% (m/v) crystalline cellulose containing 100 µL of 10% tannic acid in the reaction buffer (20 mM sodium acetate, 0.02% (m/v) NaN_3 and 0.1 mM EDTA at pH 5.0) into 0.5 ml of crude protein solution obtained from the faecal inoculum and incubated for 72 h at 38 ºC to determine

exocellulase activity. The enzyme reaction was stopped by boiling at 100ºC, and 400 µL of sample solution was analysed for reducing sugars using the DNS method. Specific enzyme activity was measured in µg glucose/µg crude protein.

4.2.3.3 Hemicellulase activity

Hemicellulase activity was assayed as described by (Fon et al., 2014a) except for the addition of tannic acid. Hemicellulase was measured by pipetting 0.6 ml of 0.1% (m/v) xylan solution containing 100 µL of 10 % tannic acid in the reaction buffer (20 mM sodium acetate, 0.02% (m/v) NaN_3 and 0.1 mM EDTA at pH 5.0) into 0.4 ml of crude protein solution and incubated at 38ºC for 2 h to determine hemicellulase activity. The enzyme reaction was stopped by boiling at 100 ºC, and 25 µL of sample solution was analysed for reducing sugars using the DNS method. Specific enzyme activity was measured in µg xylose/µg crude protein.

4.2.4 Determination of reducing sugars by Dinitrosalicylic Method

The concentration of reducing sugar (glucose or xylose) released in the reaction was determined using either glucose or xylose standards as described by the DNS method (Miller, 1959). Glucose or xylose solution containing different sugar concentrations (1 to 150 µg and 5 to 40 µg, respectively) in a reaction buffer (20 mM sodium acetate, 0.02% (m/v) NaN_3 and 0.1 mM EDTA at pH 5.0) were used to prepare standard solutions. Dinitrosalicylic reagent 600 µL was pipetted into 400 µL of standard solution, incubated in boiling water for 5 min and cooled on ice along with the blank. Absorbance versus the concentration of the standards were plotted to construct xylose and glucose standard curves. The same procedure was followed to determine enzyme specific activities which are the amount of reducing sugar (glucose or xylose) released from reaction. Dinitrosalicylic reagent (600 µL) was pipetted into 400µL of enzyme substrate solution and absorbance at 540 nm. The amount of reducing sugar released was calculated from the linear equation generated from glucose or xylose plots. The concentration of reducing sugar released was measured either as µg glucose/mg crude protein or µg xylose/mg crude protein.

4.2.5 Chemical analysis of *Acacia sieberiana*

Acacia sieberiana was used for *in vitro* degradability study. Dry matter (DM), crude protein (CP), neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL) and condensed tannin (CT) were analysed for *Acacia sieberiana*. Nitrogen (N) concentration was measured by the Kjeldahl method described by Basha (2012) using the nitrogen analyser

system. The chemical components (NDF, ADF and ADL), cellulose and hemicellulose were determined according to the Van Soest et al. (1991b) principles using the ANKOM fibre method. The acid-butanol proanthocyanidins assay was used to determine CT (Makkar and Goodchild, 1995). The dry matter of *Acacia sieberiana* was as follows; DM (94.7%), CP (12.7%), NDF (65.8%), ADF (51.5%), ADL (39.3%), condensed tannin (6.2%), cellulose (12.2%) and hemicellulose (14.2%).

4.2.6 *In vitro* digestibility and gas production

In vitro degradability and the gas produced during fermentation was measured using a computerized pressure transducer system as described by Nsahlai et al. (2011) (section 3.2.8). About 1 g of ground (pass through 1 mm sieve) *Acacia sieberiana* dry matter was transferred into 250 mL Duran bottles containing 67 mL salivary buffer. The tannin concentration of the feed was made up to 10% by pipetting 100ul of tannic acid containing 3.8 mg into the sample solution containing 6.2 mg of tannin in 1g of *Acacia sieberiana*. Faecal inoculum (33 mL) was then added into the bottle while flushing with CO_2 to maintain anaerobiosis. The bottles were tightly closed with stoppers and placed in an incubator for 72 h at 39℃ with blanks lacking substrate only. After incubation, the contents of each reach was centrifuged at 8,000 × *g* for 15 min at 4℃ and dried for 72 h at 60. Apparent degradability (APD), true degradability (TD), neutral and acid detergent fibre degradability (NDFdeg and ADFdeg) and cellulose degradability (HEMdeg) were measured. Metabolisable energy (ME) was calculated using a formula described by (Afshar et al., 2011) in chapter 3 (section 3.2.8) using the gas produced and microbial yield. *In vitro* degradability was replicated thrice for each animal with five pseudo repeats for each run.

4.2.7 Statistical analysis

The Statistical Analysis System (SAS 9.3, 2013) was used to determine gas production from the regression equation described by Campos et al. (2004). The effects of tannin on APD, TD, NDFdeg, ADFdeg, HEMdeg, microbial yield, and gas parameters obtained from the regression equation were evaluated using linear analysis of variance (ANOVA). Significance between individual means was identified using Turkey multiple ranges test and means difference was considered significant at $P< 0.05$.

4.3 Results

4.3.1 Enzyme assays

The effect of tannin on microbial enzyme specific activity is shown in Table 4.1. The addition of tannic acid positively (P<0.05) influenced enzyme specific activities in all ecosystems (Table 4.1). Exocellulase and endocellulase activities were generally higher in the presence of tannic acid but for the goat, exocellulase activity was lower (P<0.05). The highest (P<0.05) exocellulase and endocellulase specific activities were observed in impala and A1 while it was moderate for A2 and Kudu. Hemicellulase specific activities were higher (P<0.05) in the presence of tannin acid. Impala again showed higher (P<0.05) hemicellulase activities than any other ecosystem.

Table 4.1 Effect of condensed tannin on fibrolytic enzyme activities

Inoculum source	**Enzyme specific activities**					
	Hemicellulase (µg xylose/mg)		Endocellulase (µg glucose/mg)		Exocellulase (µg glucose/mg)	
T (%)	0	10	0	10	0	10
Goat	30.0^{c}	50.4^{c}	99.9^{a}	134.6^{b}	65.9^{d}	53.6^{e}
Giraffe	30.1^{c}	85.1^{ab}	99.6^{c}	170.4^{b}	72.7^{bc}	105.0^{cd}
Kudu	29.8^{d}	83.7^{b}	95.8^{c}	163.8^{b}	72.9^{bc}	110.7^{bcd}
Impala	30.9^{a}	100.4^{a}	93.1^{d}	259.7^{a}	80.2^{a}	148.4^{b}
A1	30.2^{b}	75.9^{b}	95.2^{c}	230.9^{a}	76.0^{ab}	136.9^{ab}
A2	30.1^{c}	85.1^{ab}	96.0^{c}	166.1^{b}	70.0^{bc}	122.4^{ab}
A3	29.7^{d}	89.8^{ab}	97.0^{b}	125.3^{b}	71.0^{bc}	116.8^{bc}
A4	31.1^{a}	58.8^{c}	96.0^{c}	172.1^{b}	62.0^{d}	86.5^{d}
SEM	0. 3	1.96	0.12	7.17	0.85	3.46
P value	0.05	0.05	0.05	0.05	0.05	0.05

A1-Giraffe+Kudu, A2-Giraffe+Impala, A3-Kudu+Impala, A4- Giraffe+Kudu+Impala, SEM-standard error of the means, T-Tannin, ^{a,b,c,d,e} Means in the same column with different superscripts are different (P<0.05).

4.3.2 *In vitro* degradability

The effect of condensed tannin on *Acacia sieberiana* degradability is presented in Table 4.2. *In vitro* DM degradability of *Acacia sieberiana* treated with tannic acid varied greatly with ecosystems (P<0.05). The effect of tannin on Apparent degradability was highest (P<0.05) in giraffe, A1, A3 and lowest in goat, kudu and impala. The effect of tannin on True degradability was highest in A1, moderate in giraffe, A2, A1 and kudu but remained the same in A4 and

goat. Addition of tannic acid significantly decreased (P<0.05) microbial yield in wild animals and their consortia. The results from NDFdeg, ADFdeg and HEMdeg varied (P<0.05) among those treated with tannic acid as well those that were not treated. A significant increase in hemicellulose degradability was observed in the presence of tannin for A3, A1 and impala while it decreased for A4, A2 and goat. Metabolizable energy (ME) was significantly (P<0.05) reduced in the presence of tannin.

