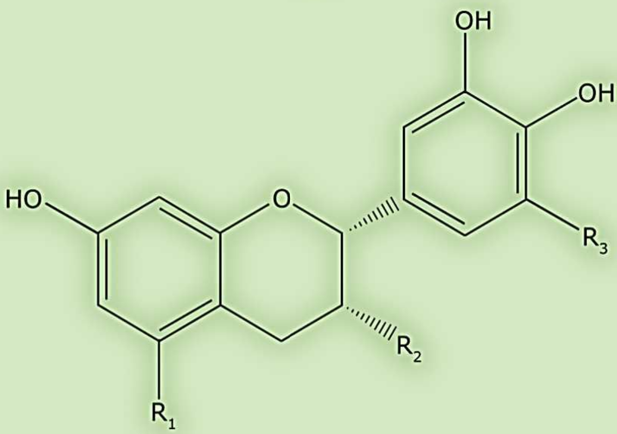
Browse species from Ethiopia:
their role in methane reduction and nematode control in goats



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Summary

The aim of the research reported in this thesis was to evaluate browse species collected from Ethiopia for preference by goats, and for their *in vitro* anthelmintic and methane (CH_4) reduction properties. During the conduct of the studies observations were made warranting a further aim, to compare *in vitro* fermentation patterns of browse species using inocula from goats and cows kept on identical dietary regime.

The preference of browse species using dry matter intake (DMI) as a proxy and in combination with polyethylene glycol (PEG), relationships between browse species intake and chemical composition were determined in Chapter 2. Air-dried leaves of *Acacia etbaica*, *Cadaba farinosa*, *Capparis tomentosa*, *Dichrostachys cinerea*, *Dodonaea angustifolia*, *Euclea racemosa*, *Maerua angolensis*, *Maytenus senegalensis*, *Rhus natalensis* and *Senna singueana* were used. Two cafeteria trials, each lasting 10 days were conducted using goats receiving a daily ration of grass hay and wheat bran, without (trial 1) or with (trial 2) the inclusion of PEG. Preference measured as the first 10 min browse DMI differed significantly among browse species and with PEG (P<0.0001). Browse with higher tannin content, *D. cinerea*, *R. natalensis* and *A. etbaica* were the most preferred species regardless of PEG presence. Preference appeared to be based on digestible fibre fraction, hemicellulose rather than tannin levels in the browse species.

Extracts of the 10 browse species were evaluated for their anthelmintic activity against *Haemonchus contortus* (Chapter 3). The larval exsheathment inhibition assay (LEIA) was applied using *H. contortus* third stage larvae (L_3) in a dose dependent manner with extract concentrations of 0, 150, 300, 600, 1200 µg/ml phosphate buffered saline (PBS). The role of polyphenols in the inhibition against L_3 was evaluated using polyvinylpolypyrrolidone (PVPP). All browse extracts significantly ($P \leq 0.0001$) inhibited larval exsheathment in a dose dependent manner with the dose required to inhibit 50% of the L_3 (EC_{50}) being highest in *C. farinosa* and lowest in *E. racemosa* and *M. senegalensis*. Polyvinylpolypyrrolidone treated *A. etbaica*, *C. tomentosa*, *M. angolensis*, *R. natalensis* and *D. cinerea* were different (P<0.001) from the control (only PBS), indicating that larval inhibition was largely due to non-phenolic compounds. Absence of significant differences between PVPP treated *E. racemosa*, *M. senegalensis*, *D. angustifolia* and *S. singueana*, and control suggest that inhibition was mostly attributable to tannins and other polyphenols. Browse species anthelmintic property against *H. contortus* L_3 was due to the presence of phenolic and non-phenolic compounds.

In vitro gas production (GP), CH_4, volatile fatty acids (VFA) and *in vitro* organic matter digestibility (IVOMD) of the 10 browse species were determined using PEG 6000 in Chapter 4. Proanthocyanidins (PA) were quantified using a modified HCl-butanol method and PA composition was determined by UPLC-DAD, with detection of other polyphenols by UPLC-ESI-MS/MS. Substrates were inoculated in buffered goat rumen fluid with or without PEG 6000 for 72 h to measure GP with head space gas sample measurements taken at 0, 3, 6, 9, 12, 24, 30, 48, 54, and 72 h for CH_4. At the end of incubation, VFA, ammonia (NH_3) and IVOMD were determined. Increased ($P<0.0001$) GP, CH_4 and total VFA were observed after PEG addition indicating PA were mainly involved in reducing methanogenesis and to a lower extent also overall fermentability. Prodelphinidins were the major explaining factors for this reduction but other polyphenols like quercetin, myricetin and kaempferol were also involved in CH_4 reduction. The effect of PEG addition on IVOMD was variable among browse and could be due to artefacts from the tannin-PEG complexes in the incubation residue. Proanthocyanidins were mainly responsible for the reduced *in vitro* fermentative activities with possible minor effects of other phenolic and non-phenolic components.

Due to unusual fermentation patterns observed in Chapter 4, a comparison was made between goat and cow inocula on *in vitro* gas and CH_4 production and kinetics parameters as well as VFA production in Chapter 5. Leaves of *A. etbaica, C. tomentosa, D. cinerea, R. natalensis*, freeze-dried maize and grass silage, and a concentrate were inoculated for 72 h to measure GP, in buffered inocula from goats and cows kept on an identical feeding regime. During incubation, headspace gas samples were obtained at 0, 3, 6, 9, 24, 30, 48, 54, and 72 h, and analysed for CH_4 with VFA determined at the end of incubation. A triphasic and monophasic modified Michaelis-Menten equation was fitted to the cumulative GP and CH_4 curves, respectively. Total GP and CH_4 ($P<0.0001$), half-time for asymptotic ($P<0.012$) and rate ($P<0.0001$) of GP were higher for goat inoculum. The total VFA were higher ($P<0.0001$) in goats and the proportion of individual VFA differed significantly ($P<0.002$) between animal species. Differences between goat and cow inocula were attributable to variation in the activity and composition of the microbial population, and differences were more pronounced for fermentation of browse species than grass and maize silages.

A synthesis of the results from the four research chapters is provided in the general discussion (Chapter 6). The present work highlights the browse species characteristics which can be strategically exploited in goat production systems to improve health and feed utilization efficiency.

Table of Contents

CHAPTER 1

General introduction

1.1. Background

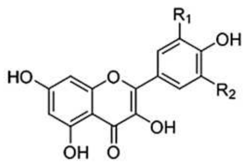
Livestock constitute a very important component of the agricultural and pastoral system in developing countries. The major socio-economic rationale of keeping livestock is its role as nourishment, an asset and safety net, source of income, employment and traction for crop production (Herrero et al., 2013; Thornton, 2010). Historically, human population growth, increased income and urbanization are the driving forces underpinning the demand for livestock products (Thornton, 2010). The response to this demand has a profound implication on existing production systems. Increasing the number and efficiency of production animals with a concomitant aim to reduce the environmental impact is the most sustainable strategy. In low input, extensive grazing systems, intensification of livestock production is often challenged by availability and quality of natural pasture and disease occurrence (Kaasschieter et al., 1992). One mechanism to lower the risk of animal loss due to nutrition and/or health constraints is to keep diverse groups of livestock species.

Sheep and goats, commonly known as small ruminants, can easily be integrated in grazing systems where resources are limited. Major advantages of small over large ruminants are their small size and thus lower feed requirement and a shorter generation interval allowing flock establishment within a short period of time. Goats in particular are able to survive in harsh, dry environments (Salem and Smith, 2008), as in arid and semi-arid regions. These areas account for 25 % of the world's land area and are characterized by low, erratic and unreliable rainfall, unusual extremes in temperature, high evaporation and evapotranspiration rates limiting herbaceous species growth in rangelands (Boufennara et al., 2012; Mlambo and Mapiye, 2015). Despite the harsh environmental conditions, some browse species maintain greenness during critical periods in dry seasons and remain the sole source of feed for domestic and wild ruminants (Rubanza et al., 2005).

Browse refers to leaf and twig growth of trees and shrubs that can be ingested by herbivores (Allen et al., 2011). Browse species provide a cheap protein source to supplement tropical grasses to meet the protein needs of grazing animals (Goel et al., 2005). In addition, plant secondary metabolites (PSM) which include terpenes, phenolics (coumarin, lignin, flavonoids, tannins), nitrogen containing PSM (alkaloids, cyanogenic glucosides) and sulphur containing metabolites (Mazid et al., 2011) are widely distributed in browse species. One of the most abundant PSM in browse species are phenolic compounds which can contribute up to 50% of their organic matter (Reed, 1986).

1.2. Phenolic compounds in forages

Phenolics are widely distributed in the plant kingdom and structurally consist of an aromatic ring with one or more hydroxyl groups, and ranging from simple to highly polymerized molecules (Balasundram et al., 2006). Phenolic compounds include flavonoids, phenolic acids and tannins and have been found to be the major constituent of some tropical browse species (Mueller-Harvey et al., 1987) with divergent effects on animal nutrition (Lowry et al., 1996). Flavonoids are compounds with a low molecular weight and consist of flavonols, flavones, flavanols and isoflavones. Quercetin, kaempferol and myricetin are the most common flavonols (Fig. 1.1).



	R_1	R_2
Kaempferol	H	H
Quercetin	H	OH
Myricetin	OH	OH

Figure 1.1. Structure of the three common flavonols (Jia and Liu, 2013).

Forage tannins are among the phenolic groups that gained a growing interest for their beneficial role in ruminant nutrition, in terms of enhancing protein utilization, ruminal methane (CH_4) reduction, reducing bloat risks and as a measure for nematode control (Aerts et al., 1999; Rochfort et al., 2008). Broadly, tannins can be characterised into two groups: condensed tannins (CT) and hydrolysable tannins (HT). Tannins have a high molecular weight (500-20000 Da) with the ability to precipitate mainly protein (Frutos et al., 2004) and to a lesser extent carbohydrates and minerals. This description, however, does not cover all types of tannins and excludes tannins with low protein binding capacity, e.g. ellagitannins (Salminen and Karonen, 2011). The latter authors gave a more elaborate definition of tannins based on their chemical structures. Condensed tannins typically consist of two or more monomeric (+)-catechin or (-)-epicatechin units (Fig. 1.2a-b), which make the procyanidins (PC); while (+)-gallocatechin or (-)-epigallocatechin monomeric units (Fig. 1.2c-d) make the prodelphinidins (PD). Condensed tannins are present as oligomers (two to ten monomer units) or polymers (>10 monomer units, Fig. 1.2m) in plants. Hydrolysable tannins consist of simple gallic acid derivatives, gallotannins and ellagitannins. Simple gallic acid derivatives contain five or less galloyl groups that are most commonly esterified to either glucose (monogalloyl and pentagalloyl glucoses) or quinic acid.

Gallotannins represent gallic acid derivatives that contain six or more galloyl groups and are characterized by having one or more digalloyl groups (heptagalloyl glucose).

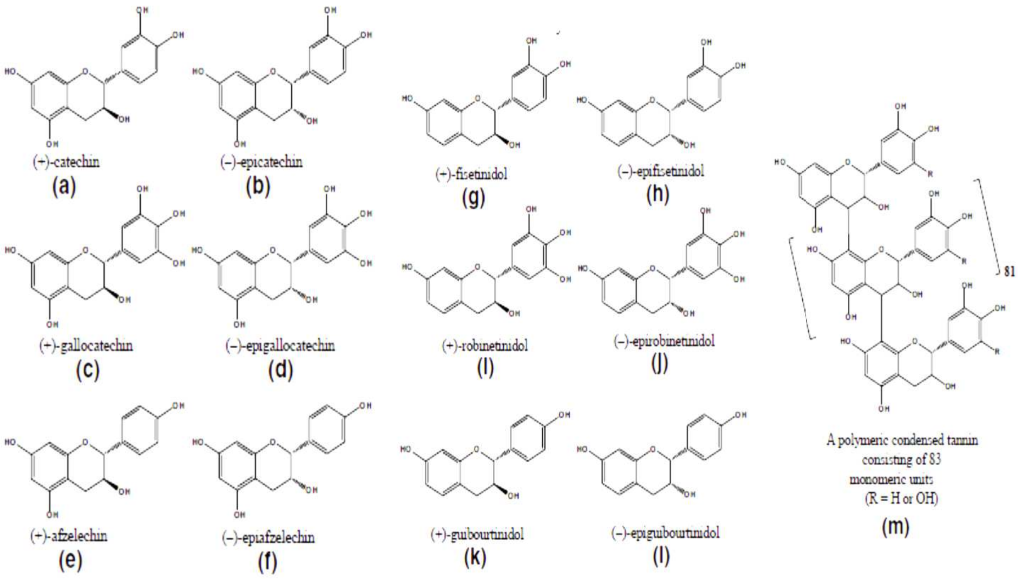


Figure 1.2. Structure of monomeric units of condensed tannins (a-f, common), (g-l, rare 5-deoxy units) and an example of polymeric condensed tannins (m) (Salminen and Karonen, 2011).

1.3. Biological activity of browse phenolics

Some phenolics such as tannins can make complexes with protein which explains most of their biological activity. Molecular size, solubility and the number of phenolic groups determine complexation formed by hydrogen bonding between the phenolic protons and the peptide carbonyl groups (Lowry et al., 1996). In general, tannins have higher affinity towards large, porous, proline rich proteins and precipitation of proteins is effective at a neutral pH (Hagerman et al., 1992). However, affinity towards protein can differ depending on tannin type. Protein digestibility in deer and sheep has been reduced by CT while no effect was observed for HT (Hagerman et al., 1992). In a recent *in vitro* study, both HT and CT were shown to reduce methane (CH_4) production, however, CT also reduced *in vitro* organic matter digestibility (Jayanegara et al., 2015). Since HT can easily be degraded by microbes, they have a lesser influence on digestibility. However, nutritional effects of tannins could not be attributed to either HT or CT groups due to the diverse nature of tannins and the presence of other plant chemical components such as protein, fibre and lignin (Mueller-Harvey, 2006).

Structural differences in terms of monomeric flavanol units, mean degree of polymerization, prodelphinidin:procyanidin (PD:PC) and *cis*-*trans* ratios were observed in sainfoin (*Onobrychis viciifolia*) accessions (Hatew et al., 2014). These differences are related to differences in the activity of CT. The PD have more hydroxyl groups compared to PC which allows binding mainly with proteins and is responsible for higher biological activities. Higher molecular weight of tannins is also related to higher protein binding capacity. Within the HT sources, comparing commercial tannic acid and gallotannins in fireweed flowers, tannin-protein complex was strong for fireweed due to the larger molecular weight compared to tannic acid (Hagerman et al., 1992). The PD:PC ratio contributes most to the variation in *in vitro* CH_4 reduction potential of different tannin sources (Huyen et al., 2016). Monomers of PD were more potent inhibitors of *in vitro* larval exsheathment of *Haemonchus contortus* and *Trichostrongylus colubriformis* than monomers of PC (Brunet and Hoste, 2006). The anthelmintic property of polyphenols other than tannins was indicated (Hoste et al., 2012). Therefore, accurate analysis of forages for tannin and other polyphenol composition and identifying the biologically active ingredient is important in nutraceutical studies of forages containing phenolics.

1.4. Browse preference by production ruminants

The term preference of an animal can be defined as a measure of relative intake of alternative forages and describes the selective behaviour of the animal, and is different from palatability. Palatability refers to acceptability of a forage by an animal and describes forage characteristics that invoke a sensory response (Allen et al., 2011). Eating rate at the beginning of a meal for single feed and preference tests in the case of several feeds can be used to evaluate palatability (Baumont, 1996). In the latter case, however, palatability can be confounded with preference. Therefore, preference is more an objective measure to be used in diet selection studies. When offered dietary choices, ruminants based their decision on preingestive (feed sensory characteristics) and postingestive (digestive and metabolic consequences) information. Preference results from the interaction between taste and postingestive feedback (Provenza, 1996).

Preference starts with the preingestive signals (smell, sight, odour) associated with the feed/forage causing the release of saliva, digestive enzymes and hormones in the gastrointestinal tract, and neurotransmitters involved in eating (Provenza, 1996). The chemical characteristics of the feed/forage and the physiological condition of the animal influence preference. The presence of phenolics (e.g. alkaloids, glucosinolates, tannins) usually decrease feed intake in ruminants (Provenza, 1996), and thus preference. However, intake or preference for forages containing phenolics can be higher depending on the ability of the animal to overcome toxic effects. An inconsistent relationship between browse chemical components and browse preference were reported for sheep and goat. In free ranging sheep and goats, Basha et al. (2012) observed no relationship between fibre fraction and CT composition with preference and concluded that selection of browse is species specific. Preference for browse species was established based on digestible fibre fractions than on tannin composition in goats with browsing experience (Hernández-Orduño et al., 2012). Previous experience influences feed preingestive and postingestive consequences, and hence preference.

Experience during early life develop through the neurological, morphological and physiological changes which shape behaviour of animals including preference for certain feeds (Provenza et al., 2003). In naive animals, the decision to consume a feed or feed ingredient is largely based on feed characteristics (smell, palatability); whereas, in experienced animals both palatability and postingestive consequences influence preference. Preference in ruminants is a complex process and influenced by factors such as the physico, chemical characteristics and accessibility of feed, previous experience, rumen fill, satiety, stress, reproduction cycle and malaise.

1.5. Natural browse species as forages for production animals

Natural browse is considered advantageous over cultivated forages in low input extensive production systems. Cultivated forages are considered land and labour intensive, hindering possible adoption by resource poor farmers (Mekoya et al., 2008). Moreover, in dry areas, seedlings from cultivated browse have poor survival and slow growth rates. Therefore, with proper management practice in place, natural browse is promising for sustainable forage availability (Coppock and Reed, 1992) in areas where exotic forages cannot be established. The forage value of browse species depends on nutritional composition and the capacity of the animal to maintain an optimal nutrient intake level while avoiding detrimental effects of browse phenolics, in particular tannins. In the past, tannins were often regarded as only antinutritional (Goel et al., 2005; Mueller-Harvey, 2006; Rubanza et al., 2005), associated with the characteristics of binding proteins, cellulose, hemicellulose and minerals rendering them less digestible.

The formation of tannin-mucoprotein complexes in the mouth causes an astringent sensation which reduces feed intake (Goel et al., 2005). Nonetheless, depending on tannin level and chemical characteristics, the type of animal species involved (Rochfort et al., 2008), animal nutrient requirements and dietary nutrients (Waghorn, 2008), tannins can have beneficial effects. When ruminants are fed a high quality forage, 56-

65% of the protein reaching the rumen will be deaminated which exceeds microbial protein synthesis resulting in excretion of surplus levels of NH_3 (20-35%) in the urine (Patra and Saxena, 2011).

Protein degradation in the rumen is considered as wastage in ruminants. It is, thus, vital to reduce ruminal protein degradation (increase rumen escape protein) and increase its subsequent availability in the lower digestive tract. Condensed tannins can be used as protein protectant in the rumen because of their ability to form complexes with proteins. Tannin-protein complexes are stable between pH ranges of 3.8 and 8 and dissociation occurs in the abomasum (pH, 2.5-3) and duodenum (pH, 8) (Frutos et al., 2004). It has been widely accepted that dietary CT levels below 50 g/kg dry matter (DM) have beneficial effects in ruminants by increasing rumen escape protein. Reduction in feed intake, digestive efficiency and animal performance can be observed with CT levels of 6-12% of DM (Aerts et al., 1999; Frutos et al., 2004). However, the 50 g/kg DM threshold tannin level was based on temperate forages for ruminants consuming these forages (Mueller-Harvey, 2006) and may not be applicable to tropical tannin containing forages (Mlambo et al., 2004). In addition to their protein sparing function, browse species can be used as a source of protein in animals kept on protein deficient herbaceous grazing lands. Crude protein level of 9-17% in Acacia fruits (Mlambo et al., 2008) and 15-25% in leaves of various browse species (Osuga et al., 2008) were reported.

Tannin binding agents such as polyethylene glycol (PEG) and polyvinylpyrrolidone (PVPP) have been used over the past four decades to attenuate detrimental effect of tannins so as to increase utilization of tannin containing forages (Goel et al., 2005). The practical applicability of tannin attenuating agents in low input production systems is doubtful. The forage value of tannin containing browse species can be increased by recruiting ruminant species capable of utilizing these forages, and by employing appropriate feeding strategies, e.g. feeding a mixture of browse species to overcome nutrient imbalance and also to dilute the potential risks of consuming high levels of plant secondary metabolites (e.g. tannins) (Salem and Smith, 2008). In most mammals, the production of tannin binding salivary proteins, proline-rich and histatin-rich, is considered as a first line of defence against tannins at moderate intake levels (Muir, 2011). These salivary proteins inactivate tannins more than dietary proteins and this reduced fecal nitrogen loss (Goel et al., 2005). Another mechanism to overcome detrimental effects of tannins, e.g. in goats, is by harbouring tannin tolerant or tannin-protein complex degrading gastrointestinal microflora (Bhat et al., 1998; Goel et al., 2005).

1.6. Anthelmintic properties of forage polyphenols

The problem of gastrointestinal nematodes (GIN) in small ruminants remains widespread in both temperate and tropical climates (Hoste et al., 2005), although variation in the prevalence and dominance of the different helminth species differ based on climatic conditions (Torres-Acosta and Hoste, 2008). The incidence of GIN is high in grazing systems during seasons favourable for the survival and development of nematode eggs to the infective larval stage (L_3). The average survival rate of the L_3 in a tropical or subtropical climate is short (1-3 months) compared to 6-18 months in a temperate climate (Torres-Acosta and Hoste, 2008). The control of GIN largely relies on the use of synthetic anthelmintic drugs. However, accessibility or affordability limits the use of anthelmintic drugs in low input production systems. On the other hand, the extensive use of synthetic anthelmintic drugs in intensive production systems (Hoste et al., 2005; Oliveira et al., 2011) has raised consumer concerns of drug residues in food products (Hoste et al., 2012). It also contributed to the development of anthelmintic resistant parasite strains. Alternative, natural anthelmintic sources, such as plant polyphenols, have received more attention in recent years (Oliveira et al., 2011).

Tannins are the most studied group of plant polyphenols natural components with promising anthelmintic properties *in vitro* and *in vivo* in goats (Hoste et al., 2005). The presence of tannins in some common tropical and temperate forages provides the opportunity to exploit these forages as nutraceuticals. There are two hypotheses behind the observed anthelmintic properties of tannins: direct effect by modulating nematode biology, and by boosting host immunity resulting from the increased rumen escape protein to the lower tract (Hoste et al., 2005). The activity of tannins depends on the source, concentration and composition and there appears to be a wide variability among tannins in anthelmintic activity. Recently, reports have also emerged on the anthelmintic properties of polyphenols other than tannins (Hoste et al., 2012).

1.7. Forage polyphenols and enteric methane mitigation

Ruminal fermentation of ingested feed results in the production of microbial biomass, volatile fatty acids (VFA), hydrogen, carbondioxide (CO_2) and methane (CH_4). Acetic acid, butyric acid and propionic acids are the major VFA and the relative proportion is related to dietary composition and the microbes involved in fermentation. Both CO_2 and CH_4 serve as an oxygen and hydrogen sink respectively as a natural process during ruminal fermentation in the rumen. The production of CH_4 is, however, associated with 2-12% of dietary gross energy loss in ruminants and contributes to 11-17% of the global greenhouse gasses (Beauchemin et al., 2009; Gerber et al., 2013). Nutritional strategies are among the many enteric CH_4 mitigation strategies, including increasing the proportion of concentrates in rations, increased use of dietary lipids, use of antimicrobials (e.g. ionophores) (Beauchemin et al., 2008) and nitrate (Klop et al., 2016). However, the practical application of some of these methods could be limited due to toxicity risks or costs.

Alternative use of PSM to mitigate methane has received more attention. Pasture forages rich in PSM such as tannins are of practical importance in extensive, low input ruminant production systems. The mode of action of tannins on CH_4 reduction differs depending on the type of tannin. Hydrolysable tannins have direct inhibitory effects on microbes, while CT act through reduction of fiber digestion with a subsequent lower CH_4 production (Jayanegara et al., 2015). Within the CT group, differences in monomeric units and structure determine the magnitude of CH_4 reduction *in vitro* (Hatew et al., 2014; Huyen et al., 2016).

1.8. Goat production and nutrition in Ethiopia

According to the Central Statistical Agency (CSA, 2015), there are 29.1 million goats in Ethiopia of which 15% are found in the Tigray region. Approximately 2 million goats are exported yearly and 1.1 million goats supplied to the domestic market in Ethiopia (Hirpa and Abebe, 2008). Goats are kept under extensive traditional systems as part of the mixed crop-livestock, specialized pastoral and agropastoral systems (ILRI, 2010). Extensive systems are characterized by limited use of purchased inputs and low productivity per animal (ILRI, 2010). The increasing human population and the resultant need for food crops in Ethiopia are forcing the conversion of grazing lands into croplands (Hirpa and Abebe, 2008). Under such circumstances keeping small ruminants such as goats instead of large ruminants could match the limited available feed resource.

In Ethiopia, goats predominantly obtain feed from grazing lands with only little production of improved forage crops (Yami, 2008). Insufficient feed supply due to the gradual shrinking of pasture lands and a high stocking rate are major problems. Proper utilization of natural browse by plantation, selective bush clearing, and the cut and carry system were suggested to alleviate problems associated with feed availability (Yami, 2008). Gastrointestinal nematode infection, mainly from the abomasal worm *H. contortus* is of high significance in goats (Zewde and Lidetu, 2008). Considering the significant contribution of browse species in the diet of goats in Ethiopia, their efficient utilization for increased goat production can be facilitated by understanding the nature, nutritional and anthelmintic effects of phenolic constituents.

1.9. Aims and scope of the thesis

The overall objective was evaluation of selected browse species for a) preference by goats, b) *in vitro* anthelmintic property against *H. contortus* and c) CH_4 reduction potential. During the conduct of the study involved with aim c, unusual observations were made adding a further aim d) comparison of *in vitro* fermentation patterns of browse species using inocula from goats and cows kept on identical dietary regime.

Preference of 10 browse species by goats was evaluated in a cafeteria style experiment using browse DMI as a proxy in the presence and absence of PEG. Browse species were ranked based on preference and relationships between tannin/phenol composition and browse intake are established (Chapter 2). The browse species were further evaluated for anthelmintic activity *in vitro* in the larval exsheathment inhibition assay using anthelmintic sensitive strain of *H. contortus*. Relationships between inhibition activity and browse polyphenol content and composition are described (Chapter 3). The effect of browse tannins on *in vitro* gas, CH_4 and other fermentation parameters was evaluated using goat rumen inocula. The activity of tannins was monitored using PEG. Proanthocyanidins and other polyphenol composition and concentration were used to describe fermentative activities of browse species (Chapter 4). Results of this chapter indicated a peculiar fermentation characteristics and whether this observation was due to substrate type or the goat inoculum was examined by comparing browse fermentation in goat and cow inocula *in vitro* (Chapter 5) with a selection of tropical browse and temperate feedstuffs. Results of all experiments are discussed in the light of their relationship and practical implication on the use of tannin/polyphenol containing forages and based on overall findings, conclusions are provided (Chapter 6).

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CHAPTER 2

Preference of goats (*Capra hircus* L.) for tanniniferous browse species available in semi-arid areas in Ethiopia

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Abstract

The objectives of the study were to determine browse species preference of goats using dry matter intake (DMI) as a proxy, compare preference when offered in combination with polyethylene glycol (PEG) and to establish relationships between browse species intake and chemical compositional data. Air-dried leaves of *Acacia etbaica*, *Cadaba farinosa*, *Capparis tomentosa*, *Dichrostachys cinerea*, *Dodonaea angustifolia*, *Euclea racemosa*, *Maerua angolensis*, *Maytenus senegalensis*, *Rhus natalensis* and *Senna singueana* were used. Two cafeteria trials, each lasting 10 days were conducted using four local mature male goats of 2-2.5 years receiving a daily ration of grass hay (4% of body weight) and 200 g wheat bran. In trial 1, goats were offered 25 g of each browse species for a total of 30 min with intake, time spent on consumption and the number of visits to specific browse species recorded at 10 min intervals. In trial 2, the same procedure was followed except that 25 g of PEG 4000 was added to the daily wheat bran ration. Crude protein and neutral detergent fibre in browse species ranged from 69.0-245.5 and 159.8-560.6 g/kg dry matter (DM), respectively. Total phenols and total tannins contents ranged between 3.7-70.6 and 2.5-68.1 mg tannic acid equivalent/g DM, respectively, and condensed tannins 1.7-18.4 Abs_{550nm}/g DM. Preference indicators measured in the first 10 min of browse species intake differed significantly among browse species and with PEG ($P<0.0001$). Principal components explained 69.9% of the total variation in browse species DMI. Despite the high tannin levels, *D. cinerea*, *R. natalensis* and *A. etbaica* were the most preferred species regardless of PEG presence. Tannin levels at the observed browse species DMI did not determine preference, instead, preference appeared to be based on hemicellulose. Determining browse species preference is essential to exploit them to improve nutrient utilization and control parasites in goats.

2.1. Introduction

Trees and shrubs commonly known as browse significantly contribute to ruminant nutrition in arid and semi-arid areas of the world. Browse species make up a large proportion of goats diet under traditional farming systems in Ethiopia. Average crude protein (CP) and neutral detergent fibre (NDF) content of nine browse species were 145 and 334 g/kg DM, respectively, making them nutritionally superior to five tropical grasses with 76 g/kg DM CP and 754 g/kg DM NDF, both harvested at the vegetative stage from semi-arid Ethiopia (Yayneshet et al., 2009). Browse species are also recognized for possessing plant secondary metabolites (PSM) such as polyphenols which may account for 50% of the organic matter (Reed, 1986). Over the past four decades, condensed tannins (CT) have received much attention for their beneficial roles in ruminant nutrition. *In vitro* and *in vivo* anthelmintic properties in sheep (Brunet et al., 2007), *in vitro* (Bezabih et al., 2014) and *in vivo* (Puchala et al., 2005) CH_4 reduction in goats and dairy cows (Huyen et al., 2016a) are the widely reported positive attributes of certain tannin-containing tropical and temperate forages. On the other hand, CT concentrations of more than 5% DM have been reported to reduce voluntary feed intake, digestibility and lower productivity of grazing ruminants (Min et al., 2003). However, this threshold level was based on temperate forages and did not account for the type of ruminant species nor the dietary niche or experience with tannin-containing forages of the animal.

Tannin concentrations and structural characteristics vary widely among species (Abdulrazak et al., 2000) and tannin sources (Huyen et al., 2016b). The wide variation in characteristics among browse species provides choices for browsing herbivores such as goats. Preference involves the interaction between sensory input (taste, smell, sight) and postingestive feedback (Provenza, 1996). The latter is non-cognitive and influenced by early life experience. Herbivores routinely exposed to tanniniferous diets usually adapt in terms of behaviour and physiology while satisfying their nutrient requirements. A common adaptation to tannin ingestion in domestic and wild herbivores is increased secretion of proline-rich proteins in saliva to form a complex with tannin (Shimada, 2006). Intrinsically, the saliva of goats is high in proline-rich proteins regardless of previous exposure to tannins (Ventura-Cordero et al., 2015). Another mechanism of herbivores is diet diversification, which also explains the trade-off between ingesting nutritious feed and minimizing detrimental effects of PSM ingestion (Alonso-Díaz et al., 2010). Chemical and physical characteristics of feed, physiological stage and previous dietary experience determine preference in goats (Morand-Fehr, 2003). Preference studies can be used as a tool to evaluate tropical tannin-containing forages (Alonso-Díaz et al., 2010) and the use of a tannin binding agent, polyethylene glycol (PEG) enables the investigation of feeding behaviour of goats browsing on tannin-containing woody species (Decandia et al., 2008). Landau et al. (2002) reported an increased intake of lentisk (*Pistacia lentiscus* L.), a tannin-rich forage, by naturally browsing Mediterranean goats having free access to PEG 4000 flakes compared to their control counterparts that did not receive PEG. In another study, supplementation of 50 g/day of PEG 4000 to Sarda goats browsing in lentisk (*P. lentiscus* L.) dominated Mediterranean scrubland increased the proportion of lentisk in the diet compare to the PEG unsupplemented group (Decandia et al., 2000).

Although the nutritional value of some browse species in the Tigray region of Northern Ethiopia has been studied (Melaku et al., 2010; Yayneshet et al., 2009), information on the preference of these browse species by goats is scarce. Knowledge of the browse species preference of goats is important to develop strategies for effective utilization of browse species growing in sub-Saharan Africa. Accordingly, the objectives of the present study were (1) to determine browse species preference using dry matter intake as a proxy (2) to compare preference for the same browse species when PEG is included in the diet and (3) to establish relationships between browse species intake and browse species components (total tannins, CT, total phenols and gross nutrient composition).

2.2. Materials and methods

2.2.1. Description of study area

Leaves from trees and shrubs were collected from two area exclosures around Abiy Addi town (13°37′23″N and 39°00′06″E) in the Tembein district in northern Ethiopia at an altitude range between 1917 and 2275 m above sea level. The exclosures are protected communal areas where natural vegetation is allowed to recover from previous disturbances by humans and animals since the 1990s (Yayneshet et al., 2008; Yayneshet, 2011). The average annual rainfall during 2003-2012 was 485 mm with average minimum and maximum temperature of 13.5 and 30.1°C, respectively (Tigray Regional Meteorological Agency, personal communication). Subsistence mixed crop-livestock farming is commonly practiced with goats and cattle being the most common livestock species.

2.2.2. Browse species and management

Browse species investigated were *Acacia etbaica* (shrub/tree, Fabaceae), *Cadaba farinosa* (shrub, Capparidaceae), *Capparis tomentosa* (shrub, Capparidaceae), *Dichrostachys cinerea* (shrub, Fabaceae), *Dodonaea angustifolia* (tree, Sapindaceae), *Euclea racemosa* (shrub, Ebenaceae), *Maerua angolensis* (tree, Capparidaceae), *Maytenus senegalensis* (shrub/tree, Celasteraceae), *Rhus natalensis* (tree, Anacardiaceae) *and Senna singueana* (tree/shrub, Fabaceae). From each exclosure, leaves (~1 kg per plant) from 15 phenologically similar (vegetative growth stage) mature plants of the respective species were collected by hand-clipping in October 2014, following the long rainy season (July-September) to allow collection of sufficient material. Immediately after collection, a composite sample was made per plant species and leaves were air-dried under shade and stored, pending further treatment.

2.2.3. Experimental animals management

Four mature male goats of similar age (2-2.5 years) and 14.4±1.07 kg (mean±SD) live weight of the same local breed were bought from local farmers grazing the goats at the same locality from where browse species were obtained. Transport and handling of goats were according to the federal animal handling guideline of the Ministry of Agriculture and Rural Development, Ethiopia. Goats were assigned randomly to individual pens (1.5×1.0 m) located next to each other in a closed housing with sufficient ventilation, and day light at the animal facilities of Mekelle University, Ethiopia. Dry, course wheat straw was used as bedding. Three weeks before commencing the experiment, goats were treated against internal and external parasites using a broad spectrum anthelmintic, Ivermectin injection (Hebel Yuanzheng Pharmaceutical Co., Ltd) and oral administration of Albendazole (Ashish life science Pvt., Ltd.). Vaccination against anthrax (0.5 ml) and multivitamin injection was given subcutaneously. All dosage rates were applied as specified by the manufacturers. During the three weeks before the start of the experiment, each day goats were offered native grass hay *ad libitum* and 200 g wheat bran. Clean water and salt licks were available free of choice before and throughout the experiment.

2.2.4. Treatments and measurement

A cafeteria test was employed using the four goats to study preferences of the 10 browse species. Goats were re-familiarised to all browse species and adapted to eating from 10 identical yellow, removable plastic troughs (38×11×10 cm) prior to the start of the first trial. The adaptation period lasted for 10 days during which two browse species, each 25 g/day was offered simultaneously for 30 min for two consecutive days. Each browse species was randomly distributed over five of the 10 troughs that were positioned side by side on a diagonal platform (68 cm and 43 cm above ground). The order of browse species offer and randomization was kept the same for all goats during the adaptation period. Each time one observer was positioned on the opposite side of each goat's row of troughs to accustom the goat to the observer's presence. At the end of the 30 min adaptation, all browse species were removed and grass hay was provided from a separate feeder positioned opposite of the row of troughs. Goats were tethered using a rope with a sufficient length (1.3 m) to easily reach all troughs and the feeder. The adaptation period was followed by two trials which lasted 10 days period each. In trial 1, grass hay (4% of body weight) and 200 g of wheat bran were divided into two equal portions offered at 8.00 and 15.00 h. During each of the following 10 days, at 10.00 h, the unconsumed grass hay was withdrawn and 25 g of each browse species was provided once for a total of 30 min which was divided into 10 min intervals for intake measurement.

The 10 min measurement ensures that browse species selection is based on browse species characteristics and not on availability (Jansen et al., 2007). It also allows goats to express a pattern of choice during the subsequent 10 min intervals. After each 10 min interval, troughs were removed, browse species were collected and weighed by a digital balance (Voltcraft, CTS-10, Hirschau, Germany) weighing 3 decimal places, before being returned to their previous position in the row of troughs for the next 10 min preference test. Weighing of the browse species took 15 min. Feed intake was calculated as the difference between the quantities offered and remaining as measured. The location of a particular browse species in a trough was randomly determined each day to avoid conditioned learning (i.e. association between feeder position and browse species) (Alonso-Díaz et al., 2008). During browse species preference measurement periods, two observers positioned in front of the goats recorded the length of time a particular browse species was consumed, with two goats observed alternating each day. Intake per visit was calculated as the ratio of total intake of browse species during the 30 min per total number of visits to specific browse species during this time.Visits were recorded only when there was actual consumption of browse species.

Intake rate was calculated as the total amount of specific browse species consumed during 30 min per total time spent eating. The eating time was recorded when goats visited browse species and actual consumption occur. After trial 1, goats were maintained on grass hay (4% of body weight) and wheat bran (200 g) for 10 days whereafter trial 2 was conducted which followed the same procedure except that 12.5 g of PEG 4000 was included in the 100 g wheat bran which was provided twice daily. Over the experimental periods, goats spent only 5-10 min to consume all wheat bran with no difference observed when PEG was included. Samples of browse species, wheat bran and grass hay were collected daily until the end of the two trials, pooled by species/feed type, ground to pass a 1-mm sieve and stored at room temperature awaiting chemical analysis.

2.2.5. Chemical analysis

Feed samples were analysed for dry matter (DM), ash/organic matter (OM) and N following AOAC (1990) protocols with DM determined by oven drying samples (105°C, 3 h), ash determined by incinerating samples in a muffle furnace (550°C, 3 h) and N determined by the Kjeldahl method. Crude protein was calculated by multiplying N with a factor of 6.25. Neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) were analysed according to Van Soest and Robertson (1985). Hemicellulose was calculated as NDF-ADF and cellulose as ADF-ADL.

Total phenols (TP) and total tannins (TT) were determined according to the method of Makkar (2003). Briefly 100 mg of finely ground browse species sample was weighed in a glass test tube and 5 ml 70% aqueous acetone added to extract phenolics. This was done on ice in an ultrasonic bath for 2×5 min whereafter samples were centrifuged, using an Eppendorf centrifuge 5417R, Eppendorf AG, Germany, for 10 min at 5580 g. The supernatant was collected into a glass tube and 0.05 ml diluted with 0.45 ml distilled water before 0.25 ml Folin-Ciocalteu reagent and 1.25 ml sodium carbonate solution was added. After mixing, absorbance of the solution was determined at 725 nm. Tannic acid was used to prepare a standard curve to determine total phenols as tannic acid equivalent (TA eq). Polyvinylpolypyrrolidone (PVPP) was used to bind tannins for total tannins determination. In a test tube, containing 100 mg of PVPP, 1 ml of distilled water and 1 ml of the supernatant, from aqueous acetone extraction mentioned-above, was added and kept at 4°C for 15 min before centrifugation at 5580 g for 10 min. The supernatant containing the simple phenolics other than tannins was collected and absorbance read as before and expressed as tannic acid equivalent. The difference between TP and simple phenols was taken to represent TT expressed as TA eq.

Condensed tannins were determined by the method of Grabber et al. (2013). In brief, acetone-HCl-butanol reagent was prepared from 40 mg of ammonium iron (III) sulfate dodecahydrate, 3.3 ml distilled water, 5 ml 12 M HCl, 42 ml n-butanol and 50 ml acetone. Finely ground browse species sample (30 mg) was weighed into a 25 ml thick-walled glass tube with screw cap and 15 ml of the acetone-HCl-iron reagent added. After heating in a water bath (2.5 h, 70°C) and cooling for 45 min, the reaction mixture was decanted into a 2 ml micro-centrifuge tube which was subsequently centrifuged at 16825 g for 2 min, using a table top centrifuge (Z383, HERMLE, Germany). The supernatant was decanted into quartz cuvettes and absorbance read at 550 nm. Acetone-butanol-iron solution was used as a blank, and as a diluent to keep absorbance reading below 0.6. Condensed tannin values were expressed as absorbance reading per g DM.

Table 2.1. Chemical composition of browse species, grass hay and wheat bran used in cafeteria experiments.

Feed ingredient	DM (g/kg)	Ash	CP	NDF	ADF	ADL	HEMI	CELL	TP*	TT*	CT†
		(g/kg DM)									
Browse species											
A. etbaica	919	69.4	107.2	315.6	242.4	118.3	73.2	124.1	70.6	68.1	11.2
C. farinosa	909	123.0	219.7	255.3	131.0	62.3	124.3	68.7	3.7	2.5	1.7
C. tomentosa	923	109.2	219.9	270.5	188.9	103.5	81.6	85.4	10.2	8.1	6.8
D. cinerea	908	72.5	144.8	427.1	303.4	144.2	123.7	159.2	41.3	38.9	9.3
D. angustifolia	922	53.5	107.8	297.6	216.2	81.9	81.4	134.3	66.2	61.1	16.8
E. racemosa	909	53.1	69.0	560.6	500.6	244.0	60.0	256.6	29.7	28.6	18.4
M. angolensis	910	139.9	245.5	159.8	106.2	30.5	53.6	75.7	7.2	4.6	3.3
M. senegalensis	892	71.0	81.8	467.4	426.3	213.6	41.1	212.7	46.5	32.7	16.9
R. natalensis	915	76.5	113.4	450.9	303.4	134.5	147.5	168.9	44.9	34.5	17.7
S. singueana	923	71.9	140.6	295.4	231.8	69.7	63.6	162.1	41.6	38.5	6.9
Grass hay	926	90.6	73.7	710.5	408.6	62.6	301.9	346.0	-	-	-
Wheat bran	905	46.1	160.7	435.0	129.2	38.0	305.8	91.2	-	-	-

*Calculated as mg tannic acid equivalent/g DM.

†Expressed as Abs_{550nm}/g DM.

ADF, Acid detergent fibre; ADL, Acid detergent lignin; CELL, Cellulose; CP, Crude protein; CT, Condensed tannins; DM, Dry matter; HEMI, Hemicellulose; NDF, Neutral detergent fibre; TP, Total phenols; TT, Total tannins; ADF-ADL=Cellulose; NDF-ADF=Hemicellulose; -, not determined.

2.2.6. Data analysis

Data on dry matter intake (DMI), intake rate and intake per visit were analysed by ANOVA using the PROC MIXED procedure in SAS 9.3 (SAS, 2010) using the model:

$$Y_{ijkl} = \mu + G_i + B_j + P_k + D_l + (B \times P)_{jk} + \varepsilon_{ijkl}$$

where Y_{ijkl}= the dependent variable, μ= the overall mean, G_i= animal effect (i = 1 to 4), B_j= browse species effect (j= 1 to 10), P_k= the effect of PEG (k= + or -), D_l= the effect of day (l= 1 to 10), $(B\times P)_{jk}$= the interaction between browse species and PEG and ε_{ijkl}= the residual error term. Browse species and PEG intake were fixed variables with animal considered as random factor and day used as the repeated measure. The compound symmetry covariance structure was used which generated the least fit statistics according to the Akaike information criterion (AIC) and Bayesian information criterion (BIC). PROC PLM of SAS was used to separate least square means.

Discrimination of the browse species based on browse species chemical components and the relationships among browse species components were established using principal component analysis (PCA) of SAS. Pearson correlation output from PCA analysis was also used to establish relationships between browse species chemical components, and with DMI in the absence and presence of PEG at a statistical significance set at *P*<0.05. Multiple regression analysis was performed using PROC REG procedure of SAS with selection of variables based on backward elimination to determine the best predictor of browse species DMI from browse species chemical components. The independent variables were, CP, ash, hemicellulose, cellulose, ADL, CT, TT and TP. The criteria for selection of variables were a low Mallow's Cp-criterion and high coefficient of determination (R^2).

2.3. Results

All goats remained healthy throughout the experiment. Dry matter intake, intake per visit and intake rate were analysed using data from all goats. Table 2.1 shows the chemical composition of browse species, grass hay and wheat bran. Browse species with the exception of *E. racemosa* contained 81.8 to 245.5 g/kg DM CP, which exceeded that of grass hay (73.7 g/kg DM). Neutral detergent fibre content ranged from 159.8 g/kg DM in *M. angolensis* to 560.6 g/kg DM in *E. racemosa* while grass hay had a higher value (710.5 g/kg DM). *Maerua angolensis* had the lowest ADF and ADL values, while higher values were observed for *E. racemosa*. Condensed tannins concentration was highest in *E. racemosa* (18.4 Abs_{550nm}/g DM) and lowest in *C. farinosa* (1.7 Abs_{550nm}/g DM), whereas TT content was highest in *A. etbaica* (68.1 mg TA eq/g DM) and lowest in *C. farinosa* (2.5 mg TA eq/g DM). Crude protein was negatively correlated with cellulose (*R*=-0.873; *P*=0.001), ADL (*R*=-0.762; *P*=0.011), CT (*R*=-0.817; *P*=0.004), TP (*R*=-0.774; *P*=0.009) and TT (*R*=-0.729; *P*=0.017) (data not shown in table). Positive relationships were found between ADL and cellulose (*R*=0.878; *P*=0.0008) and ADL with CT (*R*=0.744; *P*=0.014) (data not shown in table). Condensed tannins were positively related to TP (*R*=0.650; *P*=0.042) and tended to be positively related to TT (*R*=0.575; *P*=0.082) (data not shown in table). A strong positive relationship was found for TT and TP (*R*=0.984; *P*<0.0001) (data not shown in table).

Browse species DMI during the first 10 min differed (*P*<0.0001) among browse species and with PEG (Table 2.1). Comparable and higher intakes were observed for *A. etbaica, D. cinerea* and *R. natalensis*. A significant (*P*=0.008) interaction was observed between browse species and PEG, but with the exception of *A. etbaica* (*P*=0.012), inclusion of PEG did not affect browse species intake.

Browse species intake as measured during the three consecutive 10 min periods is shown in Fig. 2.2. During the first 10 min, goats predominantly consumed *D. cinerea*, *R. natalensis* and *A. etbaica* regardless of PEG presence. Remaining portions of these browse species were eaten during the second 10 min, but also with increasing intake of *M. senegalensis* and *E. racemosa*. In comparison, only negligible quantities of the remaining browse species, predominantly *E. racemosa*, were consumed during the last 10 min.