Table 4.2 Effect of condensed tannin on dry matter degradability of *Acacia sieberiana*

Inoculum source	Tannin	APD	TD	NDFdeg	ADFdeg	HEMdeg	MY	ME	GAS
	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(MJ/kg DM)	(ml/g DM)
Goat	0	25.6	45.3	27.9	19.3	64.9	19.7	26.4	70.4
	10	23.5	47.9	32.6	25.8	61.7	25.5	15.1	29.9
	Increment	-2.1	2.6	4.7	6.5	-3.2	5.8	-11.3	-40.5
Giraffe	0	15.2	48.3	31.9	24	65.6	33.1	21.5	14.2
	10	48.4	55.8	41.6	36	66	7.3	16.7	41.9
	Increment	33.2	7.5	9.7	12	0.4	-25.8	-4.8	27.7
Kudu	0	24.4	49.9	33.9	24.9	72.8	25.5	20.3	21
	10	19	56.3	42.3	36.8	66.3	37.3	15	28.8
	Increment	-5.4	6.4	8.4	11.9	-6.5	11.8	-5.3	7.8
Impala	0	38.3	52.7	37.6	29.8	71.5	14.4	22.3	51.8
	10	45.1	57.7	44.2	37.4	73.5	12.6	19.1	58.9
	Increment	6.8	5	6.6	7.6	2	-1.8	-3.2	7.1
A1	0	11.7	52.4	37.2	30.3	67	40.7	30.3	65.9
	10	38.7	60	47.4	42.3	69.5	21.5	16.6	40.8
	Increment	27	7.6	10.2	12	2.5	-19.2	-13.7	-25.1
A2	0	24.3	48.6	32.2	25.5	60.7	24.3	24	53
	10	43.9	55.9	41.8	39.5	51.8	12	17.2	45
	Increment	19.6	7.3	9.6	14	-8.9	-12.3	-6.8	-8
A3	0	12.7	43.5	25.5	17.9	58.6	30.8	23.6	42.3
	10	40.1	57.1	43.4	38.1	66.3	17.1	14.9	24.5
	Increment	27.4	13.6	17.9	20.2	7.7	-13.7	-8.7	-17.8
A4	0	20.1	49.6	33.5	25.9	66.3	29.5	26.7	61.9
	10	28.7	49.3	33.2	26.2	63.4	20.6	16.5	39.9
	Increment	8.6	-0.3	-0.3	0.3	-2.9	-8.9	-10.2	-22
SEM		2.63	0.69	1.31	0.95	1.19	1.24	0.41	2.69
P-value		0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05

A1-Giraffe+Kudu, A2-Giraffe+Impala, A3-Kudu+Impala, A4-Giraffe+Kudu+Impala, APD-Apparent degradability (%DM), TD-True degradability (%DM), NDFdeg-neutral detergent fibre degradability (%DM), ADFdeg-acid detergent fibre degradability (% DM), HEMdeg-hemicellulose degradability(% DM), MY-Microbial yield (%DM), ME-Metabolizable energy (MJ/kg DM), Gas (ml/g DM), SEM-Standard error of means, [a,b,c,d,e] Means in the same row with different superscripts are significantly different (P<0.05).

4.4 Discussion

Plant cell walls are degraded by a combination of bacteria, fungi and protozoa (Pérez et al., 2002). These microorganisms provide a broad array of enzymes that degrade a wider range of substrates. Among these enzymes are cellulases and hemicellulases which are important in harnessing energy from fibrous forages (Wang and McAllister, 2002). Tannins reduce the activity of fibrolytic enzymes *in vitro* (Ozkose et al., 2011a) but the current study observed an increase in enzyme activity when tannin was introduced. This was confirmed by high enzyme activities at 10% concentration, thus these enzymes seem to have developed some kind of adaptation in the presence of tannic acid e.g. binding to tannin and still remaining active (Paul et al., 2004). However, because the enzyme mixtures were crude, there is a possibility that these extracts might contain enzymes that can degrade tannic acid or tannin binding protein or the presence of tannin may activate the efficiency of the enzymes due to their natural environmental adaptations. The total endocellulase activity was significantly higher than exocellulase activities. This might be due to the fact that both endocellulase and exocellulase have the potential to degrade soluble carbohydrates while the insoluble structure is degraded only by exocellulase.

Hemicellulase activities were very low and the results were very similar to those observed by Ozkose et al. (2011a). Poor hemicellulase activities suggest that hemicellulases could have been more susceptible to tannic acid inhibition than cellulases. There is also a second suggestion which states that hemicellulases are more vulnerable to tannin binding because of the solubility of their substrates as opposed to exocellulase that binds to their substrate. The differences in enzyme activities between microbial ecosystems were expected due to the diversity of microbes within or among herbivore species and systems from animals feeding in different geographical locations (Fon, 2006). In addition, microbial ecosystems from the wild and their consortia possessed high microbial activities than domestic herbivores. This is probably due to the fact that wild animals browsed randomly with no manipulation of their diets hence were exposed to different types of tannins and concentrations which imply that microbes might have developed an adaptive mechanism to survive.

According to the results found in this study, microbial ecosystems from both wild and domestic herbivores appeared to empower a superior performance in the presence of tannins. Although Silanikove et al. (1996), reported an adaptation of goats on a large amount of tannins without suffering any ill effects. This study demonstrates that wild herbivores could be

harbouring microbes that are more tolerant and active. It has also been reported that tannins, when consumed at higher concentrations, can be toxic and lead to death of animals (Frutos et al., 2004) but its benefits when present in small quantities cannot be underestimated (Gordon et al., 2002; Mlambo and Mapiye, 2015; Palonen, 2004). The results for *in vitro* incubations with tannins were slightly higher than those of the control. Tannins stimulated an increased in APD, TD, NDFdeg, and ADFdeg from both wild and domestic microbial ecosystems. Again, the explanation for this was not clear although it was suggested that the presence of tannic acid could have a positive impact on ruminant microbes. This positive impact could be by stimulating an increase in fibrolytic secretion by microbes to increase the chances of free enzymes, tannin binding protein to bind tannins rather than enzymes or the presence of tannic acid tolerant microbes as suggested by many researchers (Makkar, 2003; Paul et al., 2004). However, the increase in dry matter digestibility in this study contradicts that of Mohammadabadi et al. (2010), who reported that forages containing more than 5% condensed tannin resulted in reduced feed digestibility.

Tannins had inhibitory effects on hemicellulose degradability from Kudu, A1, A4 and goat. Hemicellulose degradability decreased with increased levels of tannin (from 6.2% in control feed to 10% in treatment) which adds more emphasis to the effect of tannin on soluble carbohydrate enzymes. Besides *in vitro* degradability, gas produced from giraffe, kudu and impala microbial ecosystems tended to increase when tannin was increased while those from consortia (A1, A2, A3 and A4) and goat decreased. A similar study by Barman and Rai (2008) also found an increase in gas production at a 10% tannin level.

A decrease in gas production might also indicate a reduction in the rumen activity but was not the case as demonstrated by TD which implies that the gas might have been converted to other by-products. However, the decreased gas in consortia, was accompanied by relatively higher TD, which implies that the consortia could be essential for reducing enteric gas production as well as energy loss. Reduction in gas also means decreasing the occurrence of bloat and increasing the amount of *by pass* into the small intestine thus improving animal performance (Vieira and Borba, 2011). It is also believed that a reduction in gas production could be caused by tannin-binding which may decrease microbe attachment to some feed particles hence reducing fermentation rate (Makkar, 2003). Metabolisable energy (ME) was influenced by condensed tannins. A decrease in ME somehow emphasises the negative effect of tannins on nutrient digestibility. The results from these study were not very conclusive but

showed that both wild herbivores and consortia could be harbouring potential fibrolytic microbes with relatively high tannin tolerance. The hypothesis was rejected as a 10% increase in tannic acid did not suppress the fibrolytic activities of rumen microbial ecosystems in the majority of wild animals and consortia.

4.5 Conclusion

Both enzyme and *in vitro* fermentation studies showed that microbial ecosystems from wild herbivores may be harbouring potential fibrolytic microbes that are relatively tannin acid tolerant as demonstrated by their exocellulase specific activities and true digestibility. Giraffe, impala, kudu and consortia produced less gas than goat microbial ecosystems. The impala and A1 consortia showed the highest potential to be used for inoculating domesticated goat to improve browse fermentation in future studies. Therefore the next study will investigate the effect most active microbial ecosystems have on goat fermentation. However, other future experimentation on the effect of the type of tannins on microbial ecosystems will be encouraged to ascertain the tannin tolerance observed.

Chapter 5

The effect of microbial consortia from wild herbivores on goat rumen fibrolytic activity

Abstract

Acacia species are often recommended as a protein supplement during the long dry season or in winter because they are readily available and maintain their protein content throughout the year. However, *Acacia species* or browses are often limited by their high tannin concentrations which are also detrimental to fibrolytic microbes hence a decrease in digestibility especially in domesticated goats. Therefore this study evaluated the effect of *in vitro* inocula from wild herbivores (impala, kudu and giraffe) on *in vitro* fermentation, cellulases and hemicellulase activities of domestic goats. Consortia were created by mixing faecal inoculum from impala, giraffe and kudu with that of goat (N1 (goat + impala, 1:1), N2 (goat + kudu, 1:1), N3 (goat + giraffe+ kudu, 1:1:1) and N4 (goat + giraffe + kudu + impala, 1:1:1:1)). Crude protein enzyme extracts (CPZ) from fresh faecal samples were precipitated by 60% ammonium sulphate and assayed for exocellulase, endocellulase and hemicellulase activities by incubating with crystalline cellulose, carboxymethyl cellulose and xylan at 38ºC with an optimum pH of 5.5 to 6.5 for 1, 2, and 48 h, respectively. *In vitro* degradability was carried out by transferring 33 ml of faecal inoculum into 67 ml salivary buffer containing 1 g of *Acacia sieberiana* and 10% tannin before incubating for 72 h at 38ºC. Apparent degradability (APD), true degradability (TD), neutral detergent fibre degradability (NDFdeg), acid detergent fibre degradability (ADFdeg), cellulose degradability (CELLdeg), hemicellulose degradability (HEMdeg) and MY were calculated. Manipulation of goat enzyme activities with enzymes from the wild had a positive ($P<0.05$) influence on goat fibre degradability. Hemicellulase and endocellulase activities were highest ($P<0.05$) in N1 while N4 showed the highest exocellulase activity. Microbial ecosystem N3 had the highest ($P<0.05$) TD, NDFdeg and ADFdeg while N1 showed the highest degradability for hemicellulose and cellulose. Microbial yield also varied among the microbial ecosystems but was highest for N2, goat and N4. Therefore it was concluded that microbial activities from wild herbivores could have introduced new microbes that were able to survive in goat ecosystems and improve its general fibrolytic potential slightly. These results showed that *in vitro* microbial ecosystems from wild herbivores have a potential to improve browse utilisation in domestic goat microbial ecosystems.

Keywords: Consortia, Tannin, *In vitro*, cellulase, Crude protein

5.1 Introduction

Goats can access their nutrition from consuming a wider range of plant species. Based on their feeding behaviour, goats thrive well in arid and semi-arid regions due to their ability to feed on different woody species to satisfy their needs for nutrients (Motubatse et al., 2008). Goat production is mainly found in the subsistence sector in southern Africa (Ndlovu, 1998). The main constraint in small ruminant production is the insufficiency ,and unavailability of quality feed especially during the dry season (Uguru et al., 2014). Therefore, browses particularly the *Acacia species* is recommended as an important feed resource as it maintains a high nutritive value throughout the year (Ndlovu, 1998). This is because their leaves, twigs and pods have a fairly high concentration of protein (Motubatse et al., 2008). However, the forages may contain a lot of anti-nutritional factors (tannin and lignin) which may act as inhibitors for some rumen microbes (Jakhesara et al., 2010).

These anti-nutritional compounds are primarily produced by the plant to protect them against insects and animal herbivory (Dai and Mumper, 2010). When consumed, these compounds turn to limit the growth of different types of microbes hence causing a decrease in rumen fermentation and animal performance (Jakhesara et al., 2010). Tannins exert their secondary effect on the rumen environment by binding to enzymes and rumen microbial ecosystems which can potentially inhibit their activities since tannin binding to enzymes does not necessary mean enzyme inhibition (McMahon et al., 2000; Paul et al., 2004). However, animals that base their diet on tannin rich feed may develop different mechanisms to survive or tolerate the negativity of tannins (McSweeney et al., 2001).

Therefore for plants to be useful as feed, they should resist attack by environmental microbes while growing in the field but be susceptible to penetration, colonisation and digestion by microorganisms inside the rumen (McAllister et al., 1994). Ruminants have a unique capability to consume ligno-cellulosic feeds as a major constituent of their diet to get energy for their survival but recent studies have shown that this fibrolytic potential is strongly affected by anti-nutritional compounds such as tannin and lignin (Santra and Karim, 2003). They also have the ability to convert low quality protein into high quality protein as well as non-protein nitrogen into high quality protein (Varga and Kolver, 1997). Different approaches should be done to improve ruminant fibre utilisation since more than 50% of the forage intake is eliminated out as faeces (Paul et al., 2004). This may involve *in vitro* microbial trans-

inoculation and inoculation of the rumen with probiotics such as potential fibrolytic microbe groups from different sources. This different microbial consortia population could be made up of bacteria, fungi and ciliate protozoa (Wang and McAllister, 2002). However, digestion of feed by ruminants can be restricted by the ability of enzymes to gain access to target substrate through inhibition arising from the presence of tannins.

Therefore to improve goat utilisation of poor quality roughages or winter browses with relatively higher tannin especially in *Acacia species*, rumen manipulation with microbes from wild herbivores that have evolved with their fibrolytic potential and tannin tolerance is essential. It has been postulated that wild animals will contain microbes with higher fibrolytic potential and tannin tolerance due to the variation in tannin concentration and type. This was observed in our previous chapter where microbial consortia showed a higher true degradability, NDF degradability and exocellulase enzyme activities. Therefore the present study was designed to assess the effect of microbial inocula from wild herbivores (impala, kudu and giraffe) on *in vitro* fermentation and cellulase activities of domestic goats. It was hypothesised that in vitro inoculation of goat microbial ecosystems with inoculum from impala, kudu and giraffe will not have an effect on rumen fermentation and fibrolytic activity.

5.2 Materials and methods

5.2.1 Faecal collection and inoculum preparation

Fresh faecal samples (within 5 min of defecation) were collected from goat, giraffe, kudu and impala and prepared as described above in chapter 3 (section 3.2.1) in the laboratory of the Department of Agriculture, University of Zululand. The different active microbial ecosystems were used to inoculate the goat system to create the following microbial ecosystems; N1 (goat + impala, 1:1), N2 (goat + kudu, 1:1), N3 (goat + giraffe + kudu, 1:1:1) and N4 (goat + giraffe + kudu + impala, 1:1:1:1)). Faecal inocula were prepared by mixing 60g faecal sample with 250ml of warm salivary buffer solution (2L of warm Solution A ($NaHCO_4$ 19.60g, Na_2HPO_4 7.40g, KCl 1.14g and MgCl.6H2O 0.26g)) containing 2 mL of Solution B (5.3g CaCl. $2H_2O$ in 100 ml distilled water)) that was pre-warmed at 38ºC prior to use before squeezing through four layers of cheese cloth to get filtrate (inoculum). Microbial ecosystems N1, N2, N3 and N4 were mixed in the right proportions prior incubation.

5.2.2 Crude enzyme extraction, dialysis, concentration and quantification

Crude protein enzyme extraction, dialysis and concentration as well as enzyme quantification, was carried out as described in chapter 3 (section 3.2.2, 3.2.3 and 3.2.4 respectively) for goat, kudu, impala and giraffe.