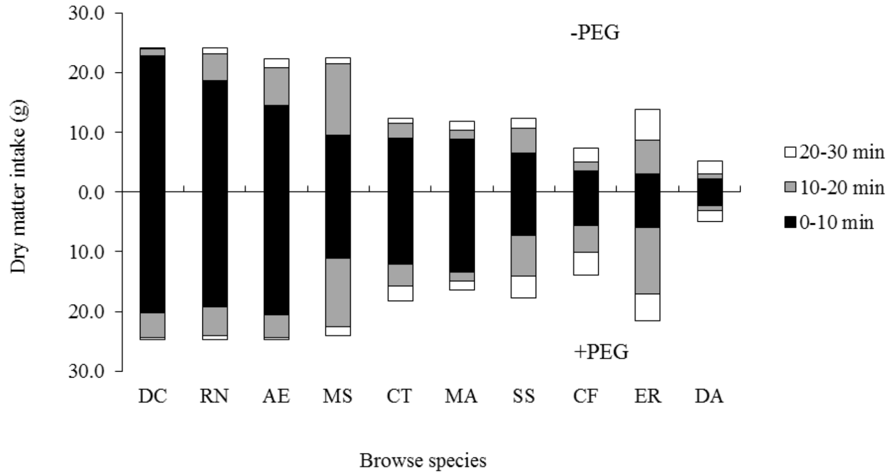

Figure 2.1. Mean browse intake of goats during three subsequent 10 min interval in the absence (-) and presence (+) of polyethylene glycol (PEG). DC, *D. cinerea*; RN, *R. natalensis*; AE, *A. etbaica*; MS, *M. senegalensis;* CT, *C. tomentosa;* MA, *M. angolensis*; SS, *S. singueana*; CF, *C. farinosa*; ER, *E. racemosa*; DA, *D. angustifolia*.

At the end of the 30 min, significant ($P<0.0001$) differences in browse species intake were observed (Table 2.2). Intake was increased by PEG inclusion ($P<0.0001$) and days ($P<0.0003$) as shown in Table 2.2. An interaction existed between browse species and PEG ($P=0.0001$). Comparable quantities of *A. etbaica*, *D. cinerea*, *M. senegalensis* and *R. natalensis* were consumed irrespective of PEG inclusion. PEG significantly increased ($P\leq0.036$) the intake of *E. racemosa*, *S. singueana*, *C. tomentosa* and *C. farinosa*. Intake per visit varied significantly among browse species ($P<0.0001$) and showed a significant browse species and PEG interaction ($P=0.006$). Intake per visit was highest in *D. cinerea*, *R. natalensis* and *M. senegalensis* in the absence of PEG. However, none of the browse species showed significant differences in intake per visit in the presence and absence of PEG. Intake rate was different ($P\leq0.027$) among browse species, PEG treatment and day. Similar intake rates were observed among browse species but with lowest rate of *C. farinosa* and *D. angustifolia* in the absence of PEG. In the presence of PEG, *S. singueana* had the highest and *E. racemosa* the lowest intake rate.

Table 2.2. Mean dry matter intake, intake per visit and intake rate of browse species by goats in the absence (-) and presence (+) of polyethylene glycol (PEG).

Item	Intake (g DM/10 min)*			Intake (g DM/30 min)†			Intake per visit (g DM/visit)†			Intake rate (g DM/min)†		
	(-)	(+)	*P*	(-)	(+)	*P*	(-)	(+)	*P*	(-)	(+)	*P*
Browse species												
Dichrostachys cinerea	22.8^{a}	20.3^{a}	0.988	24.3^{a}	24.8^{a}	1.000	9.4^{a}	6.2^{a}	0.522	11.3^{a}	11.0^{ab}	1.000
Rhus natalensis	18.6^{ad}	19.2^{ab}	1.000	24.2^{a}	24.8^{a}	1.000	8.5^{a}	7.8^{a}	1.000	7.8^{a}	9.7^{ab}	1.000
Acacia etbaica	14.5^{ade}	20.6^{a}	0.012	22.4^{a}	24.9^{a}	0.986	8.5^{a}	5.3^{ab}	0.473	8.5^{a}	13.3^{ab}	0.985
Maytenus senegalensis	9.5^{bce}	11.1^{bde}	1.000	22.5^{a}	24.1^{a}	1.000	6.0^{ab}	4.7^{b}	1.000	8.2^{a}	8.7^{ab}	1.000
Capparis tomentosa	9.1^{bce}	12.2^{bd}	0.895	12.4^{bc}	18.3^{bc}	0.014	2.3^{bc}	3.7^{bc}	0.999	8.3^{a}	11.7^{ab}	0.999
Maerua angolensis	8.8^{cde}	13.5^{bc}	0.218	11.8^{bc}	16.5^{cd}	0.173	3.5^{b}	4.4^{b}	1.000	6.5^{a}	7.9^{ab}	1.000
Senna singueana	6.6^{def}	7.3^{de}	1.000	12.5^{bc}	17.9^{bc}	0.036	2.7^{bc}	4.9^{b}	0.961	7.5^{a}	16.0^{a}	0.231
Cadaba farinosa	3.5^{df}	5.7^{d}	0.998	7.5^{cd}	14.0^{d}	0.003	2.5^{bc}	3.3^{bc}	1.000	3.9^{b}	8.5^{ab}	0.987
Euclea racemosa	3.1^{f}	5.9^{d}	0.949	13.9^{b}	21.7^{ab}	<0.0001	3.3^{bc}	5.4^{ab}	0.981	6.5^{a}	5.1^{b}	1.000
Dodonaea angustifolia	2.2^{f}	2.2^{e}	1.000	5.2^{d}	5.0^{e}	1.000	1.9^{bc}	1.5^{c}	1.000	5.3^{b}	7.2^{ab}	1.000

	SEM	*P*	SEM	*P*	SEM	*P*	SEM	*P*
Browse species	1.186	<0.0001	1.296	<0.0001	0.613	<0.0001	1.778	0.009
PEG	0.965	<0.0001	1.113	<0.0001	0.274	0.722	1.252	0.005
Day	1.186	0.235	1.296	0.0003	0.613	0.041	2.081	0.027
Browse species×PEG	1.414	0.008	1.493	0.0001	0.867	0.006	2.223	0.387

	(-)	(+)	SEM	*P*
Total browse species (g DM)*	98.5	117.9	3.12	0.0002
Grass hay (g DM/day)	406.3	451.3	12.61	0.006
Wheat bran (g DM/day)	200.0	200.0	-	-

DM, Dry matter; SEM, Standard error of the mean.

Means within a column with different superscripts differ significantly (P<0.05).

* during the first 10 min.

† during 30 min.

The principal component analysis bi-plot (Fig. 2.2) shows the relative influence of browse species chemical components and intake on the clustering of browse species. The two principal components explained 69.9% of the total variation, with PC1 explaining 48.8% and PC2 21.1% of the variation. A close association between TT, TP, CT, ADL and cellulose was shown. However, the association between these parameters and intake was less evident. Hemicellulose seemed to be closely related to intake in the presence and absence of PEG. An inverse relationship was observed between CP and CT, TT, TP, ADL, cellulose. Clustering of the browse species showed three distinct groups: the most preferred but with high TT and TP content in group I, fibrous and CT rich browse species in group II, and browse species with low phenolic components but high CP and ash in group III.

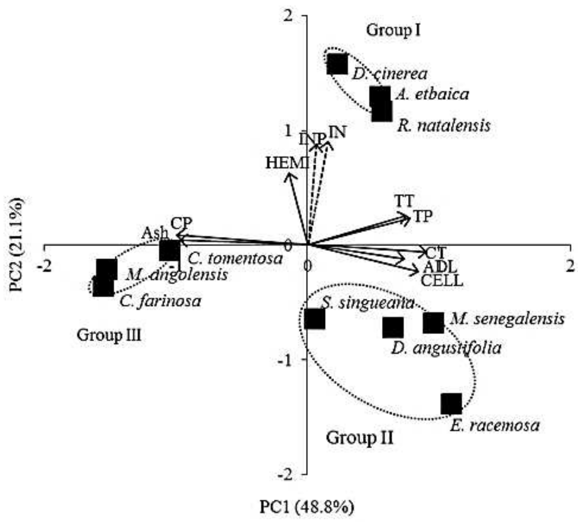

Figure 2.2. Principal component analysis (PCA) bi-plot displaying the position of browse species in relation to browse chemical components and intake. ADL, Acid detergent lignin; CELL, Cellulose; CP, Crude protein; CT, Condensed tannins; TP, Total phenols; TT, Total tannins; HEMI, Hemicellulose; IN, Browse dry matter intake in the absence of polyethylene glycol (PEG); INP, Browse dry matter intake in the presence of PEG.

Table 2.3 shows results of the multiple regression models for the prediction of browse species intake based on chemical composition including phenol and tannin components. From the phenolic groups, TP and TT contributed significantly to browse species intake in the absence and presence of PEG, respectively. The regression model shows that CP, hemicellulose, cellulose, ADL and TP in the absence of PEG positively contributed to intake. Similarly, in the presence of PEG, the same parameters contributed positively to intake except for the phenolic groups where TT replaces TP.

Table 2.3. Multiple linear regression equation for the relationship between goats' browse species intake and browse species chemical components measured during the first 10 min in the absence (-) and presence (+) of polyethylene glycol (PEG).

PEG	Intercept	CP	ASH	HEMI	CELL	ADL	TP	TT	*P*
(-)	-169.508	0.425	0.300	0.143	0.253	0.120	0.874		<0.0001
SE	25.95	0.08	0.11	0.03	0.06	0.03	0.13		
P	<0.0001	<0.0001	0.0098	<0.0001	<0.0001	0.0004	<0.0001		
(+)	-153.926	0.231	0.645	0.120	0.211	0.119		0.875	0.001
SE	36.49	0.09	0.18	0.04	0.08	0.04		0.18	
P	0.0002	0.027	0.0008	0.002	0.011	0.007		<0.0001	

ADL, Acid detergent lignin; CELL, Cellulose; CP, Crude protein; HEMI, Hemicellulose; SE, Standard error; TP, Total phenols; TT, Total tannins

2.4. Discussion

2.4.1. Browse species chemical and phenolic composition

In general, browse species have a superior protein content compared to low quality herbaceous species such as grasses in natural free range ruminant production systems (Yayneshet et al., 2009). Comparable CP values were reported for most browse species collected from semi-arid areas in Kenya and Ethiopia (Abdulrazak et al., 2000; Yayneshet et al., 2009). *Maerua angolensis* had the lowest NDF content similar to findings of Osuga et al. (2008). The low fibre content of this species in combination with its high protein content is a desirable characteristic from a nutritional point of view. In comparison, *E. racemosa* and *M. senegalensis* contained higher NDF, ADF and ADL than the other browse species as also reported by Yayneshet et al. (2009), a characteristic which can limit browse species intake or preference. The high fibre contents of these two browse species, however, may in part be due to the presence of insoluble tannin-protein complexes (Reed, 1986) which are not taken into account by currently available fibre analyses (e.g. Van Soest's detergent system). Total phenol content tended to be associated with corresponding levels of total tannins which agrees with previous observations in Acacia species (Abdulrazak et al., 2000). The presence of polyphenolic compounds in plants is widely accepted as a mechanism to deter herbivory and, generally, influences browse species selection by animals. Total tannins accounted for 60-96% of the total phenols and similar percentages of 54-98% were reported for tannin-containing tropical leaves from Kenya (Abdulrazak et al., 2000) and India (Bhatta et al., 2012). The positive correlation between fibre fractions and CT was in line with previous results in tropical browse species (Abdulrazak et al., 2000;

Kaitho et al., 1998; Melaku et al., 2010) but disagrees with reports on Spanish shrubs (Frutos et al., 2002). Differences in tannin concentration among species (Abdulrazak et al., 2000; Osuga et al., 2008) or forage maturity stage (Koupai-Abyazani et al., 1993) may explain this discrepancy. Crude protein and fibre content of the grass hay in the present study was comparable to values reported for grasses harvested from rangelands during the vegetative growth stage in a similar climatic area (Yayneshet et al., 2009).

2.4.2. Browse species preference as measured by dry matter intake

Intake measurements were divided into three consecutive 10 min intervals with the first 10 min measurement used to compare preference among browse species as the subsequent 10 min periods are confounded as unequal amounts of browse species were available to the animals. Given the range of tannin concentration in browse species, preference was not against the high tannin-containing browse species. Despite the high tannin and phenol contents, intakes of *D. cinerea*, *R. natalensis* and *A. etbaica* were higher and considered as the most preferred species, similar to the reports by Yayneshet et al. (2008). The latter authors allowed goats to browse naturally while in the study described here, goats were offered air-dried browse species. Drying increases forage cell wall fractions which may contribute to reduced intake of the same forage by ruminants.

Diet selection by ruminants is associated with reducing the risk of over ingesting toxic forage components or the need to meet nutritional requirements. Provenza (1996), however, indicated that neither could be the case and preference is related to the association between sensory input upon feed apprehension and postingestive effects which occurs involuntarily. This may partly explain the observation that goats prefer for high tannin/phenol containing browse based on a previous learned experience. Goats in the present experiment were bought from the same area where browse species were obtained, and therefore they had previous exposure to the browse species. As such, the animals were experienced and previously had learned to consume a mixture of nutritious as well as potentially toxic plants (Provenza et al., 2003). The goats' previous exposure to the browse species also suggests that a non-cognitive feedback mechanism was established based on past experience, and that decision on preference during the current experiment was based on sensory feedback signals; a non-cognitive phenomenon as described by Provenza (1996). However, in non-experienced animals the feedback mechanism has to be first established and decision on preference would not be on such mechanism. Thus, past experience and the associated adaptive behaviours of the goats with the apparent positive (or not negative) feedback may partly contribute to preferences for the high tannin browse species in the present experiment. Similar to our observation, Jansen et al. (2007) reported selection of

Acacia species by goats with high tannin content regardless of the presence of low tannin content alternatives. Moreover, these authors indicated that tannin contents of the Acacia species were tolerated to a threshold level while minimizing ADF. In the present study, selection against the lower tannin/phenol, high CP and low fibre in *C. farinosa*, *C. tomentosa* and *M. angolensis* could suggest a deliberate selection by goats for higher NDF intake while minimizing ADF intake; and also the additional benefits from tannins in increasing rumen escape protein. Since the intakes of other high tannin/phenolic rich browse species (e.g. *D. angustifolia* and *E. racemosa*) were limited, this could indicate that tannin/phenol levels were minimized to a certain threshold level.

The voluntary intake for the high tannin concentration could be attributed to the natural tendency of proline-rich protein in the saliva of goats to attenuate the negative effects of tannins. Saliva of Criollo goats from Mexico (Ventura-Cordero et al., 2015) and Damascus and Mamber goats of the Mediterranean region (Hanovice-Ziony et al., 2010) exhibited tannin binding characteristics regardless of a prior tannin stimulus indicating that goats are naturally tolerant to relatively high level of tannins. Supplementation of PEG increased total browse species and hence tannin intake as found in previous studies with goats browsing on a tannin-rich forage, *Pistacia lentiscus* L. (Decandia et al., 2000; Landau et al., 2002) as well as a quebracho tannin supplement in lambs (Titus et al., 2000). The effect of PEG in increasing intake could be related to positive effects on digestion in the rumen rather than astringency since PEG exerts effect in the rumen (Silanikove et al., 1996). However, preference for individual browse species was not affected by PEG addition as also found by Hernández-Orduño et al. (2012) in short-term trials with goats that had previous browsing experience.

2.4.3. Browse species intake, intake per visit and intake rate during 30 min

The higher consumption of *A. etbaica*, *D. cinerea* and *R. natalensis* during the first 10 min, regardless of PEG presence, is likely to have had effect on the intake of the remaining browse species. In the second 10 min, increased consumption of *M. senegalensis*, *E. racemosa* followed by *S. singueana* was observed. It is noteworthy that the goats still maintained a preference for browse species with high tannin levels supporting the existence of a tannin intake threshold level (Jansen et al., 2007). The increase in intake for *M. senegalensis* and *E. racemosa* in the second 10 min suggests transition to more fibrous browse species as the second choice, despite the accompanied high tannin levels. When tannin intake is kept below the threshold level, intake of tannin-containing forages is a function of the fibre fraction (Alonso-Díaz et al., 2008; Jansen et al., 2007). The significant increase in *E. racemosa* intake after PEG addition might be associated with its high tannin content neutralized by PEG. Although the concentration of CT in *S. singueana*, *C. tomentosa* and *C. farinosa* are relatively low, their level of intake increased significantly after inclusion of PEG.

The presence of PEG reverses the beneficial role of tannin-protein complex in supplying protein to the lower tract and therefore, it appeared that goats targeted browse species with high protein but still with reasonable NDF contents, such as *S. singueana*, *C. tomentosa* and *C. farinosa*. Besides, in these species preference may be influenced more by tannin composition, structural configuration and degree of polymerization associated with sensory properties (Robichaud and Noble, 1990). Although *M. angolensis* had the highest CP content, its consumption was not increased by PEG and this could be associated with its lower NDF content. The effect of day mainly arose from an increasing trend in intakes of *E. racemosa*, *S. singueana*, *C. tomentosa* and *C. farinosa*.

The interaction between browse species and PEG indicates an effect of PEG on intake differed with species of browse. Of note is that the highly preferred species had higher intake per visit showing goats maximize intake of the preferred browse species per visit. This result is contrary to the general characteristics of herbivores to reduce polyphenolic intake by reducing meal size (Torregrossa and Dearing, 2009). The discrepancy could suggest that tannin/phenol levels of the browse species did not influence intake per visit. The relationship between intake and intake rate revealed that goats spent more time on the most preferred species and, therefore, targeted species with higher intake rates as reported with grasses (Illius et al., 1999).

2.4.4. Principal component analysis for browse species components and intake prediction

The principal component analysis clustered browse species into three groups. The positive association between TP and tannin contents with fibre fractions was in line with earlier work on a range of browse species (Kaitho et al., 1998). The inverse relationship between CP and CT, TT and TP could be due to the formation of tannin-protein complexes increasing the proportion of indigestible CP (Reed, 1986). A close relationship between browse species intake and hemicellulose was observed which may suggest that the preference for browse species was rather based on the digestible fibre fraction. The positive association between tannin-containing forages intake and their digestible fibre fractions was reported in goats (Alonso-Díaz et al., 2008).

The contribution of CT and TT was not evident in the prediction of browse species intake in the absence of PEG. This result also substantiates the observation that discrimination of browse species by goats was not primarily based on tannins, under the conditions of the present experiment. However, TP contributed to the prediction of

DMI indicating phenolic components other than tannins may have influence on browse species intake. In the presence of PEG, the contribution of TT to browse species DMI could be associated with the increase in intake resulting from neutralization of tannins. The association between browse species intake and phenols was in line with results of Alonso-Díaz et al. (2008). However, the positive association between intake and TP was contrary to the findings of Alonso-Díaz et al. (2008) which could be explained by the apparent preference of goats for high tannin/phenol browse species in the present experiment.

2.5. Conclusions

The 10 browse species investigated were distinct in chemical composition (including phenols and tannins) and contained a high crude protein content allowing supplementation of grass hay. Independent of PEG supplementation, the goats preferred high tannin-containing browse species, *D. cinerea*, *A. etbaica*, and *R. natalensis*. Our results suggest the possibility that goats tend to ingest a certain level of tannins while targeting maximization of intake of other nutrients such as digestible fibre fraction (e.g. hemicellulose) and crude protein. Therefore, tannin content did not primarily determine the preference of goats that have been pre-exposed to tannin-containing forages early in life.

2.6. Acknowledgments

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CHAPTER 3

The *in vitro* anthelmintic properties of browse species against *Haemonchus contortus* is determined by the polyphenol content and composition

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Abstract

The aims of the present study were to (a) evaluate the anthelmintic activity of 10 East African browse extracts, (b) examine their role in inhibition of *Haemonchus contortus* larval exsheathment, (c) establish relationship between inhibition of larval exsheathment and browse extract polyphenol composition. Acetone/water (70/30%) extracts of air-dried leaves of *Acacia etbaica*, *Cadaba farinosa*, *Capparis tomentosa*, *Dichrostachys cinerea*, *Dodonaea angustifolia*, *Euclea racemosa*, *Maerua angolensis*, *Maytenus senegalensis*, *Rhus natalensis* and *Senna singueana* were used. The larval exsheathment inhibition assay (LEIA) was applied using *H. contortus* third stage larvae (L_3) and extract concentrations of 0, 150, 300, 600, 1200 μg/ml phosphate buffered saline (PBS) with four replicates per concentration. Data were analysed using the PROC MIXED procedure of SAS. Polyvinylpolypyrrolidone (PVPP) was used to evaluate whether polyphenols were involved in L_3 exsheathment inhibition. All browse extracts significantly ($P \leq 0.0001$) inhibited larval exsheathment in a dose dependent manner. The dose required to inhibit 50% of the larvae (EC_{50}) was highest in *C. farinosa* and lowest in *E. racemosa* and *M. senegalensis*. Significant differences ($P<0.001$) between the control and PVPP treated *A. etbaica*, *C. tomentosa*, *M. angolensis*, *R. natalensis* and *D. cinerea* indicates that larval inhibition was largely due to non-phenolic compounds. For *E. racemosa*, *M. senegalensis*, *D. angustifolia* and *S. singueana*, PVPP treatment reversed inhibition activity and in these extracts, inhibition was mostly attributable to tannin and other polyphenols (kaempferol, quercetin and myricetin based aglycones and glycosides). Overall, browse extracts have anthelmintic property against *H. contortus* and larval inhibition resulting from the presence of phenolic and non-phenolic compounds.

3.1. Introduction

Economic losses due to infections with gastrointestinal nematodes (GIN) represent a major challenge for small ruminant producers in outdoor grazing systems (FAO, 2002). Impaired productivity in infected goats and sheep results from reduction in voluntary feed intake with the associated decrease in nutrient availability, nutrient absorption and efficiency (Coop and Kyriazakis, 2001). Sheep and goats have important economic roles in tropical and subtropical climates (FAO, 2002; Jackson et al., 2012), and the combination of high temperature and rainfall in these areas provides a conducive environment for the development of the infective larval stage of nematodes (Waller, 1997). Until recently, the control of parasitic GIN in small ruminants has mainly been based on the quasi-exclusive reliance through the use of available synthetic, commercial drugs (Jackson et al., 2012; Oliveira et al., 2011). However, the rapid development of anthelmintic resistance, which is defined as the decline in heritable sensitivity of parasites upon drug application (Conder and Campbell, 1995) in parasite populations, is resulting in failure of the efficacy of these drugs (Jackson et al., 2012). *Haemonchus contortus* is the most commonly occurring resistant parasite due to its highly prolific nature, prompting establishment on pasture within a short time (Conder and Campbell, 1995; Waller and Chandrawathani, 2005). The use of anthelmintic drugs also raises public health concern (Oliveira et al., 2011). Moreover, in developing countries, the use of anthelmintic drugs may be limited due to inaccessibility or high cost (Debela et al., 2012). Forages possessing plant secondary metabolites (PSM) have potential use as alternative natural treatments used either as herbal drugs or nutraceuticals (Oliveira et al., 2011). Such an approach is of practical value under low input small ruminant production systems by providing a practical, sustainable (Sokerya and Preston, 2003; Max et al., 2007) and affordable (Salem and Smith, 2008) alternative control of GIN.