5.2.3 Enzyme assays

5.2.3.1 Endocellulase activity

Endocellulase activity was assayed following the method described by Fon et al. (2014a) except for the addition of tannic acid and the formation of goat enzyme consortia with CPZ from wild herbivores to form the following systems; N1 (goat +impala, 1:1), N2 (goat + kudu, 1:1), N3 (goat + giraffe + kudu, 1:1:1) and N4 (goat + giraffe + kudu + impala, 1:1:1:1). Endocellulase was measured by pipetting 0.5 ml of 0.5% (m/v) containing 100 µL of 10 % tannic acid in the reaction buffer (20 mM sodium acetate, 0.02% (m/v) NaN_3 and 0.1 mM EDTA at pH 5.0) into 0.5 ml of crude protein solution obtained from the faecal inoculum and incubated for 2 h at 38℃ to determine endocellulase activity. The enzyme reaction was stopped by boiling at 100℃, and 50 µL of sample solution was analysed for reducing sugars using the DNS method. Specific enzyme activity was measured in µg glucose/µg crude protein.

5.2.3.2 Exocellulase activity

Exocellulase activity was assayed as described by Fon et al. (2014a) in chapter 4 (section 4.2.3.2) except for the formation of goat enzyme consortia which was carried out as described above under endocellulase activity section 5.2.3.1.

5.2.3.3 Hemicellulase activity

Hemicellulase was assayed as described by Fon et al. (2014a) in chapter 4 (section 4.2.3.3) except for the formation of goat enzyme consortia which was carried out as described above under endocellulase activity (section 5.2.3.1).

5.2.4 Determination of reducing sugars by Dinitrosalicylic acid Method

The quantification of reducing sugar (glucose or xylose) released in the reaction was determined using both glucose and xylose standards as described by the Dinitrosalicylic method (Miller, 1959) in chapter 4 (section 4.2.4).

5.2.5 Chemical analysis of *Acacia sieberiana*

The chemical composition of *Acacia sieberiana* used in the *in vitro* fermentation study was determined as previously described in chapter 3 (section 3.2.7). The chemical composition of *acacia sieberiana* was as follows; DM (94.7%), CP (12.7%), NDF (65.8%), ADF (51.5%), ADL (39.3%), condensed tannin (6.2%), cellulose (12.2%) and hemicellulose (14.2%).

5.2.6 *In vitro* degradability

In vitro degradability was determined following the method described by Nsahlai et al. (2011). Approximately 1 g of ground (pass through 1 mm sieve) *Acacia sieberiana* dry matter was transferred into 250 mL Duran bottles containing 67 mL salivary buffer. The tannin concentration of the feed was made up to 10% by pipetting 100ul of tannic acid containing 3.8 mg into the sample solution containing 6.2 mg of tannin in 1g of *Acacia sieberiana*. The different inocula were produced as follows prior to inoculation; N1 (goat +impala, 16.5:16.5 mL), N2 (goat + kudu, 16.5:16.5 mL), N3 (goat + giraffe + kudu, 11:11:11 mL) and N4 (goat + giraffe + kudu + impala, 8.25: 8.25: 8.25: 8.25 mL)). Faecal inoculum (33 mL) was then added into the bottle while flushing with CO_2 to maintain anaerobiosis. The bottles were tightly closed with stoppers and placed in an incubator for 72 h at 39℃ with blanks lacking substrate only. After incubation, the contents of each reach was centrifuged at 8,000 × *g* for 15 min at 4℃ and dried for 72 h at 60. Apparent digestibility (APD), true digestibility (TD), neutral and acid detergent fibre degradability (NDFdeg and ADFdeg) and cellulose degradability (HEMdeg) were measured.

5.2.7 Statistical analysis

A Statistical Analysis System (SAS 9.3, 2013) was used to determine dry matter digestibility from the regression equation (Campos et al., 2004). Apparent degradability, TD, NDFdeg, ADFdeg, HEMdeg, microbial yield, obtained from the regression equation were evaluated using linear Analysis of Variance (ANOVA). Significance between individual means was identified using Turkey multiple ranges test and means difference was considered significant at $P < 0.05$.

5.3 Results

5.3.1 Enzyme assays

Manipulation of goat enzyme activities with microbes from the wild had an influence ($P<0.05$; Table 5.1) on goat ecosystems.

Table 5.1 Hemicellulase, endocellulase and exocellulase activities of crude protein extracts

Inoculum source	**Enzyme specific activities (µg reducing sugar/mg)**		
	Xylanase	Endocellulase	Exocellulase
Goat	30.7^{c}	91.0^{e}	46.1^{c}
N1	58.3^{a}	268.7^{a}	58.3^{ab}
N2	34.8^{c}	191.4^{b}	67.8^{a}
N3	44.7^{b}	169.1^{c}	53.8^{bc}
N4	45.9^{b}	149.2^{d}	61.3^{ab}
SED	2.19	4.58	2..64
P value	0.05	0.05	0.05

Xylanase activity=µg xylose/mg, Endocellulase activity = µg glucose/mg, Exocellulase activity = µg glucose/mg, N1 (goat + impala), N2 (goat + kudu), N3 (goat + giraffe+ kudu), N4 (goat + giraffe + kudu + impala), SD-Standard deviation, ^{a,b,c,d,e} Means in the same row with different superscripts are significantly different ($P<0.05$).

Crude enzyme extract from N1 had the highest ($P<0.05$) hemicellulose and endocellulase enzyme activities. The lowest hemicellulase and endocellulase were observed in goat ecosystems. The highest exocellulase activity was recorded in N2 while the lowest was recorded in goats.

5.3.2 *In vitro* degradability

The results obtained from *in vitro* degradability of *Acacia sieberiana* by goat microbial inoculum and microbial consortia are presented in Table 5.2. *Acacia sieberiana* degradability varied ($P <0.05$) between goat and the combined microbial ecosystems. *In vitro* NDFdeg, ADFdeg, TD and APD were higher ($P < 0.05$) high in N3 while hemicellulose and cellulose digestibility were high in N1 compared to goat ecosystems. Microbial yield varied ($P < 0.05$) between microbial ecosystems with the highest yield observed in goat and N2. Apart from N4, TD was higher in N1, N2 and N3 than goat.

Table 5.2 Effect of microbial consortium from wild herbivores on goat degradability of *Acacia sieberiana*

Inoculum source	Degradation parameters						
	NDFdeg % DM	ADFdeg % DM	HEMdeg % DM	CELLdeg % DM	APD % DM	TD % DM	MY % DM
Goat	32.5^{a}	25.9^{b}	61.7^{b}	37.8^{b}	23.5^{c}	48.9^{b}	25.3^{ab}
N1	32.2^{a}	22.7^{b}	82.6^{a}	80.3^{a}	38.0^{b}	50.1^{ab}	12.2^{bc}
N2	35.2^{ab}	24.5^{b}	77.2^{ab}	78.3^{a}	23.3^{c}	51.0^{ab}	27.7^{a}
N3	45.9^{a}	40.9^{a}	67.8^{ab}	26.7^{b}	53.8^{a}	59.0^{a}	5.2^{c}
N4	31.4^{a}	24.0^{b}	25.0^{c}	42.7^{b}	19.9^{c}	44.4^{b}	24.5^{ab}
SEM	2.4	2.2	2.9	2.6	2.4	1.9	2.1
P value	0.05	0.05	0.05	0.05	0.05	0.05	0.05

N1-Impala+Goat, N2-Kudu+Goat, N3-Giraffe+Kudu+Goat, N4-Giraffe+Kudu+Impala+Goat, APD-Apparent degradability (% DM), TD-True degradability (% DM), NDFdeg-neutral detergent fibre degradability (% DM), ADFdeg-acid detergent fibre degradability (% DM), HEMdeg-hemicellulose degradability (% DM), MY-Microbial yield (% DM), A4- Giraffe+Kudu+Impala, SD-Standard deviation, ^{a,b,c,d,e} Means in the same row with different superscripts are significantly different ($P<0.05$).

5.4 Discussion

Ruminants have the potential to convert up to 40% lignocellulosic forages to products of high nutritional value such as glucose. However, lignin is not the only anti-nutritional factor as tannin has also been found to play a major role in browse digestibility especially in domestic goats. Browses are often used in winter as protein supplements when pasture composition are limited in protein but its digestibility is affected by its high tannin concentrations. The extent of fibre degradation in the rumen depends upon the capability of individual microbes present in the microbial consortium (Sahu et al., 2004). Therefore, the present study was conducted to improve goat's fibre utilisation in browse species by inoculating with microbial ecosystems from wild herbivores. Goat enzyme activities were improved in the presence of enzyme consortia. This improvement could be due to the presence of evolved fibrolytic microbes that are relatively tolerant to significant concentrations of tannin influenced by their browsing behaviour in natural habitats.

Wild herbivores spent more time feeding on browses which may contain higher concentrations of cellulose as well as variable concentrations and type of tannins. Therefore, wild microbial ecosystems are more adaptable and active on tanniferous forages. Goats showed poor enzyme activities which were associated with its mixed browsing and grazing abilities and concentrate supplementation most often by owners. Goats sometimes depend on either

mixed diets, browse or graze on grass plant influenced by the time of the year (Oyeleke and Okusanmi, 2008). N1 had the highest hemicellulase and endocellulase activities when compared to other ecosystems. High concentrations of endocellulase and hemicellulase show that N1 were most active against soluble carbohydrates than insoluble carbohydrate. The high activities of endocellulase over hemicellulase on substrate were associated with an abundance of dry forages. Therefore, microbes were highly adapted to fibre rich feed since they were harvested in winter. However, an increase in hemicellulase and cellulase activities provides evidence of synergistic mechanisms among microbes that have evolved for efficient digestion of the plant cell wall. The improvement of goat microbial activities shows that cellulolytic organisms from wild herbivores secrete extremely diverse cellulases influenced by their natural substrates and diverse plant cell walls (Zhang and Zhang, 2013).

In vitro dry matter degradability of *Acacia sieberiana* was affected positively by the addition of inocula from wild herbivores into goat microbial inoculum. Improvement on fibre degradability may have resulted from improved enzyme activities in the present consortia. N3 showed the highest TD, NDFdeg and ADFdeg while its enzyme activities were average. This probably indicates that microbes from N3 ecosystems were more adaptable to tannin containing substrate. The lowest NDFdeg and ADFdeg observed in N1, N2 and N4 might be due to accumulation of certain end products of fermentation in the reaction mixture which might have inhibited further degradation of feed (Sahu et al., 2004).

In addition, the reasons for incomplete fibre digestion could be due to anti-nutritive compounds present in the substrate. However, N1 had the highest cellulose and hemicellulose degradability and this may be due to its high enzyme activities. Therefore, N1 microbial ecosystem was able to improve goat plant cell wall polysaccharide degradation. According to the results found in the present study, microbes from the wild are more fibrolytic compared to those from domestic herbivores. This was evident by their potential ability to improve the fibre digestion of browsed species in goat microbial consortia. Therefore microbial activities from wild herbivores might have introduced new microbes with higher fibrolytic potential which are relatively tolerant to small increments of tannin. This finding supports the positive synergism between rumen microorganisms observed by (Wang and McAllister, 2002). Therefore the hypothesis was rejected as in vitro inoculation of goat microbial ecosystems with inoculum from impala, kudu and giraffe increased rumen fermentation and fibrolytic activity.

5.5 Conclusions

It was concluded that microbial consortia from wild herbivores had the potential to improve browse utilisation in domesticated goats. Therefore, efficient degradation of complex browses in the rumen requires coordinated activities of rumen microbial ecosystems that will accelerate fibre degradation in the presence of relatively higher amounts of tannins. Microbial consortium from N3 showed the degradability (NDFdeg, ADFdeg and TD) while N1 had high cellulose and hemicellulose degradability. Therefore, microbial consortium from N1 and N3 showed the highest potential to improve goats' ability to utilise browses efficiently in the presence of 10% condensed tannin. However, it might be possible that the inhibitory effect of tannin on N4 might have prevented them from attaining their fibrolytic potential hence the need to investigate the effect tannin binding agents such as PEG and PVP have on goats' consortia fermentation.

Chapter 6

The effect of polyethylene glycol on *in vitro* digestibility of tanniferous browses by goat microbial consortia

Abstract

Tannin content in browse species exerts a significant negative effect on ruminant feed intake and digestion. Polyethylene glycol 4000 (PEG 4000) can help to alleviate the adverse effects of tannin rich feed on ruminant nutrition. This study evaluated the effect of PEG 4000 on goat microbial consortia *in vitro* degradability of tanniferous feeds. Microbial consortia were created by mixing faecal inoculum from goat, impala, giraffe and kudu as follows; N1 (goat + impala, 1:1), N2 (goat + kudu, 1:1), N3 (goat + giraffe+ kudu, 1:1:1) and N4 (goat + giraffe + kudu + impala, 1:1:1:1). For microbial consortia cellulase and hemicellulase activities, crude protein enzyme extracts (CPZ) were precipitated from fresh faecal inocula by 60% ammonium sulphate and assayed for hemicellulase, endocellulase and exocellulase activities by incubating with xylan, carboxymethyl cellulose and crystalline cellulose containing 100 µL of 10 % tannic acid and 50 µL of 5 % (PEG 4000) respectively. *In vitro* degradability was carried out by transferring 33 ml of faecal consortia inoculum into 67 ml salivary buffer containing 1 g of *Acacia sieberiana*, 10% tannin and 5 % PEG4000 before incubating for 72 h at 38ºC. Apparent degradability (APD), true degradability (TD), neutral detergent fibre degradability (NDFdeg), acid detergent fibre degradabilty (ADFdeg), cellulose digestibility (CELLdeg), hemicellulose degradability (HEMdeg) and MY were measured. An increase ($P < 0.05$) in enzyme activities was observed with the addition of PEG. The highest ($P < 0.05$) endocellulase, hemicellulase and exocellulase activities influenced by PEG were observed in N1 followed by N4. The results showed that PEG generally increased ($P < 0.05$) *in vitro* degradability of *Acacia sieberiana*. Microbial consortium N1 showed the highest increment in degradability when PEG was added for TD, NDFdeg, ADFdeg and CELLdeg. The second highest for the same parameters was observed in N2, followed by N4 while the lowest was observed in N3. For HEMdeg PEG effect was not significant ($P > 0.05$) among ecosystems but were classified as N3, N2, N4 and N1 based on the relative increment observed. Improvement in fibre and *Acacia sieberiana* digestibility after inclusion of PEG clearly emphasised the negative effect of tannins on browses. Therefore, there is a need for constantly researching novel technologies that will decrease its effect and improve tanniferous browse digestibility especially in domestic browsers.

Key words: Polyethylene glycol, Tannin, Microbial consortia, Digradability, Cellulases

6.1 Introduction

Ruminant production is affected by insufficient supply of feed in terms of quality and quantity especially in winter. Therefore farmers depend on forage legumes especially browse plants to supplement animals during the dry season. However browse species may contain high levels of polyphenolic compounds including tannins which may result in low intake and digestibility of feed (Osuga et al., 2005). It is well-known that the presence of tannins has been related to subordinate biological availability of different nutrients and at high consumption may also cause toxicity or death of animals (Makkar et al., 1995). It has been reported that condensed tannin values above 50 g/kg DM can have a serious anti-nutritional effect when fed to ruminants e.g. toxic and suppression of digestibility (Yisehak et al., 2014).

The anti-nutritive effect of condensed tannin is associated with binding to dietary proteins and digestive enzymes to form complexes which may not be readily digestible (Krebs et al., 2007). Therefore the effects of condensed tannins on forage quality can be observed from its inhibitory role on palatability, the growth rate, the digestion of fibre fraction and the utilisation of nitrogen (Frutos et al., 2004). Growth rate of animals can be reduced due to a reduction in feed intake caused by high fibre and phenolic content (Frutos et al., 2004).

Some researchers have suggested the use of chemical binding reagents to eliminate tannins from binding feed (Mlambo et al., 2003; Silanikove et al., 1996; Souri et al., 2015). Among these chemicals, polyethylene glycol (with molecular weight 4000 and 6000) has been recommended (Krebs et al., 2007; Souri et al., 2015). Polyethylene glycol (PEG) has been reported to have a higher affinity to condensed tannins and inhibiting the formation of tannin-protein complexes (Souri et al., 2015; Yisehak et al., 2014). Therefore PEG can help to alleviate the adverse effects of tannin from tannin rich feeds. This is because PEG is thought to break already formed tannin-protein complexes due to its higher affinity to tannin compared to protein (Besharati and Taghizadeh, 2011).