Forages containing condensed tannins (CT) are the most widely studied models as nutraceuticals, with combined beneficial effects on nutrition and health of small ruminants (Hoste et al., 2012; 2015). Previous *in vivo* and *in vitro* studies with a range of tannin-containing forages have demonstrated anthelmintic effects against *H. contortus* by reduction of larvae establishment (Brunet et al., 2008; Oliveira et al., 2011) and reduction in egg excretion in goats e.g. when given quebracho tannins (Paolini et al., 2003). These anthelmintic properties are attributable to the ability of PSM such as CT to boost host resilience and/or modulate nematode biology. Although CT were proven to have anthelmintic properties, their mode of action, which also seems to vary on the diverse nature of tannins depending on the source and chemical structure, still remain insufficiently identified.

Condensed tannins (proanthocyanidins, PA) represent one of the common groups of tannins, and are oligomers and polymers of flavan-3-ol monomer units. Proanthocyanidins could be grouped into six main classes (Salminen and Karonen, 2011). Procyanidins (PC) are the most common PA containing (+)-catechin with 2*R*,3*S* stereochemistry and (-)-epicatechin with 2*R*,3*R* stereochemistry as monomeric units; while prodelphinidins (PD) consist of gallocatechin and epigallocatechin monomeric units. Propelargonidins (PP) contain afzelechin and epiafzelechin, profisetinidins (PF) fisetinidols and epifisetinidols, prorobinetinidins (PR) robinetinidols and epirobinetinidols, and proguibourtinidins (PG) guibourtinidols and epiguibourtinidols. The PF, PR and PG, possess the rarer monomeric 5-deoxy units of CT in their structure (Salminen and Karonen, 2011). In addition to the six main PA classes, some less common PA classes also exist. Some previous work reported the association between PA composition and anthelmintic properties. A marked reduction on *H. contortus* L_3 exsheathment *in vitro* has been associated with the PD monomers and with the galloyl derivatives of tannins (Brunet and Hoste, 2006). Quijada et al. (2015) reported that the building units of PA and the polymer size influenced anthelmintic properties in *in vitro* larval exsheathment of *H. contortus*.

Another important tannin group consists of hydrolysable tannins (HT) which are esters of gallic acid and a polyol, which in most cases is D-glucose. Hydrolysable tannins are divided into three subclasses, i.e. simple gallic acid derivatives, gallotannins and ellagitannins. Variability of HT in the size, type and monomeric unit linkages have also been associated with anthelmintic properties measured by the inhibition of egg hatching and larval motility of *H. contortus* (Engström et al., 2016). Moreover, the presence of co-occurring polyphenols or non-phenolic compounds in tannin-containing forages may contribute to the anthelmintic properties of plant extracts (Azando et al., 2011; Azaizeh et al., 2013; Barrau et al., 2005). Polyphenols of interest in anthelmintic studies other than tannins, may include, quinic acid derivatives, quercetin-based flavonol glycosides, kaempferol-based flavonol glycosides and myricetin-based flavonol glycosides. Therefore, for the efficient exploitation of polyphenol composition of tannin-containing forages, the simultaneous analysis of PA concentration and composition and the other phenolic compounds is required. Such information would also enable to make comparisons on anthelmintic properties of polyphenols across studies.

The objectives of the present work were (a) to evaluate the anthelmintic activity of a range of browse extracts from eastern Africa using an *in vitro* assay measuring the exsheathment of *H. contortus* L_3 larvae in a dose dependent manner, (b) to examine the role of tannins and other polyphenols in the inhibition of *H. contortus* exsheathment using a tannin binding agent, polyvinylpolypyrrolidone (PVPP), (c) to establish the relationship (if any) between browse polyphenol content and composition with the inhibition of L_3 exsheathment, so as to provide a model to better understand the anthelmintic activity in relation to polyphenol composition.

3.2. Materials and methods

3.2.1. Browse collection and handling

Leaves (Table 3.1) of *Acacia etbaica*, *Cadaba farinosa*, *Capparis tomentosa*, *Dichrostachys cinerea*, *Dodonaea angustifolia*, *Euclea racemosa*, *Maerua angolensis*, *Maytenus senegalensis*, *Rhus natalensis* and *Senna singueana* were collected by hand-clipping from semi-arid Tigray region of Ethiopia at the end of the long rainy season (July-September) in 2014. Immediately after collection leaves were air-dried under shade, stored and ground to pass a 1-mm sieve. Specimens of the browse species were mounted on a placard and sent to the national herbarium institute of Addis Ababa University, Ethiopia for identification.

3.2.2. Extraction procedure

Ground browse samples were extracted with 70/30% acetone/water (1/4, w/v). The supernatant has been further treated with dichloromethane (1/3, v/v) to remove lipids and chlorophyll, freeze dried and stored until use.

3.2.3. Chemical analysis

For chemical analysis, ground browse samples, were freeze-dried (Christ beta 2-8 LD plus, Martin Christ, Germany) and ground into a fine powder (Retsch MM200, Sigma-Aldrich, US). For each browse, approximately 20 mg of sample was weighed in triplicate in Eppendorf, vortexed with 1.4 ml of acetone/water (4:1, v/v) for 5 min and allowed to macerate at 4^{o}C overnight. Then the samples were extracted in a planar shaker for 3 h and centrifuged at 14000 rpm for 10 min. The residues were further extracted with 1.4 ml of acetone/water (4:1, v/v) for 3 h and centrifuged at 14000 rpm for 10 min. The extracts were combined and concentrated into the water phase and freeze-dried. The freeze-dried extracts were dissolved in 1 ml of water.

Proanthocyanidins contents were analysed as previously described by Grabber et al. (2013). The analysis of polyphenols was conducted according to Engström et al. (2014). Total PA were analysed directly from the browse materials. Soluble PA were analysed from the browse extracts and the insoluble, i.e. bound, PA from the extraction residues. Proanthocyanidins were quantified against an external PA standard (extracted from *Calluna vulgaris*) which had a PC/PD ratio of 99:1 and a mean degree of polymerization of 4.9.

Proanthocyanidins composition was determined by an ultra-performance liquid chromatograph coupled to a photodiode array detector (UPLC-DAD, Acquity UPLC, Waters Corporation, Milford, MA, USA) and a hybrid quadrupole-Orbitrap mass spectrometer (Q Exactive™ MS, Thermo Fisher Scientific GmbH, Bremen, Germany). The browse extracts were diluted 10 times and filtered through 0.2 µm PTFE syringe filters prior the analysis. The column was an Acquity UPLC® BEH Phenyl (100×2.1 mm i.d.; 1.7 µm; Waters Corporation, Wexford, Ireland). The mobile phase consisted of (A) acetonitrile and (B) 0.1 % formic acid. The elution profile was as follows: 0–0.5 min, 0.1% A in B; 0.5–5.0 min, 0.1–30% A in B (linear gradient); 5.0–5.1 min, 30–90% A in B (linear gradient); 5.1–7.1 min, 90% A in B; 7.1–7.2 min, 90–0.1% A in B (linear gradient); 7.2–8.5 min, 0.1% A in B. The injection volume was 5 µl and flow rate 0.5 ml/min. The UV data was collected at 190-500 nm. The heated electrospray ion source (H-ESI II, Thermo Fisher Scientific GmbH, Bremen, Germany) was operated in negative ion mode. The parameters were as follows: spray voltage was set at –3.0 kV, sheath gas (N_2) flow rate at 60 (arbitrary units), aux gas (N_2) flow rate at 20 (arbitrary units), sweep gas flow rate at 0 (arbitrary units), capillary temperature at +380°C and S-lens RF level at 60. The Orbitrap was set to a resolution of 70,000 and an automatic gain of 3×106 was used. Masses were scanned at *m/z* 150–2000. Pierce ESI Negative Ion Calibration Solution (Thermo Fischer Scientific Inc., Waltham, MA, USA) was used for the calibration. The data was processed with Thermo Xcalibur Qual Browser software (Version 3.0.63, Thermo Fisher Scientific Inc., Waltham, MA, USA). Other polyphenols were analysed by UPLC-ESI-MS/MS as previously described by Engström et al. (2015), with extracts diluted 10 times and filtered through 0.2 µm PTFE syringe filters prior the analysis.

3.2.4. Larval Exsheathment Inhibition Assay (LEIA)

The larval exsheathment inhibition assay as described by Jackson and Hoste (2010) was used to measure the anthelmintic properties of the browse extracts. Briefly, approximately 1000 ensheathed infective larvae (L_3) from susceptible strains of *H. contortus* were incubated for 3 h in browse extracts at concentrations of 1200, 600, 300, 150 µg/ml in PBS, (Phosphate Buffered Saline, 0.1 M phosphate, 0.05 M NaCl, pH 7.2). A negative control (L_3 only in PBS) was added to the assay. Four replicates were included for each dose and for the control. At the end of incubation larvae were washed and centrifuged for 3 min at 1000 rpm three times. Finally, the L_3 were submitted to the process of artificial exsheathment by contact with a solution containing sodium hypochlorite (2% w/v) and sodium chloride (16.5% w/v) after dilution with PBS (1/400). Then larvae were examined under a microscope at ×200 magnification to identify the proportion of exsheathed L_3 at 0, 20, 40, and 60 min after contact with the exsheathment solution.

Percentage exsheathment was calculated as follows:

$$\text{Exsheathment \%} = \frac{\text{number of exsheathed larvae}}{\text{number of exsheathed larvae} + \text{number of ensheathed larvae}} \times 100$$

In order to confirm the role of tannins and polyphenols, polyvinylpolypyrrolidone (PVPP, 50 mg/ml PBS) was used (Barrau et al., 2005). The extracts were tested at the concentration of 1200 μg /ml PBS with or without PVPP plus a control in four replicates. First extracts were pre-incubated with PVPP for 3 h before coming in contact with L_3.

3.2.5. Statistical analysis

Data on dose response and polyphenol effect on larval exsheathment were analysed using the PROC MIXED procedure in SAS 9.3 (SAS., 2010). Extract doses were further analysed for orthogonal polynomial contrasts, linear and quadratic. Dose contrasts were unequally spaced, and hence the PROC IML procedure of SAS was used to generate coefficients. The 50% effective extract concentration to inhibit exsheathment (EC_{50}) was calculated using PoloPlus 1.0 (LeOra Software, 2002).

Table 3.1. Proanthocyanidins and other polyphenol contents of browse materials.

Browse species name		Proanthocyanidins (mg/g DM)			Galloyl derivatives	Quinic acid derivatives	Flavonol aglycones and glycosides (mg/g DM) ± SE		
Scientific	Family	Total	Soluble	Bound	(mg/g DM) ± SE		Kaempferol based	Quercetin based	Myricetin based
E. racemosa	E	≥200	101-150	51-100	2.1 ± 0.1	-	0.3 ± 0.1	1.3 ± 0.1	7.1 ± 0.1
R. natalensis	A	≥200	≥200	101-150	0.2 ± 0.1	2.0 ±0.1	0.7 ± 0.1	5.8 ± 0.1	1.8 ± 0.1
M. senegalensis	Ce	≥200	≥200	101-150	<0.1	-	4.7 ± 0.1	4.4 ± 0.1	1.3 ± 0.1
D. cinerea	F	101-150	101-150	5-50	6.9 ± 0.1	-	1.4 ± 0.1	7.7 ± 0.1	6.0 ± 0.1
D. angustifolia	S	51-100	51-100	5-50	0.1 ± 0.1	0.4 ± .1	1.1 ± 0.1	1.5 ± 0.1	-
A. etbaica	F	5-50	5-50	0-4	0.9 ± 0.1	-	0.7 ± 0.1	5.5 ± 0.1	0.2 ± 0.1
S. singueana	F	5-50	5-50	-	-	-	9.0 ± 1.6	3.0 ± 0.6	0.2 ± 0.1
C. tomentosa	Ca	0-4	-	-	-	-	2.1 ± 0.1	2.0 ± 0.1	-
C. farinosa	Ca	-	-	-	-	-	-	0.5 ± 0.1	-
M. angolensis	Ca	-	-	-	-	-	0.4 ± 0.1	0.5 ± 0.1	-

A, Anacardiaceae; Ca, Capparidaceae; Ce, Celasteraceae; E, Ebenaceae; F, Fabaceae; S, Sapindaceae.
SE,Standard error; DM, Dry matter.
-, not detected.

Table 3.2. Dose dependent effect of browse extracts on the artificial exsheathment (%) and EC_{50} values for *H. contortus* infective larvae (L_3).

Browse species	Family name	Dose (µg/ml PBS)					SEM	*P*-value		EC_{50}	Confidence interval (95%)	
		0	150	300	600	1200		Linear	Quadratic		Lower (µg/ml)	Upper (µg/ml)
E. racemosa	*Ebenaceae*	94.4	24.8	0.0	0.0	0.0	4.09	<0.0001	<0.0001	UD	-	-
R. natalensis	*Celasteraceae*	99.2	59.8	18.6	0.0	0.0	9.93	<0.0001	<0.0001	UD	-	-
M. senegalensis	*Fabaceae*	91.0	33.6	0.0	0.0	0.0	9.78	0.0001	0.0002	127.4	83.6	153.8
D. cinerea	*Anacardiaceae*	100.0	33.7	4.3	0.0	0.0	5.01	<0.0001	<0.0001	168.6	97.3	217.4
D. angustifolia	*Fabaceae*	100.0	70.2	56.9	10.1	0.0	10.39	<0.0001	0.004	179.1	159.2	200.5
A. etbaica	*Sapindaceae*	98.8	84.1	6.1	1.8	1.2	2.48	<0.0001	<0.0001	275.9	132.1	425.4
S. singuenea	*Fabaceae*	100.0	96.9	2.1	0.0	0.0	1.68	<0.0001	<0.0001	285.5	229.1	360.3
C. tomentosa	*Capparidaceae*	97.0	89.0	72.3	7.6	0.0	4.07	<0.0001	<0.0001	333.0	289.3	382.6
C. farinosa	*Capparidaceae*	98.0	96.7	97.8	89.9	62.1	4.15	<0.0001	0.047	346.1	223.4	497.6
M. angolensis	*Capparidaceae*	89.1	81.2	50.5	43.9	0.0	9.26	<0.0001	0.516	2036.6	1247.2	8342.7

EC_{50}, extract concentration required to inhibit 50% of L_3 exsheathment calculated at 60 min.
PBS, Phosphate buffered saline.
SEM, Standard error of the mean.
UD, Unable to determine because the EC_{50} values are below the detection limit applied in the assay.

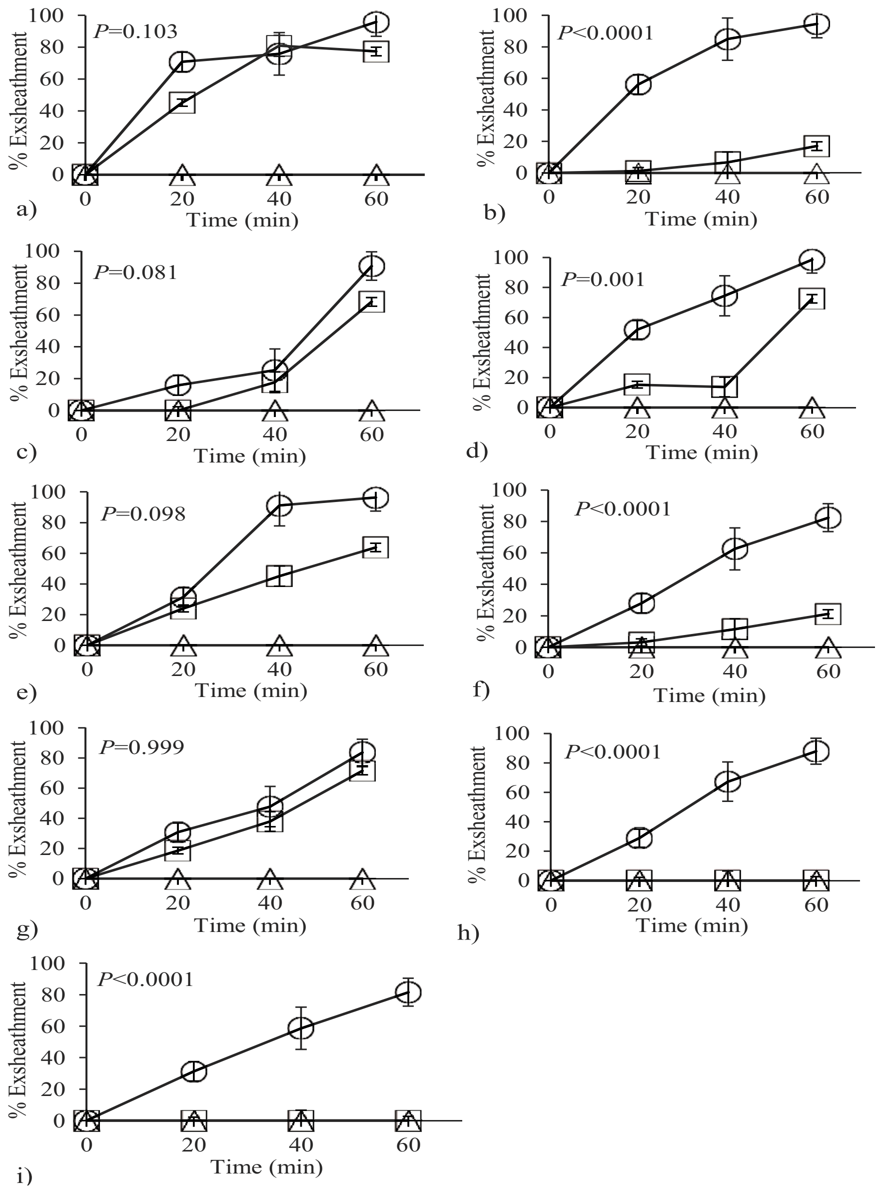

Figure 3.1. Condensed tannin and other polyphenols inhibitory activity of browse extracts (1200 µg/ml phosphate buffered saline, PBS) in the presence (☐) and absence (△) of polyvinylpolypyrrolidone (PVPP) evaluated by comparing *H. contortus* L_3 exsheathment (mean±SD, 4 replicates) and control (○, only PBS) at 60 min. *P*-values indicate the difference between PVPP treated extract and the control. (a) *E. racemosa*, (b) *R. natalensis*, (c) *M. senegalensis*, (d) *D. cinerea*, (e) *D. angustifolia*, (f) *A. etbaica*, (g) *S. singueana*, (h) *C. tomentosa*, (i) *M. angolensis*.

3.3. Results

3.3.1. Proanthocyanidins and other polyphenols in browse species

Proanthocyanidins contents varied among the different browse species (Table 3.1). Higher concentrations of PA with more than 200 mg/g of dry weight were found in *E. racemosa*, *M. senegalensis* and *R. natalensis*. Only traces of PA were found in *C. tomentosa*, while *C. farinosa* and *M. angolensis* did not contain PA. *Acacia etbaica* contained mainly PC and PD. In addition, MS data supported the presence of oligomeric PP or PF. *Dodonaea angustifolia* was found to contain mainly PC. *Dichrostachys cinerea*, *E. racemosa* and *R. natalensis* contained PC and PD. *Maytenus senegalensis* contained some procyanidins and many oligomeric PD. In proportion, *S. singueana* contained PC and PD but also PP or PF were detected.

Other polyphenols detected in the browse species are presented in Table 3.1. Gallic acid derivatives were detected in six browse species with *D. cinerea* having the highest content of 6.9 mg/g of dry weight. Ellagitannins were not detected in any of the browse species. Quinic acid derivatives were detected in *D. angustifolia* and *R. natalensis.* Kaempferol based flavonols were detected in nine browse samples and the content was highest (8.9 mg/g of dry weight) in *S. singueana.* Quercetin based flavonols were detected in all browse samples. The contents varied from 0.5 to 7.7 mg/g of dry weight. Myricetin based flavonols were detected in six samples. The highest contents were found in *E. racemosa* (7.1 mg/g of dry weight) and *D. cinerea* (6.0 mg/g of dry weight).

3.3.2. Effect of browse extract doses on exsheathment and EC_{50} values of extracts

Larval exsheathment was reduced by browse extracts and doses (Table 3.2). There was a linear (P<0.0001) decrease in exsheathment after 60 min for all browse extracts with increasing doses. The relation between doses and exsheathment was quadratic for all extracts except *M. angolensis*. At the dose of 1200 µg/ml PBS, there was a complete inhibition of exsheathment for all extracts except *C. farinosa* and *A. etbaica* with 62 and 1.2% exsheathment, respectively. At the concentration of 150 µg/ml PBS, the lowest exsheathment was observed in *E. racemosa* and the highest in *S. singuenaea*. Averaged over doses, *C. farinosa* had the highest (86.6%) exsheathment and *E. racemosa* the lowest (6.2%). The extract dose required to inhibit 50% of the L_3 (EC_{50}) is presented in Table 3.2. Activity of browse extracts on exsheathment was also reflected in the EC_{50} values with the highest EC_{50} in *C. farinosa* and values were below the detection limit for *E. racemosa* and *M. senegalensis*.

3.3.3. Larval exsheathment of *H. contortus* with or without PVPP

The different browse extracts (Table 3.2), were evaluated for the contribution of PA in the inhibition of *H. contortus* exsheathment (Fig. 3.1) with the exception of *C. farinosa* because of outlying EC_{50} values. There was a significant ($P \leq 0.001$) difference between control and PVPP treated *A. etbaica*, *C. tomentosa*, *M. angolensis*, *R. natalensis* and *D. cinerea* ($P=0.001$). No significant ($P>0.081$) differences were observed between control and PVPP treated *E. racemosa*, *M. senegalensis*, *D. angustifolia*, *S. singueana*.

3.4. Discussion

The anthelmintic properties of plant polyphenols (Hoste et al., 2015) and their mode of action (Hoste et al., 2012) have frequently been reported on both tropical and temperate forages. Depending on the plant botanical family, phenological stage and provenance, the type and concentrations of polyphenols vary accordingly. For practical application and wider use, the anthelmintic properties of different forage sources need to be evaluated. In the present experiment, 10 browse species from the semi-arid regions of Ethiopia and browsed by goats were considered. We are not aware of any previous reports on the anthelmintic property in conjunction with polyphenol composition related to these browse species which belong to six different botanical families with Fabaceae and Capparidaceae being the dominant groups. With the exception of *C. farinosa* and *M. angolensis*, the range of browse species selected possess variable concentrations of PA. Interestingly, PF or PD were found only in the family Fabaceae (*A. etbaica* and *S. singueana*). Galloyl derivatives were detected in all except *S. singueana* and the capparidaceae family. Kaempferol derivatives were present in all browse species except *C. farinosa*, while quercetin based polyphenols were present in all browse species. Myricetin based polyphenols were absent in *D. angustifolia* and Capparidaceae family.

The LEIA was chosen because of its simplicity, low cost, sensitivity, reproducibility and it allows the activity of tannin and/or flavonoids to be determined (Alonso-Díaz et al., 2011). Besides, the exsheathment of the L_3 in *H. contortus* represent the transition from the free-living to the parasitic stage (Bahuaud et al., 2006; Brunet et al., 2007) and the LEIA examines inhibitory activity at the early stage of the parasite life cycle. More than 89% of the L_3 exsheathed in the control group after 60 min. At the concentration of 1200 μg/ml, with the exception of *C. farinosa*, exsheathment was totally blocked in all extracts regardless of PA concentration. Inhibition was observed at the lowest concentration (150 μg/ml), and the highest inhibition for *H. contortus* appeared to be related to the highest PA concentration (>200 mg/g). But at the concentration of 150 μg/ml, more sever inhibition in *D. cinerea* (PA, 101-150 mg/g) compared to *R. natalensis* (>200 mg/g) could be related to its high content of galloyl derivatives or PA composition. High doses (1200 µg/ml) of tropical tanniniferous browse extracts inhibited *T. colubriformis* L_3 exsheathment in another study (Alonso-Díaz et al., 2008).