The binding effect of PEG to tannins may possibly increase the availability of certain macronutrients, particularly proteins and carbohydrates to animals (Silanikove et al., 2001). In our previous experiment, it was established that goat microbial consortia; N1 (goat + impala), N2 (goat + kudu), N3 (goat + giraffe+ kudu) and N4 (goat + giraffe + kudu + impala) showed the highest fibrolytic potential and tannin tolerance but N4 had the least TD suggesting the possibility of the vulnerability of its microbes to tannin. Therefore, this assessed at the effect of PEG 4000 on goat microbial consortia degradability of *Acacia sieberiana*.

6.2 Materials and method

6.2.1 Faecal collection and inoculum preparation

Faecal inocula were collected from goat, impala, kudu and giraffe as described in chapter 4 (section 4.2.1). Goat microbial consortia were prepared as follows; N1 (goat +impala, 1:1), N2 (goat + kudu, 1:1), N3 (goat + giraffe + kudu, 1:1:1) and N4 (goat + giraffe + kudu + impala, 1:1:1:1).

6.2.2 Crude enzyme extraction, dialysis, concentration and quantification

Crude protein enzyme extraction, dialysis and concentration as well as crude protein enzyme quantification was carried out as described in chapter 3 (section 3.2.2, 3.2.3 and 3.2.4 respectively) for goat, kudu, impala and giraffe.

6.2.3 Enzyme assays

6.2.3.1 Endocellulase activity

Endocellulase activity was assayed as described in chapter 4 (section 4.2.3.1) except for the addition of PEG4000. Endocellulase activity was measured by pipetting 0.5 ml of 0.5% (m/v) carboxymethyl cellulose solution containing 100 µL of 10 % tannic acid and 50 µL of 5 % (m/v) PEG in the reaction buffer (20 mM sodium acetate, 0.02% (m/v) NaN_3 and 0.1 mM EDTA at pH 5.0) into 0.5 ml of CPZ solution obtained from the faecal inoculum and incubated for 2 h at 38ºC to determine endocellulase activity. The enzyme reaction was stopped by boiling at 100ºC, and 400 µL of sample solution was analysed for reducing sugars using the DNS method. Specific enzyme activity was measured in µg glucose/µg crude protein.

6.2.3.2 Exocellulase activity

Exocellulase activity was assayed as described above for endocellulase activity (section 6.2.3.1). Crystalline cellulase was measured by pipetting 0.5 ml of 0.5% (m/v) crystalline cellulose solution containing 100 µL of 10% tannic acid and 50 µL of 5 % (m/v) PEG 400 in the reaction buffer (20 mM sodium acetate, 0.02% (m/v) NaN_3 and 0.1 mM EDTA at pH 5.0) into 0.5 ml of crude protein solution obtained from the faecal inoculum and incubated for 72 h at 38ºC to determine exocellulase activity. The enzyme reaction was stopped by boiling at 100ºC, and 400 µL of sample solution was analysed for reducing sugars using the DNS method. Exocellulase specific activity was measured as µg glucose/µg crude protein.

6.2.3.3 Hemicellulase activity

Hemicellulase was carried out as described for endocellulase activity (section 6.2.3.1). Hemicellulase was measured by pipetting 0.6 ml of 0.1% (m/v) xylan solution containing 100 µL of 10 % (m/v) tannic acid and 50 µL of 5 % (m/v) PEG4000 in the reaction buffer (20 mM sodium acetate, 0.02% (m/v) NaN_3 and 0.1 mM EDTA at pH 5.0) into 0.4 ml of crude protein solution and incubated at 38ºC for 2 h to determine hemicellulase activity. The enzyme reaction was stopped by boiling at 100ºC, and 400 µL of sample solution was analysed for reducing sugars using the DNS method. Hemicellulase specific activity was measured as µg xylose/µg crude protein.

6.2.3 Quantification of reducing sugars

The quantification of reducing sugar (glucose or xylose) released in the reaction was determined by using either glucose or xylose standards as described by the Dinitrosalicylic method (Miller, 1959) in chapter 3 (section 3.2.6).

6.2.4 Chemical analysis of *Acacia sieberiana*

The chemical composition of *Acacia sieberiana* used in the *in vitro* fermentation study was determined as described in chapter 3 (section 3.2.7). The chemical composition of *acacia sieberiana* was as follows; DM (94.7%), CP (12.7%), NDF (65.8%), ADF (51.5%), ADL (39.3%), condensed tannin (6.2%), cellulose (12.2%) and hemicellulose (14.2%).

6.2.5 *In vitro* degradability

In vitro degradability was determined as described in chapter 4 (section 4.2.6) except for the addition of 5% (m/v) PEG 4000. Approximately 1 g of ground (pass through 1 mm sieve) *Acacia sieberiana* dry matter was transferred into 250 mL Duran bottles containing 67 mL salivary buffer. The tannin concentration of the feed was adjusted to 10% by pipetting 100ul of tannic acid containing 3.8 mg into the sample solution containing 6.2 mg of tannin in 1g of *Acacia sieberiana*. The substrate was supplemented with and without 5 % (m/v) of PEG 4000 (5 mg was contained in 50 µL PEG solution). Goat microbial consortia were made prior to inoculation as follows; N1 (goat +impala, 16.5:16.5 mL), N2 (goat + kudu, 16.5:16.5 mL), N3 (goat + giraffe + kudu, 11:11:11 mL) and N4 (goat + giraffe + kudu + impala, 8.25: 8.25: 8.25: 8.25 mL)). Faecal inoculum (33mL) was added into the 250mL Duran bottle containing

1 g of substrate after flushing with CO_2 to maintain anaerobiosis. The bottles were tightly closed with stoppers and placed in an incubator for 72 h at 39℃ with blanks lacking substrate only. After incubation, the contents of each incubation was centrifuged at 8,000 × *g* for 15 min at 4℃ and dried for 72 h at 60. Apparent degradability (APD), true degradability (TD), neutral and acid detergent fibre degradability (NDFdeg and ADFdeg) and cellulose degradability (HEMdeg) were measured.

6.2.6 Statistical analysis

A Statistical Analysis System (SAS 9.3, 2013) was used to evaluate the effect of PEG4000 on cellulase and hemicellulase activities as well as the degradability parameters of *Acacia sieberiana* (APD, TD, NDFdeg, ADFdeg, HEMdeg and CELLdeg and microbial yield). These parameters were compared using linear Analysis of Variance (ANOVA). Significance between individual means was identified using Turkey multiple ranges test and mean difference was considered significant at $P < 0.05$.

6.3 Results

6.3.1 Enzyme assays

Table 6.1 Effect of polyethylene glycol 4000 on the degradability of crystalline cellulose, xylan and carboxymethyl cellulose by goat enzyme consortia

Inoculum source	PEG (%)	Enzyme activities (µg reducing sugar/mg)		
		Hemicellulase	Endocellulase	Exocellulase
N1	0	75.2^{e}	183.3^{dc}	88.1^{c}
	1	133.6^{a}	586.7^{a}	231.3^{a}
N2	0	78.5^{e}	191.7^{dc}	82.1^{c}
	1	130.5^{a}	510.5^{a}	184.0^{b}
N3	0	81.1^{e}	210.9^{c}	86.0^{c}
	1	122.7^{b}	489.5^{a}	204.8^{ab}
N4	0	80.3^{e}	145.5^{dc}	78.2^{c}
	1	113.6^{c}	465.9^{a}	202.9^{ab}
SEM		1.19	8.32	3.75
P-value		0.05	0.05	0.05

Xylanase activity=µg xylose/mg, Endocellulase activity = µg glucose/mg, Exocellulase activity = µg glucose/mg, N1 (goat + impala), N2 (goat + kudu), N3 (goat + giraffe+ kudu), N4 (goat + giraffe + kudu + impala), SEM-Standard deviation, ^{a,b,c,d,e} Means in the same row with different superscripts are significantly different (P<0.05).

6.3.2 *In vitro* degradability

The effect of PEG supplementation on *in vitro* degradability of *acacia sieberiana* is shown in Table 6.2. Dry matter degradability of *acacia sieberiana* treated with PEG was different (P <0.05) from that of no PEG. Addition of PEG improved (P <0.05) TD of *acacia sieberiana* leaves in N1, N2, and N4 except for N3. When PEG was added, TD, NDFdeg, ADFdeg and CELLdeg were highest in consortia N1. The lowest NDFdeg and ADFdeg values were recorded by N3 when treated with PEG. Hemicellulose degradability was not significantly different (P >0.05) when treated with or without PEG.

Table 6.2 Effect of polyethylene glycol 4000 on *Acacia sieberiana* degradability by goat consortia

		Degradation Parameters						
Inoculum source	PEG (%)	NDFdeg	ADFdeg	HEMdeg	CELLdeg	APD	TD	MY
N1	0	48.2^{b}	40.9^{a}	79.9^{a}	16.1^{c}	34.9^{c}	60.8^{b}	25.8^{b}
	1	82.6^{a}	82.4^{a}	83.4^{a}	62.1^{a}	73.0^{a}	86.8^{a}	13.9^{c}
	Increment	34.4	41.5	3.5	46.0	38.1	26.0	-11.9
N2	0	40.1^{b}	32.2^{b}	74.0^{a}	26.5^{bc}	25.3^{dce}	54.4^{b}	29.5^{b}
	1	65.3^{ab}	62.2^{ab}	79.0^{a}	65.0^{a}	60.8^{b}	73.7^{a}	13.0^{b}
	Increment	25.2	30.0	5.0	38.5	35.5	19.3	-16.5
N3	0	57.8^{b}	54.4^{b}	76.6^{a}	43.4^{abc}	30.3^{dc}	68.0^{b}	37.8^{ab}
	1	47.4^{b}	39.8^{b}	83.4^{a}	39.7^{abc}	28.0^{dc}	60.1^{b}	32.2^{ab}
	Increment	-10.4	-14.6	6.8	-3.7	-2.3	-7.9	-5.6
N4	0	48.8^{b}	40.9^{b}	80.5^{a}	58.2^{ab}	14.3^{e}	61.2^{b}	46.9^{a}
	1	58.9^{b}	52.8^{b}	85.4^{a}	75.8^{a}	19.6^{de}	68.8^{b}	43.3^{a}
	increment	10.1	11.9	4.9	17.6	5.3	7.6	-3.6
SEM		2.96	3.63	2.23	4.15	1.60	2.24	1.76
P-value		<0.05	<0.05	>0.05	<0.05	<0.05	<0.05	<0.05

N1-Impala+Goat, N2-Kudu+Goat, N3-Giraffe+Kudu+Goat, N4-Giraffe+Kudu+Impala+Goat, APD-Apparent degradability (% DM), TD-True degradability (% DM), NDFdeg-neutral detergent fibre degradability (% DM), ADFdeg-acid detergent fibre degradability (% DM), HEMdeg-hemicellulose degradability (% DM), MY-Microbial yield (% DM), SEM-Standard deviation, ^{a,b,c,d,e} Means in the same row with different superscripts are significantly different (P<0.05).

However, there were some small increments when PEG was added though not significant. Apparent degradability of a*cacia sieberiana* leaves increased with PEG addition but for N3. Microbial Yield was affected by PEG negatively as all microbial consortia recorded a decrease in microbial yield.

6.4 Discussion

Tannins are known to affect the digestibility of nutrients by forming soluble and insoluble complexes (Yisehak et al., 2014). Tannins act within the animal's digestive tract by binding to the substrate and inhibiting enzymes or exerting anti-microbial effects (Canbolat et al., 2005). Therefore the use of PEG might mitigate the negative effects of tannin on the microbial ecosystem in both wild and domestic herbivores. Addition of polyethylene glycol (PEG) on *in vitro* enzyme incubations significantly improved enzyme activities in all ecosystems. This result shows that PEG addition might have displaced protein or enzymes binding to tannin to form PEG-tannin complexes since PEG have been reported to have a higher affinity to tannin than proteins (Besharati and Taghizadeh, 2011). This displacement implies that more enzymes or substrate binding sites on rumen fibrolytic microbes will be liberated hence an increase in enzyme activity or microbial attachment hence facilitating substrate degradation. The results obtained with PEG treatment were also similar to those obtained by Souri et al. (2015).

Therefore, the use of PEG in this study verified the nutritional influence of tannin and brought prospective fermentation of *Acacia sieberiana* in the absence of tannins (Mlambo et al., 2008). An increased APD after PEG supplementation generally elucidates the negative effect of tannin on digestibility. The addition of PEG increased TD of *Acacia sieberiana*. These results correspond with the work done by Nsahlai et al. (2011), however TD values studied in the present study were generally higher. In addition, the high values obtained for true degradability suggest that PEG addition increased substrate availability to rumen microorganisms.

Inhibition of degradability in samples not treated with PEG was associated with tannin binding to proteins, digestive enzymes and microbes (binding cell wall surface proteins) (Krebs et al., 2007). Substrate incubated in the absence of PEG resulted in higher microbial yield than in the presence of PEG. Reduction in microbial yield in the presence of PEG was also reported in the work done by Getachew et al. (2000) and Nsahlai et al. (2011). The reduction in microbial yield was associated with a reduction in efficiency of microbial protein synthesis in tannin rich feeds. Microbial ecosystems from N1 gave the highest response to PEG supplementation in terms of both NDF and ADF degradability. This suggests that PEG might have increased microbial plant adhesion or fibrolytic microbial activity in N1. The least response to PEG inclusion was observed in N3, this was attributed to its low enzyme activities compared to N1 and N2 ecosystems. The potential for improving fibre digestibility in N1 and N2 microbial

ecosystems was achieved by supplementing with PEG, which resulted in an increased NDF and ADF degradability. In the absence of PEG that tannins reduced cell wall carbohydrates' digestibility by binding to bacterial enzymes and forming complexes with cell wall polysaccharides in comparison with PEG treatment. However, the present study demonstrates the possibility of improving the efficiency of tannin containing browses utilisation with PEG supplementation. Therefore the hypothesis was rejected as PEG 4000 increased goat microbial consortia degradability of *Acacia sieberiana*.

6.5 Conclusion

It was concluded that *Acacia sieberiana* digestibility by goat microbial consortia was still affected by tannin since the addition of polyethylene glycol increased TD, NDFdeg, ADFdeg and CELLdeg. The effect of PEG was most pronounced in N1 and N2 as there was a significant increase in fibre degradation upon PEG addition. Therefore, the negative effects of CTs on digestibility could be mitigated by the use of tannin binding agents such as PEG. The improvement in digestibility after inclusion of PEG clearly emphasised the importance for research into tannin-binding compounds that can be used to negate or reverse the effect of tannin.

Chapter 7

General discussion

The most expensive and largest cost of production in livestock production systems is feeding, including small ruminant production systems, especially in developing countries where there is scarcity of feed resources in the dry season (Uguru et al., 2014). Not only is availability a problem but the quality as well since most of the pasture at this time of the year is dry and deficient in protein. Therefore, farmers try to meet animal nutrient requirements during this period by providing access to browses and shrub legumes as an energy and protein supplement. Ruminant animals have developed a different and advanced microbial ecosystem for digesting fibrous feedstuffs (Wang and McAllister, 2002). However the use of shrubby vegetation by herbivores is limited mostly by the plants' defensive mechanisms put in place such as lignin, tannin and thorns or furs (Silanikove et al., 1996). Recent studies on browse fodders show that tannin plays a major inhibitory role in browse digestibility and through different mechanisms (Paul et al., 2004; Sahoo et al., 2010). These strategies include binding to dietary proteins, plant cell wall polysaccharides, gut microbes and endogenous enzymes (Bohnert et al., 2002). Therefore, strategies to improve livestock performance on browses should be well-thought-out. The purpose of this study was to scan various microbial ecosystems from the wild with the main objective of identifying potential rumen microbial ecosystems that might evolve with their ability to degrade or digest browses in the presence of tannin. Secondly, the effect of potential fibrolytic microbial ecosystems with relatively high tannin-tolerance from wild herbivores on goat rumen fibrolytic activity and browse fermentation were also investigated. It was hypothesised that microbial ecosystems from wild herbivores will improve the fibrolytic potential of domesticated ruminants to efficiently utilise tannin-containing feedstuffs (browses).