With the exception of *D. angustifolia*, PA containing extracts severely inhibited exsheathment at 300 µg/ml. The lower inhibition in *D. angustifolia* could be due to the absence of PDs in the PA. More potent inhibition of PA on the exsheathment in *H. contortus* and *T. colubriformis in vitro* were observed with a higher PD:PC ratio (Brunet and Hoste, 2006; Quijada et al., 2015). In *C. tomentosa* with the lowest PA concentration (0-4%) and *M. angolensis* lacking PA, L_3 exsheathment was blocked at 1200 µg/ml suggesting browse compounds other than PA are involved in the inhibition of L_3 exsheathment. The EC_{50} values, in general, corresponded with PA in the same direction. *E. racemosa* and *M. senegalensis* with the highest PA concentration (>20%) had the lowest EC_{50} values, below the detection limit. The EC_{50} values for the PA containing extracts were, on average, lower than the values reported by Moreno-Gonzalo et al. (2013) for heather species in *H. contortus* L_3 exsheathment *in vitro*. Interspecies differences, polyphenol concentration and composition and the strain of *H. contortus* could explain the difference.

The use of PVPP aimed at inactivating tannins and flavonol glycosides (Alonso-Díaz et al., 2008). Based on the EC_{50} value, *C. farinosa* was far less potent compared to other extracts and, therefore, excluded from the PVPP assay. There was a complete inhibition of exsheathment with and without PVPP addition for *C. tomentosa* and *M. angolensis*. However, PA are only present in small quantities in *C. tomentosa* and absent in *M. angolensis*, while none of these two extracts possess galloyl derivatives indicating PA and other polyphenols were not involved in the inhibition of L_3 exsheathment. Therefore, anthelmintic activities in the two species could be due to the presence of PSM other than PA and phenolic compounds. A partial inhibition in *A. etbaica*, *D. cinerea* and *R. natalensis* indicate that PA and flavonols were partly responsible for the inhibition, but also other biochemical components could have blocked exsheathment (Azando et al., 2011). Moreover, Alonso-Díaz et al. (2008) noted that, in addition to CT, flavonoid glycosides, other tannins and/or polyphenols can have additional anthelmintic effect in tropical browse. The partial inhibition, therefore, could be due to a higher proportion of polyphenol aglycones affecting the binding activity of PVPP. Polyvinylpolypyrrolidone preferentially binds to polyphenol aglycones than polyphenol glycosides (Laborde et al., 2006). The absence of a significant difference between PVPP treated *E. racemosa M. senegalensis* and *S. singueana* suggests, the anthelmintic effect was mainly due to PA and other polyphenols related to the presence of kaempferol, quercetin and myricetin based flavonol aglycones and glycosides.

All PA containing (≥5 mg/g) extracts, inhibited L_3 exsheathment and confirmed the anthelmintic properties of PA on *H. contortus* in other PA containing forages e.g. sainfoin extract *in vitro* (Brunet et al., 2007) and *in vivo* with quebracho extracts administration to goats (Paolini et al., 2003). One common characteristics of all PA containing extracts in the present experiment was the presence of PD, and they possibly are the main contributor of the anthelmintic effect. The anthelmintic activity of PD was identified in *in vitro* assays using different monomeric units of PA. Brunet and Hoste (2006) used flavan-3-ols and their galloyl derivatives in a LEIA and found that PD monomers were most responsible in reducing *H. contortus* L_3 exsheathment. A similar observation was associated with the anthelmintic property of PA in *H. contortus* and *T. colubriformis* with PD monomers (Quijada et al., 2015).

Although *E. racemosa*, *R. natalensis* and *M. senegalensis* had a similar PA concentration, persistence of exsheathment in the PVPP treated *R. natalensis* appeared to be due to its content of quinic acid derivatives, which is absent in the other two species or the presence of other non-phenolic compounds. Klongsiriwet et al. (2015) reported a synergistic anthelmintic effect between tannins and quercetin, against *H. contortus* larval exsheathment. Similarly, synergy could contribute to the anthelmintic property in *R. natalensis*. Despite the relatively lower PA concentration compared to the above three species, *D. cinerea* had a lower EC_{50} and showed a partial inhibition upon PVPP addition. This could be due to its higher concentration of quercetin based flavonol aglycones and glycosides. Barrau et al. (2005) previously observed that at high concentration of 1200 µg/ml, quercetin-3-rutinoside had anthelmintic effect on *H. contortus* larval migration *in vitro*. It could also be related to the high concentrations of galloyl derivatives and myricetin based flavonol aglycones and glycosides. *Senna singueana*, with a lower PA content but a high content of kaempferol based flavonol aglycones and glycosides, showed higher inhibition with a lower EC_{50} value. This could be mainly due to the content of kaempferol based flavonol aglycones and glycosides, and Barrau et al. (2005) observed the anthelmintic effect of kaempferol-3-rutinoside at high concentration. *Acacia etbaica* had the highest EC_{50} (285.5 µg/ml) values and was a relatively less potent extract among the PA containing extracts.

Comparing the botanical families in the present experiment, Ebenacae, Fabaceae, Celasteracea, commonly contain PA and with the exception of *A. etbaica,* their anthelmintic property mainly originates from PA and polyphenol contents. In Sapindaceae (*D. angustifolia*), inhibition of exsheathment exist mainly due to PA, while, in the Capparidaceae family other plant secondary metabolites exert anthelmintic property. Larvicidal activities of some selected Anacardiaceae, Fabaceae and Ebenaceae plant families have been reported previously with *H. contortus* (Diehl et al., 2004), in agreement with results presented here.

3.5. Conclusions

Browse extracts showed anthelmintic activity against *H. contortus* L_3 exsheathment, attributable mainly to the PA contents and also the PD composition. The presence of other polyphenols such as kaempferol, quercetin and myricetin based flavonol aglycones and glycosides also appeared to contribute to the anthelmintic property for most browse species. However, the possible inhibition of exsheathment by other non-phenolic constituents was apparent as evidenced in browse species with negligible polyphenol contents, e.g. *C. tomentosa* and *M. angolensis*. In comparison, extracts of *E. racemosa* and *M. senegalensis* are highly potent inhibitors of L_3 exsheathment. Overall, the results confirmed that PA, other polyphenols and non-phenolic components in browse species may act in consortium to exert anthelmintic effect on *H. contortus*.

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CHAPTER 4

In vitro fermentation of browse species using goat rumen fluid in relation to browse polyphenol content and composition

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Abstract

The effect of browse tannins (using polyethylene glycol, PEG 6000) on *in vitro* gas production (GP), methane (CH_4), volatile fatty acids (VFA), ammonia (NH_3) and *in vitro* organic matter digestibility (IVOMD) were studied. Leaves of *A. etbaica*, *C. farinosa*, *C. tomentosa*, *D. angustifolia*, *D. cinerea*, *E. racemosa*, *M. angolensis*, *M. senegalensis*, *R. natalensis* and *S. singueana* were used as substrates in an automated *in vitro* system. Proanthocyanidins (PA) were quantified using the modified HCl-butanol method and UPLC-DAD with detection of other polyphenols by UPLC-ESI-MS/MS. Substrates were inoculated in buffered rumen fluid pooled from three goats, with or without PEG 6000 and incubated for 72 h to measure GP. During incubation, head space gas samples were taken at 0, 3, 6, 9, 12, 24, 30, 48, 54, and 72 h and analysed for CH_4. Volatile fatty acids, NH_3 and IVOMD were determined at the end of incubation. Data from three runs were averaged and analysed using the PROC MIXED procedure of SAS. Addition of PEG increased ($P<0.0001$) GP, CH_4 and total VFA indicating that PA were mainly involved in reducing methanogenesis but also fermentability. Prodelphinidins were found to be the major PA affecting fermentation. Also the contribution of quercetin, myricetin and kaempferol in CH_4 reduction were evident. Changes in the molar proportions of VFA with PEG addition indicated that PA affected fermentation pathways. The absence of PEG effect on IVOMD was due to artefacts from the tannin-PEG complexes interfering with the incubation residue measurement. Overall, the effect of tannin-containing browse on *in vitro* fermentation characteristics was mainly due to PA with possible minor effects of other phenolic and non-phenolic compounds.

4.1. Introduction

The projected demand for animal products is expected to further increase in the developing world due to the growing human population and income growth (Boland et al., 2013; FAO, 2009), requiring a corresponding increase in livestock production and productivity. In low input, extensive production systems, livestock performance is hindered mainly by nutritional constraints related to forage quantity and quality (Kaasschieter et al., 1992). The unique feature of the ruminants' digestive system enables the conversion of poor quality roughages into high quality, human edible foods through the production of volatile fatty acids (VFA) as a main source of energy, but with a concomitant carbon dioxide (CO_2) and methane (CH_4) production. Methane produced in the rumen represents 2-12% of feed gross energy loss and it also contributes to greenhouse gas emissions (11-17%) globally (Beauchemin et al., 2009; Goel and Makkar, 2012). Depending on their size and dry matter intake, sheep and goat produce 10-16 while cattle produce 16-60 kg CH_4/head per year (Hristov et al., 2013). Methanogenesis in the rumen serves as a sink for the hydrogen produced resulting from acetate and butyrate production. Methane mitigation strategies, therefore, should focus on reduction of hydrogen production while enhancing hydrogen utilization by alternative pathways towards beneficial end products (Martin et al., 2010). Methane mitigating strategies in ruminants mainly include feeding high quality digestible diets, the use of methanogenesis inhibiting substances, and improving feed efficiency and nutrient utilization through genetics (Knapp et al., 2014). The possible applicability of such interventions under practical farm management conditions can be influenced by different factors. Therefore, selection of dietary CH_4 mitigating strategies must consider available feedstuff, the livestock species (Beauchemin et al., 2009) and the production system. The use of rumen modifiers and supplementation may not be feasible in low input systems. In such systems, exploitation and manipulation of pasture forages could be more applicable to mitigate CH_4 (Buddle et al., 2011).

Browse species form an important component of pasture lands in dry regions and many have been reported to possess polyphenols mainly condensed tannins (CT) also called proanthocyanidins (PA). Goats consuming up to 100 g dry matter (DM)/d CT from *Lespedeza striata*, showed a decrease in CH_4 emission and reduced urinary nitrogen loss without significant reduction in DM intake (DMI) (Animut et al., 2008). Methane expressed as digestible DMI or g/d was lower in goats consuming 197 g CT/d compared to goats on a control diet containing negligible tannin levels (Puchala et al., 2005). However, biological effects of tannins depend on botanical origin, concentration and types. Recently, Jayanegara et al. (2015) compared the two major groups of purified tannins *in vitro* and reported stronger CH_4 suppressing effect of hydrolyzable tannins (HT) compared to CT, and the latter also significantly decreased digestibility.

However, results are not always consistent and it is not possible to generally attribute the observed beneficial effects to either of these generic tannin groups. Variability in CH_4 reduction within HT (0.3-3.4%) and CT (1.1-5.7%) sources, was reported (Pellikaan et al., 2011b). Condensed tannin composition specifically prodelphinidin:procyanidin (PD:PC) ratio and degree of polymerization were key factors in differences in *in vitro* CH_4 suppressing effects of *Onobrychis viciifolia* (Hatew et al., 2016; Huyen et al., 2016). Polyphenol groups other than tannins have also CH_4 mitigating properties. *In vitro* gas production (GP) increased while CH_4 production decreased when flavonoid-containing plant extracts were incubated with Timothy (Kim et al., 2015). The latter authors related the decrease in CH_4 to anti-protozoal properties rather than a direct effect on methanogens.

In browse species, polyphenol content may account up to 50% of the total organic matter (Reed, 1986). Although the knowledge on ruminal CH_4 reducing property of polyphenols from browse species has long been established, the relationship between phenolic composition and biological activities is only marginally studied. In the present study, emphasis was given to browse PA composition and other polyphenols to better describe the role of polyphenols as rumen fermentation modifiers using the automated *in vitro* GP technique. It was hypothesised that PA content, composition and the presence of other polyphenols determine CH_4, GP and other fermentation characteristics. The objectives were (a) to evaluate the effect of tannins (using polyethylene glycol, PEG 6000) on *in vitro* GP, CH_4, VFA and *in vitro* organic matter digestibility (IVOMD) of browse species, (b) to describe *in vitro* fermentation characteristics of browse in terms of PA and other polyphenol composition.

4.2. Materials and methods

4.2.1. Browse species chemical composition

Leaves of *Acacia etbaica, Cadaba farinosa, Capparis tomentosa, Dichrostachys cinerea, Dodonaea angustifolia, Euclea racemosa, Maerua angolensis, Maytenus senegalensis, Rhus natalensis* and *Senna singueana* were collected by hand-clipping from two area exclosures (protected areas allowed to rest from human and animal intervention) in the Tigray region of Ethiopia in October 2014. From each exclosure, leaves were collected from 15 phenologically similar plants per species, bulked into one sample per species and air dried in an open shed. Freshly cut specimens of the browse species were mounted on a placard and sent to the national herbarium institute of Addis Ababa University, Ethiopia for identification. Dry leaves were ground to pass through a 1-mm sieve and used for determination of their chemical and phenolic composition. The same samples were also used as substrates for *in vitro* incubation with rumen fluid.

4.2.2. Browse species chemical composition

Browse samples were analysed for DM, ash/organic matter (OM) and N following AOAC (1990) protocols with DM determined by oven drying samples (105°C, 3 h), ash by incinerating samples in a muffle furnace (550°C, 3 h) and N by the Kjeldahl method. Crude protein was calculated by multiplying N with the factor of 6.25. Neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) were analysed according to Van Soest and Robertson (1985).

Polyphenol analysis was conducted according to Engström et al. (2014) using an air-dried, ground and freeze dried (Christ beta 2-8 LD plus, Martin Christ, Germany) browse samples which was ground into a fine powder (Retsch MM200, Sigma-Aldrich, US). Approximately 20 mg of sample was weighed in triplicate into Eppendorfs before being vortexed with 1.4 ml of acetone/water (4:1, v/v) for 5 min and macerated at 4°C overnight. In the morning samples were extracted in a planar shaker (Heidolph promax 2020, Heidolph, Germany) for 3 h and centrifuged at 14000 rpm for 10 min. The residues were further extracted with 1.4 ml of acetone/water (4:1, v/v) for 3 h and centrifuged at 14000 rpm for 10 min. The two extracts were combined, the acetone evaporated, the sample freeze-dried and the freeze-dried extracts dissolved in 1 ml of water. Proanthocyanidins were determined according to Grabber et al. (2013), directly from the dried and ground browse materials. Soluble PA were analysed from the browse extracts and the insoluble, i.e. bound, PA from the extraction residues. Proanthocyanidins were quantified against an external PA standard which had a PD:PC ratio of 1:99 and a mean degree of polymerization of 4.9. Proanthocyanidins composition was determined by an ultra-performance liquid chromatograph coupled to a photodiode array detector (UPLC-DAD, Acquity UPLC, Waters Corporation, Milford, MA, USA) and a hybrid quadrupole-Orbitrap mass spectrometer (Q Exactive™ MS, Thermo Fisher Scientific GmbH, Bremen, Germany). The browse extracts were diluted 10 times and filtered through 0.2 µm PTFE syringe filters prior the analysis. The column was an Acquity UPLC® BEH Phenyl (100×2.1 mm i.d.; 1.7 µm; Waters Corporation, Wexford, Ireland). The mobile phase consisted of (A) acetonitrile and (B) 0.1 % formic acid. The elution profile was as follows: 0–0.5 min, 0.1% A in B; 0.5–5.0 min, 0.1–30% A in B (linear gradient); 5.0–5.1 min, 30–90% A in B (linear gradient); 5.1–7.1 min, 90% A in B; 7.1–7.2 min, 90–0.1% A in B (linear gradient); 7.2–8.5 min, 0.1% A in B. The injection volume was 5 µl and flow rate 0.5 ml/min. The UV data were collected at 190-500 nm. The heated electrospray ion source (H-ESI II, Thermo Fisher Scientific GmbH, Bremen, Germany) was operated in negative ion mode. The parameters were as follows: spray voltage was set at –3.0 kV, sheath gas (N_2) flow rate at 60 (arbitrary units), aux gas (N_2) flow rate at 20 (arbitrary units), sweep gas flow rate at 0 (arbitrary units), capillary temperature at +380°C and

S-lens RF level at 60. The Orbitrap was set to a resolution of 70,000 and an automatic gain of 3×106 was used. Masses were scanned at *m/z* 150–2000. Pierce ESI Negative Ion Calibration Solution (Thermo Fischer Scientific Inc., Waltham, MA, USA) was used for the calibration. The data were processed with Thermo Xcalibur Qual Browser software (Version 3.0.63, Thermo Fisher Scientific Inc., Waltham, MA, USA). Other polyphenols were analysed by UPLC-ESI-MS/MS as described by Engström et al. (2015). The extracts were diluted 10 times and filtered through 0.2 µm PTFE syringe filters prior the analysis.

4.2.3. Animals and diet

Nine dry female Saanen goats (41.4-62.1 kg) were divided based on similar body weight into three groups per pen (4m×2.5m) and kept indoors in a closed housing system at the animal research facility of Wageningen University & Research, the Netherlands. Goats were fed a grass silage (DM content 568 g/kg) and concentrate, offered in two equal portions at 9:00 h and 15:00 h. The total adaptation period to the diet and pen condition lasted for eight weeks. The mean (±SEM) daily grass silage intake of the goats over the 8 weeks was 834.1±21.4 g DM/goat and 200 g concentrate. The total DM intake (2% body weight) was within the recommendations of CVB (2008) to meet maintenance requirements. Goats had access to water free of choice and body weights were measured weekly. Animal handling followed the guidelines of the institutional Animal Care and Use at Wageningen University & Research (Wageningen, the Netherlands). For the *in vitro* incubations, goats were re-divided into three groups with similar average body weight across groups (51.4±0.3 kg) and each group was used in successive incubation runs.

Table 4.1. Chemical composition of browse species used in the *in vitro* experiment.

Browse species	DM (g/kg)	Ash	CP	NDF	ADF	ADL	Polyphenol contents								
							Proanthocyanidins				Galloyl derivatives	Quinic acid derivatives	Flavonol derivatives (mg/g DM) ± SE		
							Content (mg/g DM)			Composition	(mg/g DM) ± SE		Kaempferol based	Quercetin based	Myricetin based
		(g/kg DM)					Total	Soluble	Bound						
E. racemosa	909	53	69	561	501	244	+++++	+++	++	PC,PD	2.1 ± 0.1	-	0.3 ± 0.1	1.3 ± 0.1	7.1 ± 0.1
R. natalensis	915	77	113	451	303	135	+++++	+++++	+++	PC,PD	0.2 ± 0.1	2.0 ± 0.1	0.7 ± 0.1	5.8 ± 0.1	1.8 ± 0.1
M. senegalensis	892	71	82	467	426	214	+++++	+++++	+++	PC,PD	< 0.1	-	4.7 ± 0.1	4.4 ± 0.1	1.3 ± 0.1
D. cinerea	908	73	145	427	303	144	+++	+++	+	PC,PD	6.9 ± 0.1	-	1.4 ± 0.1	7.7 ± 0.1	6.0 ± 0.1
D. angustifolia	922	54	108	298	216	82	++	++	+	PC	0.1 ± 0.1	0.4 ± 0.1	1.1 ± 0.1	1.5 ± 0.1	-
A. etbaica	919	69	107	316	242	118	+	+	traces	PC,PD,PF/PP	0.9 ± 0.1	-	0.7 ± 0.1	5.5 ± 0.1	0.2 ± 0.1
S. singueana	923	72	141	295	232	70	+	+	-	PC,PD,PF/PP	-	-	9.0 ± 1.6	3.0 ± 0.6	0.2 ± 0.1
C. tomentosa	923	109	220	271	189	104	traces	-	-		-	-	2.1 ± 0.1	2.0 ± 0.1	-
C. farinosa	909	123	220	255	131	62	-	-	-		-	-	-	0.5 ± 0.1	-
M. angolensis	910	140	246	160	106	31	-	-	-		-	-	0.4 ± 0.1	0.5 ± 0.1	-

ADF, Acid detergent fibre; ADL, Acid detergent lignin; CP, Crude protein; DM, Dry matter; NDF, Neutral detergent fibre; PC, Procyanidins; PD, Prodelphinidins; PF, Profisetinidins; PP, Propelargonidins; SE, Standard error.
-, not detected; traces, 0-5 mg/g; +, >5–50 mg/g; ++, >50–100 mg/g; +++, >100–150 mg/g; ++++, >151-200 mg/g; +++++, > 200 mg/g.

4.2.4. *In vitro* incubations, methane and gas measurements

Rumen fluid from three goats was collected after euthanasia at a commercial abattoir. Immediately after slaughter, the rumen content was manually collected and filtered through double layer cheesecloth. Approximately 1L of rumen fluid was collected per goat in CO_2 filled thermos flasks and transported within 30 min to the laboratory where the rumen fluid of each goat was re-strained through a double layer of cheesecloth. Half a litre per goat was pooled and mixed with anaerobic and pre-warmed (39^{o}C) buffer solution (1:2, v/v) under continuous flushing with CO_2 as described by Cone et al. (1996).

Browse substrates (0.5 g) were weighed in triplicate into 250 ml fermentation bottles (Schott, Germany) as such or in combination with 1.0 g of PEG to counteract the effect of tannins. Pre-warmed bottles containing substrate (and PEG) were inoculated with 60 ml buffered rumen fluid under flushing with CO_2. Bottles were connected to an automated pressure evaluation system in a shaking water bath at 39^{o}C for 72 h. Within each run, one of the replicate bottles with a substrate and corresponding bottle with substrate + PEG were assigned randomly to one water bath with the other replicate bottles randomly assigned to different water baths.

A fully automated *in vitro* system (Cone et al., 1996) was used to measure cumulative GP and CH_4 production at fixed time points as described by Pellikaan et al. (2011a). Concentration of CH_4 was determined by sampling 10 µl aliquots of gas from the fermentation bottle headspace using a gas tight syringe (Hamilton 1701N, 10µ, point style 5; Hamilton, Bonaduz, Switzerland) at 0, 3, 6, 9, 12, 24, 30, 48, 54, and 72 h after incubation. The head space sample was immediately injected onto a gas chromatograph (GC; GC8000Top, CE Instruments, Milan, Italy) with CH_4 concentration quantified using a calibration sample with a known CH_4 concentration (Linde Gas Benelux, Schiedam, The Netherlands). Peak area from each measurement was obtained after integration using dedicated software for gas chromatography (Chrom-Card data system Version 2.4. 2006, Rodano, Milan, Italy).

4.2.5. Analysis at the end of *in vitro* incubation

Immediately after 72 h of incubation, fermentation was terminated and pH of the incubation fluid was measured using a calibrated portable pH meter (Hanna Instruments Model HI 9024, IJsselstein, the Netherlands). Next, a sample (0.75 ml) of the supernatant was taken (1:1, v/v) in a 10% (v/v) trichloroacetic acid solution for ammonia (NH_3) analysis. Another sample (0.75 ml) of each incubation bottle was acidified with an equal volume (1:1, v/v) of a stock solution composed of 25 ml of 85% (v/v) ortho-phosphoric acid dissolved in 200 ml Millipore water and 300 ml of a 4 g/l of 4-methyl valeric acid (internal standard) for VFA analysis. Samples were stored at -

20°C until further analysis. The remaining content was filtered and washed three times with warm distilled water in a pre-weighed glass filter crucible which was oven dried for 16 h at 70°C, followed by further drying at 103°C for 4 h and incineration at 550°C for 3 h to determine IVOMD.

4.2.6. Calculations

Metabolizable energy (ME) was estimated from 24 h GP, crude protein composition of browse species with their cellulose content calculated from ADF and ADL (Van Soest and Robertson, 1985) as a proxy for crude fibre (CF), using the formula below (Menke and Steingass, 1988),

$$\mathrm{ME} = 2.20 + 0.1357 \times \mathrm{GP} + 0.0057 \times \mathrm{CP} + 0.0002859 \times \mathrm{CF}^2$$

where, ME is metabolizable energy (MJ/kg DM), GP the net gas volume at 24 h fermentation (200 ml/mg DM), CP the crude protein (%) and CF the crude fibre (%).

The non-glucogenic ratio (NGR) was calculated according to Ørskov (1977) as:

$$\mathrm{NGR} = (\mathrm{HAc} + 2 \times \mathrm{HB} + \mathrm{HV}) \;/\; (\mathrm{HP} + \mathrm{HV})$$

where HAc is the molar percent acetic acid, HB the molar percent butyric acid, HP the molar percent propionic acid and HV the molar percent valeric acid.

Branched chain volatile fatty acids (BCVFA) were calculated as:

$$\mathrm{BCVFA}\ (\%) = \left(\frac{\text{isobutyric} + \text{isovaleric}}{\text{total VFA}}\right) \times 100$$

with isobutyric acid, isovaleric acid and total VFA expressed as mmol/g OM.

4.2.7. Experimental design and statistical analysis

Treatments were the 10 browse species incubated in triplicate with and without PEG repeated in three runs on different weeks. Per run, data were averaged and repeated measures for total gas and CH_4 production were analysed using the PROC MIXED procedure of SAS 9.3 (SAS, 2010). Browse, PEG, time and their interaction were included as fixed factors with run included as a random factor and analysed using the following model:

$$Y_{ijk} = \mu + B_i + P_j + T_k + (B \times P)_{ij} + (P \times T)_{jk} + (B \times P \times T)_{ijk} + \varepsilon_{ijk}$$

where Y_{ijk} is the dependent variable, μ the overall mean, B_i the effect of browse species (i=1 to 10), P_j the effect of PEG (j=- or +), T_k the effect of time (k=5), $(B\times P)_{ij}$ the interaction of browse with PEG, $(P\times T)_{jk}$ the interaction of PEG with time, $(B\times P\times T)_{ijk}$, the interaction of browse with PEG and with time and ε_{ijk} is the residual error term.

End time point measurement data on VFA, NH_3, IVOMD, ME and NGR were subjected to ANOVA using the PROC GLM procedure of SAS.

4.3. Results

4.3.1. Chemical composition of browse species

Variability in terms of chemical, PA and other polyphenol composition of browse species is shown in Table 4.1. Among the browse with a moderate to high PA contents (>5 mg/g), CP ranged from 69.0 g/kg DM in *E. racemosa* to 144.8 g/kg DM in *D. cinerea*. Neutral detergent fibre, ADF and ADL were highest in *E. racemosa* (560.6, 500.6, 244.0 g/kg DM, respectively). *Senna singueana* had the lowest NDF (295.4 g/kg DM) and ADL (69.7 g/kg DM) content; the lowest ADF content (216.2 g/kg DM) was found in *D. angustifolia*. Proanthocyanidins were detected in most browse species with the highest total PA concentrations (>200 mg/g DM) found in *E. racemosa*, *M. senegalensis* and *R. natalensis*. Moderate to lower PA concentrations were found in *D. cinerea* (101-150 mg/g) and *D. angustifolia* (50-100 mg/g). *Acacia etbaica* and *S. singueana* contain the lowest PA concentrations (5-50 mg/g). Only traces of PA (0-4 mg/g) found in *C. tomentosa*. The proportion of PA was higher in the soluble fraction, but substantial quantities of PA were also found in bound form in *E. racemosa*, *M. senegalensis* and *R. natalensis*.

Analysis on PA composition of the browse species revealed the presence of PC, PD and oligomeric propelargonidins (PP) or profisetinidins (PF) in *A. etbaica* and *S. singueana*. The PA in *D. cinerea*, *E. racemosa* and *R. natalensis* contained PC and PD. For *D. angustifolia*, the PA contained mainly PC. *Maytenus senegalensis* contain both PC and PD with the latter found in large proportions. Galloyl derivatives were detected in 5 of the 10 browse species and varied considerably in concentration with the highest concentration found in *D. cinerea* (6.9 mg/g DM) and lowest in *D. angustifolia* (0.1 mg/g DM). Quinic acid derivatives were only found in *D. angustifolia* (0.4 mg/g DM) and *R. natalensis* (2.0 mg/g DM). Kaempferol based polyphenols were absent in *C. farinosa* while present at 9.0 mg/g DM in *S. singueana* and 0.3 mg/g DM in *E. racemosa*. Quercetin based polyphenols were present in all 10 browse species with highest concentrations (7.7 mg/g DM) in *D. cinerea* and lowest (0.5 mg/g DM) in *C. farinosa* and *M. angolensis*. Myricetin based polyphenols were found in 6 of the 10 browse with the highest (7.1 mg/g DM) values measured in *E. racemosa*, and lowest (0.2 mg/g DM) in *A. etbaica* and *S. singueana*.

Table 4.2. *In vitro* gas production of browse species in the absence (-) and presence (+) of polyethylene glycol (PEG).

Browse species	PEG	Total gas (ml/g of incubated OM)				
		6 h	12 h	24 h	48 h	72 h
E. racemosa	(-)	49.4	64.2	113.5	147.4	160.0
	(+)	83.7*	126.0***	181.2***	213.1***	219.2***
R. natalensis	(-)	60.4	79.0	111.7	148.8	164.0
	(+)	101.3**	152.9***	190.7***	224.2***	230.7***
M. senegalensis	(-)	53.9	70.8	105.5	149.2	169.3
	(+)	111.5***	171.9***	216.6***	250.8***	260.3***
D. cinerea	(-)	54.9	77.5	102.6	138.3	150.6
	(+)	96.8***	138.0***	187.4***	220.4***	226.2***
D. angustifolia	(-)	64.8	73.8	89.7	112.3	116.8
	(+)	81.6	106.2*	157.7***	206.5***	213.5***
A. etbaica	(-)	39.0	47.4	64.9	71.8	76.4
	(+)	101.9***	149.1***	189.3***	211.4***	216.1***
S. singueana	(-)	66.9	106.9	147.1	174.0	179.1
	(+)	131.3***	176.3***	219.9***	249.8***	255.1***
C. tomentosa	(-)	129.3	181.8	224.6	259.5	272.5
	(+)	126.9	185.3	232.3	269.6	284.7
C. farinosa	(-)	106.9	171.0	233.7	279.8	290.6
	(+)	108.6	170.6	231.8	274.0	284.4
M. angolensis	(-)	145.7	213.5	265.7	311.0	317.0
	(+)	141.9	210.5	259.2	304.5	311.1

	SEM	*P*-value
Browse	4.95	***
PEG	4.73	***
Time	4.81	***
Browse×PEG	5.20	***
PEG×Time	4.95	***
Browse×PEG×Time	6.89	***

OM, Organic matter; SEM, Standard error of the mean.

*P<0.05; **P<0.01; ***P<0.001 from corresponding values without (-) PEG.

4.3.2. *In vitro* gas and methane production of browse species

Gas production differed significantly (P<0.0001) among browse species and was dependent on the presence of PEG and time of incubation (Table 4.2). Addition of PEG had no effect on the GP of *C. farinosa*, *C. tomentosa* and *M. angolensis* for any of the incubation times. Addition of PEG had an effect on the GP of *D. angustifolia* from 12 h onwards, but from 6 h onward for the other browse where PEG affected GP. The highest increase in total GP at 72 h by the addition of PEG was observed in *A. etbaica* (139.7 mg/g OM) followed by *D. angustifolia* (96.7 ml/g OM), *M. senegalensis* (91.0 ml/g OM), *S. singueana* (76 ml/g OM), *D. cinerea* (75.6 ml/g OM), *R. natalensis* (66.7 ml/g OM) and *E. racemosa* (59.2 ml/g OM (Table 4.2). The effect of PEG was different (P<0.0001) for browse with time (Table 4.2).

Table 4.3. *In vitro* methane (CH_4) production (ml/g incubated OM) and calculated CH_4 as percent total gas production (CH_4%) of browse fermentation in the absence (-) and presence (+) of polyethylene glycol (PEG).

Browse species	PEG	CH_4	CH_4%	CH_4	CH_4%	CH_4	CH_4%	CH_4	CH_4%	CH_4	CH_4%
		6 h		12 h		24 h		48 h		72 h	
E. racemosa	(-)	4.6	9.3	8.1	12.7	14.2	12.6	22.6	15.3	28.4	17.7
	(+)	9.5	11.3	18.0***	14.5	29.9***	16.5**	39.8***	18.7†	43.6***	19.9
R. natalensis	(-)	4.4	12.3	7.0	13.4	11.5	13.1	18.5	13.6	24.3	14.8
	(+)	9.1	13.7	16.0***	14.6	26.2***	16.4	37.1***	17.9***	42.3***	18.7**
M. senegalensis	(-)	6.5	7.3	9.4	8.9	13.8	10.3	20.3	12.4	25.1	14.8
	(+)	15.0	9.0	25.0***	10.8	35.4***	13.8*	44.6***	16.6	48.3***	18.4*
D. cinerea	(-)	3.4	6.2	5.4	7.1	8.6	8.5	14.0	10.1	18.1	12.0
	(+)	8.7	9.0	17.0***	12.1***	27.6***	14.8***	38.1***	14.3***	42.5***	18.8***
D. angustifolia	(-)	3.3	5.1	4.6	6.2	6.5	7.3	9.2	8.2	11.1	9.6
	(+)	5.4	6.6	9.6	9.0	16.7***	10.6†	26.5***	12.9***	32.5***	15.2***
A. etbaica	(-)	1.9	5.0	2.8	6.0	3.9	6.1	5.0	7.0	5.6	7.4
	(+)	8.9†	8.7*	18.0***	11.8***	28.0***	14.8***	36.2***	17.1***	39.3***	18.2***
S. singueana	(-)	3.5	5.2	7.6	7.1	14.1	9.5	21.0	12.0	24.2	13.5
	(+)	13.0***	10.0***	23.0***	12.9***	34.3***	15.5***	44.5***	17.8***	48.5***	19.0***
C. tomentosa	(-)	14.8	11.5	28.0	15.1	43.2	19.3	59.3	22.8	67.2	24.6
	(+)	14.8	11.6	28.0	15.0	45.3	19.5	63.6	23.6	72.6	25.5
C. farinosa	(-)	8.9	9.1	21.0	12.7	41.7	17.3	61.5	20.9	69.0	22.8
	(+)	8.4	9.0	21.0	12.5	40.9	17.3	60.1	20.8	67.4	22.7
M. angolensis	(-)	13.3	8.3	27.0	12.6	46.0	17.9	65.0	22.0	72.4	23.8
	(+)	12.8	7.7	26.0	12.2	44.7	17.6	63.3	21.9	70.7	23.7

	CH_4		CH_4%	
	SEM	P-value	SEM	P-value
Browse	0.99	***	0.47	***
PEG	0.93	***	0.44	***
Time	0.95	***	0.45	***
Browse×PEG	1.05	***	0.50	***
PEG×Time	0.99	***	0.45	***
Browse×PEG×Time	1.45	***	0.71	***

OM, Organic matter; SEM, Standard error of the mean.

$^{\dagger}P$<0.10; $^{*}P$<0.05; $^{**}P$<0.01; $^{***}P$<0.001 from corresponding values without (-) PEG.

Table 4.4. Total and molar proportions of volatile fatty acids (VFA) and non-glucogenic ratio (NGR) of browse species *in vitro* fermentation in the absence (-) and presence (+) of polyethylene glycol (PEG).

Browse species	Total VFA (mmol/g OM)		Molar proportion (% of total VFA concentration) Acetate		Propionate		Butyrate		Valerate		BCVFA		NGR	
	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)
E. racemosa	9.0	10.3***	69.7	67.5	18.0	17.3	7.9	8.7	1.4	1.8***	3.1	4.8***	4.5	4.6
R. natalensis	9.1	10.7***	72.8	69.2*	17.5	17.0	5.5	7.2***	1.6	2.0***	2.7	4.6***	4.5	4.5
M. senegalensis	9.3	11.3***	71.8	68.5	19.0	17.7	5.7	7.5***	1.2	1.7***	2.5	4.6***	4.2	4.4
D. cinerea	8.9	11.0***	70.9	66.7**	20.7	19.7	4.8	6.4***	1.3	2.1***	2.3	5.1***	3.7	3.8
D. angustifolia	8.0	10.1***	68.8	69.6	22.3	19.4†	5.5	6.6†	1.3	1.5	2.1	2.9***	3.4	4.0
A. etbaica	6.7	10.9***	70.1	66.1**	21.9	20.2	5.4	7.0***	0.8	1.9***	1.8	4.9***	3.7	3.7
S. singueana	9.7	11.7***	64.4	65.8	25.3	20.4***	6.0	6.9	1.1	1.9***	3.2	4.9***	2.9	3.7†
C. tomentosa	11.8	11.9	65.4	65.0	18.9	18.6	9.2	9.3	1.9	2.0	4.7	5.1	4.1	4.2
C. farinosa	12.7	12.7	67.4	67.8	16.4	16.3	9.5	9.3	1.9	1.9	4.9	4.8	4.9	4.9
M. angolensis	13.7	13.8	66.1	66.4	18.0	17.8	9.1	9.0	1.8	1.8	5.0	5.0	4.4	4.4
SEM														
Browse	0.35		0.67		0.40		0.23		0.07		0.25		0.10	
PEG	0.33		0.56		0.18		0.19		0.07		0.25		0.05	
Browse×PEG	0.37		0.80		0.56		0.28		0.08		0.26		0.14	
P-value														
Browse	***		***		***		***		***		***		***	
PEG	***		***		***		***		***		***		**	
Browse×PEG	***		***		**		***		***		***		†	

SEM, Standard error of the mean; OM, Organic matter; BCVFA, Branched chain volatile fatty acids.

$^{\dagger}P<0.10$; $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ from corresponding values without (-) PEG.

Table 4.5. Ammonia, *in vitro* organic matter digestibility (IVOMD) and calculated metabolisable energy (ME) of browse species fermented in the absence (-) and presence (+) of polyethylene glycol (PEG).

Browse species	Ammonia (mg/g incubated OM)		IVOMD (%)		ME (MJ/kg DM)	
	(-)	(+)	(-)	(+)	(-)	(+)
E. racemosa	42.3	64.4***	40.8	23.9***	8.7	12.4***
R. natalensis	41.8	69.5***	48.0	39.5**	8.4	12.7***
M. senegalensis	34.6	66.0***	47.9	31.6***	8.2	13.9***
D. cinerea	37.5	72.1***	51.2	71.7***	7.9	12.5***
D. angustifolia	32.8	44.5	44.3	51.7**	7.2	11.0***
A. etbaica	27.5	70.6***	48.1	50.3	5.9	12.6***
S. singueana	45.8	73.8***	66.1	69.9	10.3	14.3***
C. tomentosa	84.5	89.8	77.3	77.5	13.8	14.2
C. farinosa	94.6	92.8	79.2	80.2	14.2	14.1
M. angolensis	102.2	102.9	87.0	87.6	15.5	15.6
SEM						
Browse	7.27		0.83		0.31	
PEG	7.08		0.37		0.26	
Browse×PEG	7.50		1.17		0.36	
P-value						
Browse	***		***		***	
PEG	***		NS		***	
Browse×PEG	***		***		***	

SEM, Standard error of the mean.

$^{*}P$<0.05; $^{**}P$<0.01; $^{***}P$<0.001; from corresponding values without (-) PEG.

NS, non-significant; OM, Organic matter; DM, Dry matter.

Methane production differed significantly (P<0.0001) among browse species and was dependent also on the presence of PEG and time of incubation (Table 4.3). Addition of PEG significantly increased CH_4 production in 7 of the 10 browse species with PEG having no effect in *C. tomentosa, C. farinosa* and *M. angolensis*. Only for *S. singueana* did PEG addition significantly increase CH_4 production from 6 h onwards while for 5 other browse species, CH_4 production was significantly increased from 12 h onwards with PEG addition to *D. angustifolia* increasing CH_4 production from 24 h onwards. Figure 4.1 provides a graphical representations of total GP (Fig. 4.1A,B) and CH_4 as a percentage of total GP (Fig. 4.1C,D) for *M. senegalensis*, *D. cinerea* and *S. singueana*. Screening of these species was based on variability in PA concentration respectively (high, moderate, low), represent different fermentation patterns and their lower relative reduction in CH_4 compared to reduction in GP. All the three species had rapid increase in GP during the first 3 h (Fig. 4.1A). Between 10 and 26 h, *M. senegalensis* had a slow rate of increase in cumulative GP. There was also a decline in the rate of GP increase for *D. cinerea* after 10 h. *Senna singueana* showed relatively a slight decline in the rate of increase between 6 and 8 h. After PEG addition, however, the rate of increase in GP improved. Rapid increase in CH_4 concentrations was observed in *M. senegalensis* and *D. cinerea* during the first few hours of fermentation in the absence of PEG (Fig. 4.1C). However, CH_4 concentration increased gradually for the same browse species in the presence of PEG (Fig. 4.1D). There was a continuous increase in CH_4 concentration for *S. singueana*.

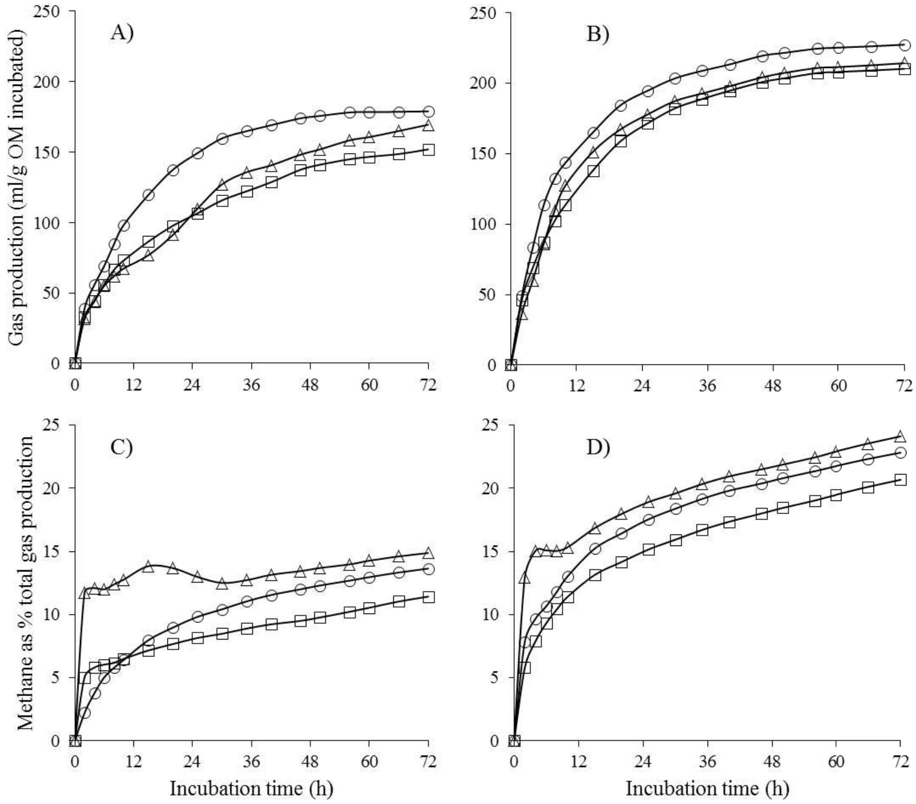

Figure 4.1. *In vitro* cumulative gas production (A, B) and methane production as a percentage of total gas production (C, D) in the absence (A, C) and presence (B, D) of polyethylene glycol. *M. senegalensis* (△), *D. cinerea* (□), *S. singueana* (○).

4.3.3. Volatile fatty acids, ammonia, metabolisable energy and organic matter digestibility

Total and individual VFA concentrations in the 72 h fermentation liquid significantly (P<0.0001) differed between browse species, PEG addition and the interaction between browse and PEG addition (Table 4.4). Total VFA significantly increased when PEG was present for all browse species except *C. farinosa*, *C. tomentosa* and *M. angolensis*. The molar proportion of acetic acid was significantly decreased in *A. etbaica* (P=0.003), *D. cinerea* (P=0.002), *M. senegalensis* (P=0.037) and *R. natalensis* (P=0.016) when PEG was present. Propionic acid decreased (P<0.0001) in *S. singueana* and showed a tendency to decrease in *D. angustifolia* (P=0.054) with the addition of PEG. The proportion of butyric acid increased with PEG in *A. etbaica* (P=0.001), *D. angustifolia* (P=0.037), *D. cinerea* (P=0.0003), *M. senegalensis* (P<0.0001) and *R. natalensis* (P=0.0002). There was an increase in the proportion of valeric acid with PEG addition as well as branched chain fatty acids (P<0.0001) in all PA-containing browse. The non-glucogenic ratio differed with browse species and in the presence of PEG (Table 4.4). Ammonia concentration differed among browse and increased significantly with PEG (P<0.0001) as shown in Table 4.5. The highest NH_3 concentration was observed in *A. etbaica* (61.0%) and the lowest in *D. angustifolia* (26.1%). *Acacia etbaica* had the lowest increase (4.4 %) in IVOMD when PEG was added. A decrease in IVOMD was observed in *E. racemosa* (P<0.0001), *M. senegalensis* (P<0.0001) and *R. natalensis* (P=0.001). The calculated ME differed among browse species (P<0.0001) and increased significantly (P<0.0001) with the addition of PEG (Table 4.5). Seven of the 10 browse showed an increase in ME with the addition of PEG with *C. tomentosa, C. farinosa* and *M. angolensis* showing no increase in ME content with the addition of PEG.

4.4. Discussion

4.4.1. Browse polyphenol composition in relation to gas and CH_4 production

Total PA or CT concentrations in seven of the 10 browse species were higher than values (52.8-98.3 mg/g DM) reported for five Acacia species (Rubanza et al., 2005), a dominant browse species in the semi-arid tropics. Flavonoids in Acacia species with uncommon characteristics of the resorcinol-type hydroxylation pattern in the A-ring constituted in profisetinidins and prorobinetinidins (Foo, 1984) were only detected in *A. etbaica* and *S. singueana*. The presence of PC and PD in PA-containing browse confirmed their wide distribution in plants (Foo, 1984). Mueller-Harvey et al. (1987) detected myricetin, quercetin and kaempferol in *C. farinosa* and *R. natalensis*. However, quercetin was lower and kaempferol was not detected in *C. farinosa*, while

R. natalensis had higher quercetin, myricetin and kaempferol in the present study. These discrepancies could be due to differences in origin, stage of maturity of plants, plant fractions investigated, season of harvest and analytical techniques.

The increase in *in vitro* gas and CH_4 production after PEG addition confirms the fermentation depressing effect of tannins as also observed in other tannin-containing forages (Bhatta et al., 2012; Getachew et al., 2002; Rubanza et al., 2005). The effect of tannins on GP is through substrate deprivation or enzyme activity inhibition in rumen microbes. The effect of PA on fermentation is more pronounced with PA containing more PD monomeric units (Huyen et al., 2016; Lowry et al., 1996). All PA-containing browse in the present experiment with the exception of *D. angustifolia* contained PD, indicating that the GP and CH_4 depressing effect partly originated from PD activity. However, the observed effect on GP was not necessarily consistent with browse PA concentration. Addition of PEG to *A. etbaica* and *D. angustifolia*, which both contain low to moderate PA concentrations, markedly increased gas and CH_4 production. Differences could be partly attributed to the biochemical nature of PA, e.g. monomeric composition, degree of polymerization, and the presence of other compounds. Therefore, for nutritional significance it is crucial to combine quantitative, qualitative analysis of PA, and evaluation of the corresponding effect on fermentation. *Acacia etbaica* has PF with a 5-deoxy group in close proximity to the interflavanol bond (López-Andrés et al., 2013), and this could lower PEG binding leading to the presence of active tannins responsible for an overall decline in fermentation. The relatively lower effect of PA, despite high concentrations in *E. racemosa*, *R. natalensis* and *M. senegalensis* could be due to the presence of highly polymerized phenolics lowering protein binding properties (Kumar, 1983). Moreover, high fibre-bound PA may lower fibre digestion and also lower PEG binding and therefore reduce effects of PEG addition.