The results obtained from chapter 3, showed that domesticated goat microbial ecosystems can digest or ferment soluble plant cell wall polysaccharides as good as wild ruminants and their consortia but were deficient in digesting non-soluble polysaccharides (cellulose). Little or no variation in digesting soluble carbohydrates was associated with forage type adaptability since domesticated animals' diet is often influenced by human (feeding concentrates) and human activities. Wild animals were more exposed to high fibre forages than domesticated animals hence were bound to adopt strategies of harnessing energy from such diets. It has been reported that forage type plays a major role in the type of rumen microbes, population, evolution and efficiency (Varga and Kolver, 1997). Thus the higher exocellulase

activity observed in wild animals was associated with microbes that have evolved with their efficiency in digesting fibre. *In vitro* digestibility from wild ruminants and their consortia showed greater potential in fermenting *Acacia sieberiana* than for goats which was also associated with microbes that have evolved in their fibrolytic potential. However, gas production was highest in goats. This confirms the adaptability of the goat microbial ecosystem in digesting soluble polysaccharides (with high gas as a characteristic by-product) than high fibre forages. High enteric gas production is not cost effective to farmers (energy loss and metabolic disorders like bloat) as well as to the environmentalist where it is a major contributor to global warming especially methane. Giraffe, impala, kudu and consortia (A1, A2, A3 and A4) does not only harbour microbes with relatively higher fibrolytic potential but also had the potential to reduce the amount of energy lost as enteric gas. About 12% of the total greenhouse gases (GHG) emitted into the atmosphere comes from agriculture while 0.28 of global methane emission comes from ruminant livestock enteric fermentation (Animut et al., 2008; Jayanegara et al., 2009). The harmful effects of GHG (global warming, climate change, ozone depletion, sea level rise and adverse diversity), calls for an urgent need to protect the environment. Therefore microbial ecosystems and their consortia could be a potential solution to cut down the percentage of enteric gases in livestock and agriculture.

Trail 2 shows that microbial ecosystems from both wild and domestic herbivores appeared to empower a superior performance in the presence of tannins. Cellulase activities were generally higher than hemicellulase activities and the results were very similar to those observed by (Ozkose et al., 2011a). This suggests that hemicellulases could have been more susceptible to tannic acid inhibition than cellulase. Paul et al. (2004), explained that hemicellulose are more soluble hence most hemicellulases are bound to be free in solution making them more vulnerable to tannin binding. Tannin-binding will imply a decrease in enzyme active sites or changes of enzyme confirmation which can potentially deactivate enzyme activity. Tannin addition increased APD, TD, NDFdeg and ADFdeg from both wild and consortia. Microbial ecosystems from the wild and their consortia possessed higher microbial activities than domestic herbivores. Again, the high activity was associated with evolved fibrolytic microbes with relatively high tannin tolerance due to continuous exposure to different types and concentrations of tannins while browsing in the thorn velds at Tala Game Reserve.

The third experiment evaluated the effect of microbial consortia from wild herbivores on goat rumen fibrolytic activity. Microbial ecosystems with high fibrolytic enzyme potential

as confirmed by the results observed in chapter 1 and chapter 2 were used to inoculate the goat microbial ecosystem. The high activities of cellulases over hemicellulase on substrate were associated with abundance of forage type in the natural environment. Since the animals were browsing fibre rich forages especially in winter when they were collected, cellulase production would have been higher and more efficient in order for the animal to be able to harness more energy to meet its energy requirements. *Acacia sieberiana* degradability was affected positively by the addition of microorganisms from wild herbivores in goat microbial ecosystems. The increase in *acacia sieberiana* degradability was due to improved activities of potential fibrolytic microbes from wild herbivores inoculated to goat ecosystems. The lowest NDF and ADF degradability on N1, N2 and N4 might be due to accumulation of certain end products of fermentation in the reaction mixture which might have inhibited further degradation of feed (Sahu et al., 2004). In addition, cellulolytic enzyme on substrate degradation could also be limited by the presence of anti-nutritional compounds (Palonen, 2004). The present study showed that wild herbivores could be regarded as important sources of highly active microbial ecosystem for improving degradation of fibrous feed in domesticated ruminants. Therefore, an introduction of microbes from one species to another can enhance efficient utilisation of tannin rich browses if the microbes can compete, survive and colonise an ecological niche. This approach is also very important and cheap because enzyme supply will be continuous if they survive and colonise an ecological niche unlike other feed additives or enzymes that are required to be fed daily.

Chapter 6 revealed that tannin limits the *in vitro* degradability of *Acacia sieberiana* as demonstrated by the increase in dry matter degradability following supplementation with PEG (Mlambo et al., 2009). The supplementation of *Acacia sieberiana* with PEG 4000 significantly increased nutrient digestibility. Improvement found after supplementing with PEG included increases in NDFdeg, ADFdeg, CELLdeg and TD. It is worth mentioning that these degradability parameters are all fibrous components of the feed which implies that tannin binding to cellulase was imminent. Increased in TD and reduction in microbial yield after PEG supplementation of *Acacia sieberiana* was similar to the work done by Nsahlai et al. (2011). A comparison between 0 % tannin and 10 % tannin indicates that nutrient value of *Acacia sieberiana* is influenced by condensed tannin (Nsahlai et al., 2011). Degradability of fibre was lower for diets not treated with PEG. This result indicates that PEG addition could have increased microbial plant adhesion or fibrolytic microbial activity. Therefore, the results of this study suggest that tannin still plays a major inhibitory role on browse digestibility even in smaller quantities. The decrease in microbial yield was similar to what was observed by Nsahlai

et al. (2011) but the explanation behind this decrease was not clear. The big question was, why did fibre degradability increase and the microbial population did not? It was postulated that there was probably an increase in microbial population but might have been lost through binding with high concentrations of both tannin and PEG. Such binding would be advantageous to the animal as it may promote flow through of protein to the abomasum hence an increase in protein uptake. The hypothesis was therefore accepted as inoculation of goat microbial ecosystem with inocula from wild herbivores improved TD, NDFdeg. ADFdeg and CELLdeg.

Conclusions

Wild herbivores and their consortia can utilise browse forages better than domestic goats. The impala and consortia (A1 and A2) showed the highest potential of improving *Acacia sieberiana* digestibility. Subjecting these microbial ecosystems to a slightly higher tannin concentration showed that wild herbivores were relatively more tolerant to small increments of tannin than domestic goats without losing their fibrolytic potential. A comparison between 0 % and 10 % tannin indicated the adaptability of rumen microbial ecosystems from wild herbivores on tannin-rich forages. This study also showed that wild herbivores could be a potential source of inocula to manipulate goat microbial ecosystems for better forage utilisation. This was evident by the increase in enzyme activities and nutrient degradability parameters observed when goat microbial consortia were formed. Manipulating goat rumen microbial population increased cellulolytic and hemi-cellulolytic activities for efficient utilisation of fibrous feed. The addition of polyethylene glycol 4000 also increased cellulase and hemicellulase activities as well as *in vitro* digestibility of *Acacia sieberiana*. This shows that both domestic and wild herbivores microbial ecosystems can only tolerate minimal changes in tannin concentrations. It also concluded that polyethylene glycol 4000 can reduce the inhibitory effects of tannins in wild herbivores than in domesticated goat as demonstrated by their higher *Acacia sieberiana* degradability.

Recommendations

- Wild herbivores and their consortia have a higher potential over domestic herbivores to utilise feed with high fibre content and tannins. It is therefore recommended to investigate the effect of microbial ecosystems from giraffe, impala, kudu and consortia on goat both *in vitro* and *in vivo* to confirm its application as a feed additive.

- Future experimentation of microbial ecosystems from giraffe, kudu, impala and goat with different types of tannins will be encouraged to ascertain their tannin tolerance.
- Microbes from wild browsing ruminants might have introduced highly active microbial species in goat rumen *in vitro*. Therefore, *in vivo* trans-inoculation is recommended for better understanding on how to improve rumen fermentation in domestic browsers.
- It is recommended to determine the tannin concentrations of different *Acacia* species and their effect in small ruminant nutrition in terms of feed intake, growth rate and production.

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