Graphical representation of the gas production pattern (Fig. 4.1) of *M. senegalensis*, *D. cinerea* and *S. singueana* show the effect of PA on the rate and production of gas. A rapid increase in GP during the first 3 h for these browse indicates minor effects of tannins on the rate of fermentation. It appeared that the effect of tannins to reduce the rate of fermentation was low in *S. singueana*, moderate in *D. cinerea* and markedly higher in *M. senegalensis*. *Mytenus senegalensis* and *S. singueana* had comparable GP but the pattern was different. These results indicate that the high PA concentrations in *M. senegalensis* influenced the rate of GP for the same browse. It may also suggest that PA is more correlated with effects on the rate of fermentation. Huyen et al. (2016) reported that PA extracts added to grass silage (4%) decreased the rate of *in vitro* substrate degradation.

Methane was markedly reduced and the percent increase after PEG addition ranged from 53% in *E. racemosa* to 601% in *A. etbaica*. Bhatta et al. (2012) also reported 10-

798% increase in CH_4% *in vitro* when PEG was added to a range of tropical tannin-containing leaves. These data demonstrate the potential CH_4 mitigation of browse. Methane reduction is accompanied by a concomitant reduction in GP. The latter indicates impaired fermentation and hence affecting rumen microbes. However, it is important to place the results in perspective considering CH_4 reduction and decline in fermentation.

Bezabih et al. (2013) evaluated 24 tropical grass species from Ethiopia *in vitro* for 72 h and reported a higher average GP (228±31 vs 145±36 ml/g OM) and CH_4 (53±8 vs 20±8 ml/g OM) values compared to values for the PA-containing browse investigated here. This comparison shows that the browse species produce lower CH_4 concentration of total gas produced per OM fermented. Tannin biological activities are dependent on concentrations but also mainly on structure (Bodas et al., 2012; Salminen and Karonen, 2011). The high PD:PC ratio in sainfoin was associated with reduced CH_4 production *in vitro* (Hatew et al., 2014; Huyen et al., 2016). The presence of PD in most PA-containing browse could result in reduction of CH_4. Prodelphinidins have more hydroxyl groups to form tannin-protein complexes with digestive or microbial enzymes compared to PC. The presence of PD- and quercetin-based polyphenols appeared to be related to the decrease in CH_4 production. Quercetin added to a grass/concentrate substrate *in vitro* did not have a significant effect on ruminal fibre degrading microbes but significantly reduced the protozoal population (Oskoueian et al., 2013).

The effect of galloyl derivatives may be dependent on its composition, e.g., the contribution of galloyl derivatives to CH_4 reduction in the present study is less evident in *D. angustifolia*. This browse lacks galloyl derivatives but could markedly reduce CH_4, in a proportion comparable to *D. cineara* which contain the highest galloyl derivatives. Since *D. angustifolia* has moderate PA consists of PC monomeric units, lower concentrations of other polyphenols, its CH_4 inhibitory activity could be due to the presence of non-phenolic compounds. Methane production was reduced by the addition of kaempferol, quercetin and myricetin to guinea grass hay/concentrate mixture *in vitro* (Oskoueian et al., 2013). The presence of kaempferol in high concentrations in *S. singueana* is likely to significantly contribute to its CH_4 lowering property and Oskoueian et al. (2013) indicated that kaempferol significantly reduces CH_4 without reducing GP. In *D. cinerea*, CH_4 depressing activity could be mainly due to the presence of higher levels of quercetin and myricetin. The latter reduces both GP and CH_4 but quercetin increased GP while decreasing CH_4 (Oskoueian et al., 2013).

4.4.2. Browse polyphenol composition in relation to *in vitro* fermentation end products

Most phenolic compounds in forages exert antimicrobial activity by lowering microbial fermentation with a shift in total and individual VFA production (Bodas et al., 2012; Oskoueian et al., 2013). The increase in the total VFA with PEG addition is in line with reports on tropical browse (Gemeda and Hassen, 2015; Getachew et al., 2002). However, the observed decrease in acetic acid in *A. etbaica*, *D. cinerea*, *M. senegalenis* and *R. natalensis* by the addition of PEG contradicts results of the latter authors. It could be due to the PA and/or other polyphenols in these browse result in a shift in the proportion of VFA. Furthermore, these browse species contain higher concentrations of quercetin. According to Oskoueian et al. (2013), degradation of quercetin produces acetic acid, favours fibre digesting microbes while inhibiting protozoa. In addition, microbes which can degrade quercetin may be less affected by PA and this may contribute to the increase in acetic acid pool (Lowry et al., 1996; Oskoueian et al., 2013), also suggesting the contribution of some polyphenols in energy metabolism in the rumen. Similar to the reports of Getachew et al. (2002) in browse species, propionate and acetate were decreased while butyrate increased in the presence of PEG. The increase in branched chain fatty acids up on PEG addition agrees with results from tropical browse investigated by Gemeda and Hassen (2015), indicating the release of protein from protein-tannin complex and the subsequent fermentation.

The increase in NH_3 concentration in PA-containing browse after PEG addition indicates tannins activity in reducing NH_3 similar to reports with hydrolysable and CT extracts (Pellikaan et al., 2011b) and tannin-containing forages (Bhatta et al., 2012) *in vitro*. Ammonia is released in the rumen as a result of protein fermentation and serves as the major nitrogen source for microbial protein synthesis. Microbial protein fermentation can be hindered for instance when protein is bound to tannins rendering it less accessible to microbial digestion. Although all PA-containing browse also possess galloyl derivatives, a large proportion of NH_3 reduction could be mainly imposed by PA due to the irreversible nature of PA-protein complex compared to hydrolysable tannin-protein complex (Bhatta et al., 2009).

The increase in GP and VFA production upon addition of PEG to tannin-containing forages indicates increased organic matter digestibility. However, *E. racemosa*, *M. senegalensis* and *R. natalensis* had lower IVOMD after PEG addition. It could be that the higher concentration of PA in these browse and the possible tannin-protein complex formation increased the proportion of the undigested fraction in incubation residue and hence lowers IVOMD values (Makkar et al., 1995; Reed, 2001). Metabolisable energy was increased by PEG addition in line with previous studies with tannin-containing forages (Gemeda and Hassen, 2015; Rubanza et al., 2005). The PA-containing browse species had also average ME values comparable to ME values of a range of tropical grass species (Bezabih et al., 2013).

4.5. Conclusions

The results show that the browse species except *C. farinosa*, *C. tomentosa* and *M. angolensis*, had CH_4 inhibitory but also fermentation depressing activity. The use of PEG confirmed that the inhibitory activity is due to PA likely mediated by the presence of PD monomeric units, common to all PA containing browse except *D. angustifolia*. High PA concentration does not necessarily imply a higher effect on fermentation and vice versa. The ratio of the difference due to PEG addition in CH_4 production, to the differences in GP show the simultaneous CH_4 mitigating potential and nutritive value of the PA containing browse. All PA containing browse decreased total VFA but with variable effects on molar VFA proportions. Quercetin based flavonols contributed to acetic acid production in some browse species. Although all PA containing browse species reduced CH_4, considering CH_4 reduction as a proportion of GP reduction, total VFA, IVOMD and ME, *S. singueana* is superior followed by *D. cinerea* with low to moderate PA, respectively. Overall, the effect of PA-containing browse could also be due to the additional inhibitory effects of other phenolic and non-phenolic compounds. Therefore, evaluation of the fermentative characteristics of browse needs to take in to account co-occurring phenolic and non-phenolic compounds in addition to tannins.

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CHAPTER 5

In vitro methane and gas production with inocula from cows and goats fed an identical diet

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Abstract

The aim of the study was to compare cow and goat rumen inocula in terms of gas production (GP) and methane (CH_4) production and kinetics parameters and volatile fatty acids (VFA) production, using tanniniferous browse and temperate feedstuffs as substrate. Air-dried leaves of *Acacia etbaica, Capparis tomentosa, Dichrostachys cinerea, Rhus natalensis*, freeze-dried maize silage and grass silage, and concentrate were used as substrates for *in vitro* incubation. Substrates were inoculated for 72 h to measure GP, in buffered rumen fluid from individual 6 goats and 6 cows kept on identical roughage/concentrate diets. Headspace gas samples were taken during incubation at 0, 3, 6, 9, 24, 30, 48, 54, and 72 h, and analysed for CH_4. Volatile fatty acids were determined at the end of incubation. A triphasic (GP) and monophasic (CH_4) modified Michaelis-Menten equations were fitted using the non-linear least square regression procedure in SAS. Data were averaged per treatment per run and analysed using the PROC MIXED procedure of SAS. Total GP and CH_4 were higher (P<0.0001) in goat inoculum than cows across substrates. The half-time for asymptotic GP was lower (P<0.0001) in phase 1 and higher (P<0.012) in phase 2; and the maximum rate of GP was higher (P<0.0001) in phase 1 and phase 3 (P<0.0001) for goats compared to cows. Methane production and as percentage GP were higher in goats (P<0.0001) and the half-time tends (P=0.059) to be at a latter time for goats compared to cows. The maximum rate of CH_4 production was higher (P=0.008) in goats. The total VFA were higher (P<0.0001) in goats. The higher fermentative activity in goat inoculum was attributable to differences in the microbial population between the two species. The differences between goats and cows were more pronounced in fermentation of browse species than grass and maize silages.

5.1. Introduction

The stepwise degradation of carbohydrates, proteins and other organic polymers in the rumen results in the formation of volatile fatty acids (VFA), carbon dioxide (CO_2), hydrogen (H_2) and ammonia (NH_3). The synthesis of methane (CH_4) from CO_2 and H_2 is a key process in maintaining optimal partial pressure of H_2 for normal rumen fermentation (Morgavi et al., 2010). Acetic, propionic and butyric acids are the major VFA and the relative proportion of each in the rumen is influenced by dietary composition. The production of propionic acid utilizes H_2 and competes with methanogenesis while acetic and butyric acid formation results in the production of CH_4. Plant secondary metabolites, e.g. tannins can inhibit enteric CH_4 production which may be beneficial in reducing both feed gross energy loss and greenhouse gas emissions (Beauchemin et al., 2008; Goel et al., 2012).

Utilization of tannin-rich forages by ruminants is enhanced in the presence of rumen microbes with the ability to tolerate tannins (McSweeney et al., 2001) and the tannin-binding activity of proline-rich proteins in the saliva (Jones et al., 2001). Previous reports have shown the superior ability of goats compared to other domestic ruminants in fermenting tannin-rich and fibrous forages *in vitro* (Camacho et al., 2010; Odenyo et al., 1999) and *in vivo* (Ferreira et al., 2016). Goats have specialized groups of microbes contributing to the capacity to utilize more fibrous- and tannin-rich diets, compared to cows and sheep (Ferreira et al., 2016). Although interspecies differences in the fermentative activity of inocula from ruminants have long been established, only limited information is available on the comparison of goat and cow rumen fluid within the effectiveness of tannin-containing forages (Bueno et al., 2015; Odenyo et al., 1999). In a previous study (Chapter 4), a marked decline in *in vitro* methane concentration was observed in browse species fermented in inoculum from goats not adapted to tannin diets. It was, however, unclear whether the observed fermentation patterns were due to the goat inoculum or were specific to the browse species used. In order to distinguish between the latter two, a range of substrates: tropical tannin-rich browse and temperate feedstuffs (grass silage, maize silage and concentrate) were used in *in vitro* incubations with rumen inocula from goats and cows, kept on an identical diet. It was hypothesised that rumen fluid from goats has a higher fermentative activity compared to cows with a concomitant increase in CH_4 concentration. The present study was, therefore, designed to compare the effect of goat and cow rumen inocula on *in vitro* gas, CH_4 and VFA production using tropical browse and temperate feedstuffs as substrates.

5.2. Materials and methods

5.2.1. Chemical composition of substrates used in *in vitro* incubation

Air-dried leaves of *Acacia etbaica, Capparis tomentosa, Dichrostachys cinerea, Rhus natalensis*, freeze-dried maize silage and grass silage, and concentrate were used as substrates for *in vitro* incubation. Leaves were collected by hand-clipping from two area exclosures (protected areas allowed to rest from human and animal intervention) in the Tigray region of Ethiopia in October 2014. From each exclosure, leaves were collected from 15 phenologically similar plants per species, bulked into one sample per species and air dried in an open shed. Freshly cut specimens of the browse species were mounted on a placard and sent to the national herbarium institute of Addis Ababa University, Ethiopia for identification. All the samples were ground to pass through a 1-mm sieve. Organic matter (OM) determination of browse species (AOAC, 1990) and maize, grass and concentrate (ISO, 2002) was achieved by incinerating samples in a muffle furnace (550°C, 3 h). Nitrogen (N) content of all the feedstuffs was measured by the Kjeldahl method (AOAC, 1990). Crude protein (CP) was calculated as N×6.25.

Table 5.1. Chemical composition of substrates used in *in vitro* incubation and diets fed to goats and cows.

Substrate	OM	CP	NDF	PA
		(g/kg DM)		(mg/g DM)
Browse				
Acacia etbaica	930.6	107.2	315.6	5-50
Capparis tomentosa	890.8	219.9	270.5	0-4
Dichrostachys cinerea	927.5	144.8	427.1	101-150
Rhus natalensis	923.5	113.4	450.9	≥200
Concentrate	924.9	172.5	206.2	-
Grass silage	921.1	124.0	546.0	-
Maize silage	964.0	71.0	353.0	-

Diet chemical composition: Grass silage: DM=396 g/kg product; chemical composition (g/kg DM): OM=911, CP=166, NDF=470, ADF=263, ADL=16, EE=41.

Maize silage: DM=328 g/kg product; chemical composition (g/kg DM): OM=962, CP=72, NDF=371, ADF=206, ADL=15, EE=31, Starch=349. Concentrate: wheat semolina, linseed, palm kernel expeller, rapeseed meal 00, soybean meal 44/7, B-resistant soybean, soy hulls, sunflower seed meal ARG, beet pulp Du 15-20 % Su, chalk, mixing salt, mono calcium, palm oil, vinasse, urea, digestarom dairy, maize MV, PRX AR 202 MELKVEE B BASIS, safMannan, molasses, malt sprouts, AR unique buffer, diamond V XPC ULTRA. DM = 873 g/kg product; chemical composition (g/kg of DM): OM=914, CP=198, NDF=236, ADF=131, ADL=20, EE=42, starch=263.

ADF, Acid detergent fibre; ADL, Acid detergent lignin; CP, Crude protein; DM, Dry matter; EE, Ether extract; NDF, Neutral detergent Fibre; OM, Organic matter; PA, Proanthocyanidins.

Neutral detergent fibre (NDF) was analysed for browse species (Van Soest and Robertson, 1985) and maize, grass and concentrate (Van Soest et al., 1991) after pre-treatment with a heat stable amylase and expressed exclusive of residual ash (aNDFom).

Total proanthocyanidins (PA) were determined according to Grabber et al. (2013). The OM, CP, NDF and PA composition of the substrates used are presented in Table 5.1.

5.2.2. Animals and rumen inoculum collection

Six Saanen dry goats weighing 60±8.5 (±SD) kg and 6 rumen cannulated Holstein dairy cows in mid to late lactation were used as inoculum donors. Goats were kept in pens (2 goats/pen), while cows in one group in a free stall. All the animals were housed at the animal research facility of Wageningen University & Research, the Netherlands. Both groups of animal species were adapted to a grass silage/maize silage (50:50, product basis) roughage and concentrate diet for a four week period before the start of rumen fluid collection. During this period, the roughage to concentrate ratio for goats (1.5±0.26) was adjusted based on cows (1.6±0.15), to ensure similar ratios between the two species. Average roughage intake per cow per day (15.5±1.4) kg DM was calculated as average value from the group of cows during the four weeks period. Each cow received 9.7 kg DM concentrate daily. Dry matter intake of goats was adjusted based on the concentrate to roughage intake ratio of the cows. The daily DM intake of goats was 795±82.8 g DM roughage and 513±89.9 DM concentrate during the four weeks. Cows and goats were fed according to the recommendation of CVB (2008). Goats and cows had access to water free of choice. Animal handling was done in accordance with European Union directive 2010/63/EU and was approved of by the Institutional Animal Care and Use Committee of Wageningen University & Research (Wageningen, the Netherlands).

For each run of *in vitro* incubation, rumen fluid was collected separately from two goats in a pen after euthanasia at a commercial slaughter house, and from two cows through the cannula. Briefly, immediately following euthanasia, the rumen was removed from the gastrointestinal tract of each goat, cut on the side and the total content strained through a double layer of cheesecloth, and the fluid collected in a CO_2 filled thermos flask that was transported to a laboratory within 0.5 h. Rumen fluid from the cows was sampled at the same time as the goat rumen fluid and directly transferred into a CO_2 filled thermos flask from the cannula. Inocula from individual animals were transported to the laboratory where rumen fluids were strained through a double layer of cheesecloth. Inoculum from the individual animals was used separately in *in vitro* incubations.

5.2.3. *In vitro* incubation

The strained rumen fluid was mixed with anaerobic and pre-warmed (39°C) buffer solution (1:2, v/v) under continuous flushing with CO_2 as described by Cone et al. (1996). Each substrate (approximately 0.5 g) was inoculated in triplicate per individual animal inoculum in 60 ml buffered rumen fluid in a pre-warmed 250 ml fermentation bottle (Schott, Germany). Each rumen fluid substrate combination was incubated in triplicate and bottles were randomly assigned to a different water baths. Bottles were connected to an automated pressure evaluation system in a shaking water bath at 39°C for 72 h. During each incubation, one blank bottle containing only buffered rumen fluid was incubated per inocula from individual animals. Average gas produced in the blank incubations was 61.2 ml (goats) and 13.9 ml (cows). Incubations were done in three runs conducted on three different days.

5.2.4. Gas, methane and fermentation end products measurement

A fully automated *in vitro* system (Cone et al., 1996) was used to measure cumulative gas production (GP) and CH_4 production at fixed time points as described by Pellikaan et al. (2011). At 72 h of incubation, pH was measured using a portable pH meter (Hanna Instruments Model HI 9024, IJsselstein, the Netherlands). After pH measurement, 0.75 ml of supernatant from each incubated bottle was acidified with an equal volume (1:1, v/v) of a stock solution composed of 25 ml of 85% (v/v) ortho-phosphoric acid dissolved in 200 ml Millipore water and 300 ml of a 4 g/L 4-methyl valeric acid, and used for VFA analysis. In order to enhance baseline separation during the VFA analysis, the gas chromatograph used hydrogen as a carrier gas instead of helium according to Pellikaan et al. (2011). Isocaproic acid was used as an internal standard. Results for individual VFA were expressed as molar percent of total VFA (TVFA=sum of C2, C3, C4, isoC4, C5, isoC5 VFA) with the latter expressed as mmol/g OM. The VFA samples were stored at -20°C until further analysis.

Concentration of CH_4 was determined by sampling 10 µl aliquots of gas from the fermentation bottle headspace using a gas tight syringe (Hamilton 1701N, 10µ, point style 5; Hamilton, Bonaduz, Switzerland) at 0, 3, 6, 9, 24, 30, 48, 54, and 72 h after incubation. Upon collection, the gas sample was immediately injected and analysed by gas chromatography (GC; GC8000Top, CE Instruments, Milan, Italy). Methane concentration was calculated against a calibration curve developed using a standard with a known CH_4 concentration (Linde Gas Benelux, Schiedam, The Netherlands). Peak area from each measurement was obtained after integration using dedicated software for gas chromatography (Chrom-Card data system Version 2.4. 2006, Rodano, Milan, Italy).

Cumulative CH_4 production was calculated by the sum of the increase in headspace CH_4 concentration between two successive valve openings and the amount of CH_4 vented from the bottle (Pellikaan et al., 2011), according to the following equation:

$$CH_4 = \sum_{i=1}^{i=n+1} \{V_{HS}(C_{i+1} - C_i) + G_{i+1} \times C_{i+1}\}$$

where CH_4 is the cumulative CH_4 production (ml/g of incubated OM); V_{HS} is the bottle headspace volume (ml); C_i, C_{i+1} are CH_4 concentration in the bottle headspace gas at i and $i+1$ valve openings, respectively; G_{i+1} is the amount of gas (ml) vented at $i+1$ valve opening; and n is the total number of valve openings.

5.2.5. Curve fitting and calculations

A triphasic and monophasic modified Michaelis-Menten equation was fitted to the cumulative GP and CH_4 production curves, respectively (Groot et al., 1996) using the non-linear least square regression procedure in SAS (Bauer et al., 2001).

$$OMCV = \sum_{i=1\ or\ 3}^{n} \frac{A_i}{1+\left(\frac{C_i}{t}\right)^{B_i}}$$

where $OMCV$ is either the gas or CH_4 production corrected for OM incubated (ml/g of incubated OM); A_i is the asymptotic gas production (ml/g of incubated OM); B_i the switching characteristics of the curve; C_i is the half-time or time at which half of the asymptotic gas production is reached; and t is the time (h). The maximum rate of gas production (R_{max}, ml/h) was calculated based on the method as described by Bauer et al. (2001).

5.2.6. Experimental design and statistical analysis

A completely randomized design was used to evaluate the effect of inoculum source (goat and cow) using seven different substrates. Inoculum source and substrates were included as fixed factors and run as a random factor using the following model:

$$Y_{ij} = \mu + F_i + S_j + (F \times S)_{ij} + \mathcal{E}_{ij}$$

where Y_{ij}= the dependent variable, μ= the overall mean, F_i= the effect of inoculum source (i =1 or 2), S_j= the effect of substrate (j= 1 to 7), $(F\times S)_{ij}$= the interaction of inoculum source and substrate, and $\mathcal{E}_{ij}$= residual error term. Incubated bottles were averaged per treatment per run and individual animals were considered as the experimental unit. The PROC MIXED procedure of SAS 9.3 (SAS, 2010) was used. The Tukey-Kramer's multiple comparison procedure in the LSMEANS statement in SAS was used to test differences among means with significant differences identified at a probability value of 5%.

5.3. Results

5.3.1. Inoculum effects on gas, methane production and kinetics parameters

There was an effect of inoculum ($P<0.0001$) on the cumulative GP at 72 h of incubation (Table 5.2). Total gas production was significantly higher ($P<0.0001$) in goat inoculum than cows' across the seven substrates. Average GP were 348.2 and 239.4 ml/g OM in goat and cow inoculum, respectively. Effects on the asymptotic gas production during the 1st (A1), 2nd (A2) and 3rd (A3) phases were observed ($P<0.0001$) with higher values for goat inoculum. The first phase (A1) corresponds to the fermentation of the rapidly fermentable fractions during the first 3 h of incubation, A2 to fermentation of the non-soluble fraction during 3-20 h of incubation, and A3 to the microbial turnover (Cone et al., 1997). The half-time for asymptotic gas production differed significantly ($P<0.012$) between goat and cow inocula in the first (C1) and second (C2) phase. The rate of gas production was significantly ($P<0.0001$) different in the first (R_{max1}) and third (R_{max3}) phases between inocula source. Differences ($P<0.0001$) between inocula in the asymptotic CH_4 production and calculated CH_4 percentage (CH_4%) of total GP were also observed (Table 5.3). On average, goat inoculum produced more CH_4 (71.5 ml/g OM) than cow (44.6 ml/g OM) and CH_4% was also higher in goat inoculum. An effect of inocula on R_{max1} was observed with higher rates for goat inoculum ($P=0.008$).

Table 5.2. The effect of goat and cow rumen inocula on gas production and kinetics parameters.

Substrate	Inoc	GP (ml/g OM)	GP kinetics parameters											
			A1	A2	A3	B1	B2	B3	C1	C2	C3	R_{max1}	R_{max2}	R_{max3}
A. etbaica	goat	181.1***	69.7*	73.6	37.8*	1.13	1.88***	3.73**	0.7	8.1	24.6	71.4	5.8	1.6
	cow	93.8	29.3	50.9	13.7	1.22	2.78	11.97	0.5	9.0	20.4	39.9	4.5	1.5
C. tomentosa	goat	370.3***	131.2*	171.9***	67.2**	0.97	2.02*	3.90	1.0	6.1	25.2	138.2*	18.5	2.7***
	cow	247.2	91.7	117.8	37.7	1.02	2.48	3.18	1.3	4.9	30.6	57.7	17.9	1.1
D. cinerea	goat	265.3***	83.4***	112.9***	68.9***	0.15	1.87**	3.55	0.9	8.2	26.6	71.2	8.8	2.4***
	cow	129.1	33.4	64.6	31.2	1.17	2.38	3.27	0.7	8.8	26.6	37.1	5.3	1.0
R. natalensis	goat	264.0***	86.7**	97.7*	79.6**	1.12	1.75*	3.80	0.6	9.0	27.6	111.3*	6.8	3.0**
	cow	157.2	41.8	69.3	45.6	1.10	2.23	3.80	0.7	8.7	27.5	40.9	5.6	1.6
Concentrate	goat	485.7***	158.2	248.2*	79.4***	1.07***	2.17	4.88	1.0*	7.0	22.5	132.7*	24.1	4.4***
	cow	375.8	125.7	213.0	37.2	1.33	2.38	3.67	1.4	6.1	21.3	58.3	25.0	1.7
Grass silage	goat	419.3***	123.2**	209.8	86.3**	1.07	2.17	4.13	0.8	8.1	23.8	119.3	17.7	3.9***
	cow	322.7	77.8	188.7	56.3	1.13	2.48	3.77	1.1	7.4	25.2	52.5	18.9	2.3
Maize silage	goat	451.4***	129.6*	233.4	88.4***	1.07	2.10	4.50	1.2***	7.1	23.7	98.7*	22.2	4.4***
	cow	350.1	88.6	207.0	54.5	1.12	2.30	3.67	2.3	5.8	24.3	30.2	25.0	2.2
SEM														
Substrate		10.07	6.29	5.13	3.90	0.03	0.08	0.90	0.06	0.21	1.21	11.26	0.61	0.16
Inoc		6.90	4.37	2.74	2.62	0.01	0.05	0.47	0.03	0.11	0.79	7.72	0.32	0.10
Substrate×Inoc		13.52	8.40	7.39	5.27	0.04	0.10	1.30	0.09	0.30	1.65	15.22	0.88	0.23
P-value														
Substrate		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.003	0.010	<0.0001	<0.0001	0.0002	0.007	<0.0001	<0.0001
Inoc		<0.0001	<0.0001	<0.0001	<0.0001	0.001	<0.0001	0.306	<0.0001	0.012	0.722	<0.0001	0.590	<0.0001
Substrate×Inoc		0.459	0.943	0.143	0.577	0.013	0.002	0.004	<0.0001	0.001	0.094	0.447	0.011	<0.0001

GP, Gas production; Inoc, Inoculum; OM, Organic matter.

A1, A2, A3, represent asymptotic gas production in different phases (ml/g OM); B1, B2, B3, represent switching characteristics of the curve in different phases; C1, C2, C3, represent half time in different phases (h); R_{max1}, R_{max2}, R_{max3} represent the rate of maximum gas production in different phases (ml/h).

SEM, standard error of the mean; *$P<0.05$, **$P<0.01$, ***$P<0.001$, indicate significant differences between inoculum within substrate.

Table 5.3. The effect of goat and cow rumen inocula on methane production, methane percentage and kinetics parameters.

Substrate	Inoc	Methane		Methane production kinetics parameters		
		(ml/g OM)	% gas production	B	C	R_{max1}
A. etbaica	goat	25.6	13.9	0.93*	15.4	18.7**
	cow	9.8	10.4	2.75	11.4	4.9
C. tomentosa	goat	90.8***	24.5	1.08	17.6	6.1
	cow	60.1	24.3	1.03	23.8	4.2
D. cinerea	goat	51.1***	19.0	0.95	35.0	3.5
	cow	20.0	15.3	0.83	18.1	1.9
R. natalensis	goat	51.4**	19.1	0.93	39.2	2.8
	cow	25.4	15.7	0.81	28.3	2.1
Concentrate	goat	106.7***	21.9	1.15	12.0	7.1
	cow	75.2	20.0	1.32	8.8	5.7
Grass silage	goat	81.9**	19.5	1.33	12.4	4.4
	cow	60.0	18.6	1.23	12.2	4.1
Maize silage	goat	92.8***	20.5	1.20	12.3	5.7
	cow	62.0	17.8	1.18	9.9	4.8
SEM						
Substrate		3.50	0.70	0.24	4.95	1.75
Inoc		2.78	0.49	0.14	4.22	1.31
Substrate×Inoc		4.46	0.93	0.34	5.82	2.21
P-value						
Substrate		<0.0001	<0.0001	0.102	<0.0001	0.003
Inoc		<0.0001	<0.0001	0.199	0.059	0.008
Substrate×Inoc		0.220	0.203	0.059	0.237	0.034

B, represent switching characteristics of the curve in different phases; C, half time; Inoc, Inoculum; OM, Organic matter; R_{max1}, rate of maximum methane production; SEM, Standard error of the mean.
**P<0.05, **P<0.01, ***P<0.001, indicate significant differences between inoculum within substrate.

5.3.2. Substrate effects on gas production, methane and volatile fatty acids

Gas production differed (P<0.0001) among substrates (Table 5.2). In both goat and cow rumen inocula, GP was lower with browse species (*A. etbaica*, *D. cinerea*, *R. natalensis* and *C. tomentosa*) compared to grass silage, maize silage and concentrate (temperate feedstuffs). There were differences (P<0.0001) among substrates in CH_4 production and percentage (Table 5.3). On average, fermentation of browse resulted in lower CH_4 production (41.8 vs 79.8 ml/g OM) and percentage (17.8 vs 19.7%) compared to silages and concentrate. However, lower TVFA (13.2 vs 14.4 mmol/g OM) and higher propionic acid (19.7 vs 18.9%) were observed in browse.

Table 5.4. Volatile fatty acid production of substrates in cow and goat rumen fluid.

Substrate	Inoc	TVFA	Acetic	Propionic	Butyric	Valeric	BCVFA	A:P
		(mmol/g OM)	as % TVFA					
A. etbaica	goat	10.7*	61.2	23.1	8.1	4.3	3.3	2.7
	cow	8.3	63.0	23.2	7.0	4.5	2.3	2.7
C. tomentosa	goat	19.3***	60.4	17.5*	11.2	5.9	5.0*	3.5
	cow	16.0	61.1	20.1	9.6	5.8	3.5	3.1
D. cinerea	goat	15.4***	62.0	16.7	10.7	5.6	4.9*	3.7
	cow	12.6	63.9	18.4	9.1	5.2	3.4	3.5
R. natalensis	goat	13.5***	65.6	17.5***	8.3	4.5	4.2***	3.8
	cow	9.9	67.0	20.9	6.1	4.0	2.0	3.2
Concentrate	goat	17.6***	62.0	18.9	9.2	5.2	4.7	3.3
	cow	14.5	62.5	20.5	8.3	5.1	3.4	3.1
Maize silage	goat	17.0***	60.6	18.4***	10.9	5.7	4.4*	3.3**
	cow	14.2	59.8	22.9	9.1	5.5	2.8	2.6
Grass silage	goat	13.1***	67.8	15.2	8.1	4.7	4.2***	4.5
	cow	10.1	69.6	17.2	6.6	4.5	2.1	4.1
SEM								
Substrate		0.37	0.43	0.46	2.12	2.92	0.81	0.10
Inoc		0.28	0.23	0.36	2.10	2.91	0.79	0.08
Substrate×Inoc		0.47	0.61	0.57	2.15	2.93	0.83	0.13
P-value								
Substrate		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Inoc		<0.0001	0.0024	<0.0001	<0.0001	0.223	<0.0001	<0.0001
Substrate×Inoc		0.796	0.250	0.0008	0.748	0.965	0.322	0.024

A:P, Acetic: Propionic ratio; BCVFA, Branched chain volatile fatty acids; Inoc, Inoculum; OM, Organic matter; SEM, Standard error of the mean; TVFA, Total volatile fatty acids.

$^{**}P$<0.05; $^{**}P$<0.01; $^{***}P$<0.001, indicate significant differences between inoculum within substrate.

5.3.3. Inoculum effects on volatile fatty acids

The production of TVFA, and the molar proportion of acetic, propionic, butyric and branched chain volatile fatty acids (BCVFA) were different (*P*<0.002) between goat and cow inocula (Table 5.4). Overall, the production of TVFA (15.2 mmol/g OM), the proportion of butyric (9.5%) and BCVFA (4.4%) were higher in goat inoculum. The acetic to propionic ratio (A:P) was also significantly higher (*P*<0.0001) in goat inoculum.

5.3.4. Interactions of inoculum and substrates

Interactions between substrate and inoculum were observed for half-time GP parameters, C1 and C2 (*P*<0.001). Values of C1 were similar between goat and cow inocula for browse species, but higher in cow inoculum for the silages and concentrate. Similar values for C2 were observed despite inoculum type, but values were higher in goat inoculum for silages and the concentrate; whereas C2 was higher in cow inoculum but lower in goat inoculum for browse. Interactions also existed for R_{max2} and R_{max3} ($P<0.011$) with higher values in cow inoculum for browse. For the silages and concentrate, high values for R_{max2} were observed but not for R_{max3}. The proportion of propionic acid was affected by the interaction between substrate and inoculum.

5.4. Discussion

Ruminants differ in the diversity and number of ruminal microorganisms, the interaction of which determines feed degradation, CH_4 formation and productivity (Hegarty, 2004; Henderson et al., 2015). The variation in cumulative GP between goat and cow inocula must be due to differences in rumen microbial community between animal species (Bueno et al., 2015). The rumen microbial profiles of goats were distinct from cows when kept under similar dietary conditions (Lee et al., 2012). The finding of a higher GP for goat inoculum disagrees with data of Bueno et al. (2015) where incubation with cow (Holstein) inoculum produced more gas than Saanen goat inoculum. The difference could be due to the duration of *in vitro* incubation (24 h vs 72 h). In the result presented here a significant contribution of fermentation during 20-72 h to cumulative GP was observed in goat inoculum.

Our observation on the interspecies differences in *in vitro* cumulative GP is supported by previous reports for cows and buffalo (Calabró et al., 2004), sheep and buffalo (Calabró et al., 2005), goat and cows (Bueno et al., 2015) and goat and sheep (Ammar et al., 2008) inocula. The increase in GP in goat inoculum in the present study reflects increased microbial activity. The source of inoculum also affected fermentation kinetics parameters. The observed higher GP during different phases for goat inoculum indicates its superiority in terms of fermenting the soluble and non-soluble fractions in the substrate. The proportion of gas produced during the first phase (32.4 vs 28.9%) and third phase (21.3 vs 17.5%) was higher in goat inoculum compared to cows. Similarly, increased GP during the early hours of *in vitro* fermentation was also observed in goats compared to cow rumen inoculum (Camacho et al., 2010). Partitioning of gas production into different phases assists to understand fermentation characteristics of incubated feedstuff. The same observation could also be used to compare results with degradability of substrates in *in vitro* and *in situ* systems (Cone et al., 1997). The highest R_{max} was observed during the first phase with 82% in goat and 74% in cow inoculum.

This observation is in line with the observation that R_{max} is high due to fermentation of the rapidly fermentable fractions during phase 1 (Cone et al., 1997). The higher R_{max3} in goat inoculum, which would be associated with microbial turn over (Cone et al., 1997), indicates higher microbial growth rates.

The substrates used in the present study can be grouped in to tropical browse species (*A. etbaica*, *C. tomentosa*, *D. cinerea*, *R. natalensis*) and temperate feedstuffs (grass silage, maize silage and concentrate). The difference in GP for the first phase between goat and cow inocula was higher in browse (43.7 ml/g OM) than temperate feedstuffs (39.6 ml/g OM). Differences in GP between the two inocula during the second phase when browse was used as a substrate (38.4 ml/g OM) were higher than the temperate feedstuffs (27.6 ml/g OM) indicating that the microbial flora in goat rumen fluid has a greater ability to ferment non-soluble fractions. On average, the effect of inoculum source on GP was more pronounced in browse species (114.8 ml/g OM), known for their phenolic/tannin composition, than for temperate feedstuffs (102.6 ml/g OM). The greater differences between cow and goat to ferment browse substrates were in line with results of Camacho et al. (2010) who observed higher browse fermentation *in vitro* in goat inocula (128.4 ml/g DM) compared to cow inocula (104.7 ml/g DM) adapted to the same browse diet. Such peculiar characteristics may be related to the feeding habits or animal species specific adaptations.

Gas production, OM disappearance and extent of degradation have been reported to be different for cereal, high quality forage and shrubs when incubated in sheep and red deer rumen fluid adapted to alfalfa hay/concentrate diet (Hervás et al., 2005). Differences among ruminants in morphological, physiological and behavioural characteristics shape the microbial community structure (Henderson et al., 2015). The rate of digesta passage is faster in goats compared to cows under grazing conditions (Huston et al., 1986) and sheep in confinement (Li et al., 2008; Tsiplakou et al., 2011). In addition, there is evidence that the mean ruminal retention time affects microbial species and composition (Biddle et al., 2013). In the present experiment, since both goats and cows were adapted to a similar roughage/concentrate ratio, variation in the activity of the inoculum should be mainly due to animal species differences. A remarkable difference in R_{max2} existed in browse between cow and goat inoculum (average 8.3 vs 19.5 ml/h), but only small differences were observed for the temperate feedstuffs (average 23.0 vs 21.3 ml/h), indicating a faster rate of degradation of the non-soluble fractions in goat inoculum. The rate and extent of GP differed when diets of different quality were incubated in sheep and deer inocula (Hervás et al., 2005).

Bueno et al. (2015) reported that large ruminants (dairy and cattle) produced more CH_4 (g/DM digested) compared to small ruminats (sheep and goat). These authors suggested that the lower CH_4 in goats resulted from inferior ability to digest fibre, and conflicting results present in the literature which contradict (e.g. Ferreira et al., 2016; Odenyo et al., 1999) or support the same view (e.g. Li et al., 2008). Unlike cattle and sheep, goats were shown to possess bacteria of the *Ruminococcaceae* family (Ferreira et al., 2016), a specialized group of microbes that can degrade complex plant material (Biddle et al., 2013). This may explain the high CH_4 production in goat inoculum in the present study. The higher CH_4% in goat inoculum was consistent with results of Bueno et al. (2015), reporting 19.5% for Saanen goat and 15.9% for Holstein cow inoculum. Enteric CH_4 production reduces efficiency of energy utilization, and thus productivity in ruminants. Although observations are limited to *in vitro* results, under the conditions of the present experiment, goats appeared to emit more CH_4. However, further *in vivo* comparisons based on CH_4 produced per unit product or per unit intake need to be considered in order to evaluate the effect of CH_4 on productivity. Regardless of inoculum source, CH_4 production was markedly lower in browse species which is likely due to the presence of tannins/polyphenols affecting activity of methanogenic bacteria and protozoa (Animut et al., 2008; Beauchemin et al., 2008; Goel and Makkar, 2012).

More than 70% of the energy available to ruminants originates from VFA and as such constitute a major source of energy. The higher extent and rate of GP resulted from higher microbial activity (Silanikove et al., 1993) in goat inoculum was also reflected in the production of higher total VFA. However, the proportion of individual VFA differed between goats and cows suggesting differences in fermentative pathways (Hervás et al., 2005). The higher proportion of branched chain fatty acids in goat inoculum indicates efficient microbial growth (Silanikove et al., 1993) and subsequent turn-over. This observation was corroborated by the increased GP during the third phase related to microbial turnover.

The proportions of VFA produced influence the production of CH_4. Methane production increases with the formation of acetic acid and decreases with propionic acid. Since the proportion of acetic acid was comparable between goat and cow inocula, the higher CH_4 production in goat inoculum could be explained by the lower proportion of propionic acid, an alternative H_2 sink. A strong direct relationship between A:P ratio and CH_4 production was reported by Saminathan et al. (2015), similar to results of the present experiment. The ratio was higher in goat inoculum with a corresponding increase in CH_4.

5.5. Conclusions

Differences in the rate and extent of fermentation between goats and cows, kept on identical dietary regime, exist. Rate of substrate fermentation was faster in goat inoculum during the first and third phase of *in vitro* incubation, which both contribute significantly to a higher cumulative GP. Goat inoculum produced more CH_4 in absolute terms and as a percentage of cumulative gas production compared to cows. The microbial flora in goat inoculum was less affected by the presence of tannins/phenolic compounds in browse species. The differences between the two inocula were more pronounced in fermentation of browse than silages (grass and maize) and concentrate. These results explain previous observations (Chapter 4) where unusual fermentation patterns were observed when browse was inoculated with goat rumen fluid *in vitro*.

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CHAPTER 6

General discussion

6.1. Background and outline

Browse species constitute a large proportion of the dietary niche for browsing and grazing animals in areas where the growth of herbaceous species is limited by the amount and distribution of precipitation. Typically, this concerns arid and semi-arid areas in many countries including Ethiopia. The nutritional value of a number of browse species from semi-arid areas of Ethiopia, in terms of chemical composition, was studied previously (e.g. Melaku et al., 2010; Shenkute et al., 2012; Yayneshet et al., 2009) but these authors paid little attention to the tannin/phenol contents. Beneficial effects of tannins or other polyphenols has been researched over the past four decades (Aerts et al., 1999) and shown to be natural alternatives to improve feed utilization (e.g. protein efficiency, ruminal CH_4 reduction) and health (e.g. bloat reduction, nematode control) in ruminants.

The present work investigated tannin/phenolic-containing browse species commonly available in the Kola-tembein area of Tigray region of Northern Ethiopia. The area was selected based on the large goat population present in the region and the construction of area exclosures. The latter are areas which have been degraded previously due to overgrazing and over exploitation of wood for domestic use (fuel wood, construction), and allowed to recover from these human and animal activities to facilitate regeneration of the natural vegetation (Yayneshet et al., 2008). Two adjacent exclosures were selected for browse collection due to similarity in vegetation and ease of access via roads. Leaves of nineteen browse species were sampled at the end of the long rainy season (July-September). The presence of condensed tannins (CT) in the browse species was confirmed as absorbance values ranged from 1.1 to 657.2 Abs_{550nm}/g dry matter (DM) (data not included in the thesis). Based on the relative abundance in exclosures and variation in CT concentration, 10 browse species were selected for further investigation. At the end of the long rainy season in 2014, leaves of these 10 browse species were collected from the two area exclosures and pooled for use in four consecutive experiments. The browse species were: *Acacia etbaica, Cadaba farinosa, Capparis tomentosa, Dichrostachys cinerea, Dodonaea angustifolia, Euclea racemosa, Maerua angolensis, Maytenus senegalensis, Rhus natalensis* and *Senna singueana*. Confirmation of the taxonomy of the browse species was achieved by experts at the national herbarium institute of Addis Ababa University, Ethiopia.

The objectives of the studies reported in this thesis were to evaluate the 10 browse species from Ethiopia for a) preference by goats, b) *in vitro* anthelmintic property against *Haemonchus contortus* and c) methane (CH_4) reduction potential. During the conduct of the study involved with aim c, unusual fermentation patterns of browse species were observed adding a further aim d) comparison of *in vitro* fermentation characteristics of browse species using inocula from goats and cows kept on identical dietary regime, in order to determine whether the patterns were cause by the browse species or the inoculum.

Preference of goats as measured by DM intake as a proxy, in the presence and absence of the tannin binding agent polyethylene glycol (PEG) showed that preference was more related to the digestible fibre fraction than tannin concentration (Chapter 2). The voluntary intake of browse containing a higher tannin/phenolic content provides opportunity to exploit browse for beneficial effects in goat production and husbandry. Browse species were subsequently analysed for proanthocyanidins (PA) contents present in the soluble and fibre fractions according to Grabber et al. (2013) and for other polyphenols by UPLC-DAD-ESI-MS/MS as described by Engström et al. (2015). Results of these analyses were used in the subsequent experiments involving browse anthelmintic property against *Haemonchus contortus* and CH_4 reducing properties.

The larval exsheathment inhibition assay was used to examine anthelmintic properties of browse species extracts. Nine of the extracts were potent against exsheathment of *H. contortus* third stage larvae (L_3) attributable to the presence of phenols and non-phenolic compounds (Chapter 3) as confirmed by the use of polyvinylpolypyrrolidone (PVPP). The CH_4 reducing potential of the 10 browse species was investigated using the *in vitro* automated gas production (GP) technique in the presence and absence of PEG. Reduction in CH_4 and also GP was observed in 7 browse species containing PA. The inhibitory activities were attributable to PA but also other non-phenolic compounds appeared to be involved (Chapter 4). The peculiar *in vitro* fermentation of browse species observed in Chapter 4 led to further investigations to determine if the observed characteristics originated from the inoculum or substrate types. Comparison between goat and cow inocula revealed that differences in fermentative activity between the inocula existed and were more amplified for the browse species (Chapter 5).

6.2. Preference for browse species in experienced goats

Preference refers to the ability of animals to discriminate among diets/feed ingredients and is made based on preingestive and postingestive feedback. Goats have been shown to have a relatively limited ability to discriminate among feeds (Illius et al., 1999), and the need to ingest nutrients or to avoid toxins has been considered as reasons for their partial preference. In their review, Provenza et al. (2003) argued, based on a satiety theory, that partial preference results from feed aversions due to the interaction among flavours, nutrients and toxins. However, observed preferences for feeds could be an indication of both the characteristics of the feed, and the need of the animal such as the animal's state of nutrition (satiety), feed accessibility, familiarity with the diet and the need for self-medication, are also additional factors determining preference.

The preference of browse by goats and whether preference is influenced by browse tannins was investigated using PEG (Chapter 2). The design of the experiment was different from previous preference studies with sheep and goats in confinement (Alonso-Díaz et al., 2008; Hernández-Orduño et al., 2015), as it provided information on the sequential preference pattern during 10 min intervals once the most preferred species were consumed. The preference for the high tannin-containing browse species: *D. cinerea, R. natalensis, A. etbaica* and *M. senegalensis* was consistent during each 10 min of intake measurements (Chapter 2; Fig. 2.1), which is not in line with the aversion hypothesis (Provenza, 1996). According to this hypothesis, a decline in preference for the most preferred species occurs due to aversions. The switch in browse preference in the present study was more determined by the relative availability of the most preferred browse species, than due to aversions. It can be hypothesised that for aversions to be shown, goats should have *ad libitum* access to browse species.

The experience animals acquire in the embryonic stage of development via *in utero* mechanisms, continue post-partum with the interaction of the genome with the environment, shaping dietary habits (Provenza et al., 2003). Phenolic compounds in browse may deter herbivory and thus result in a reduced intake and preference. Naive animals are in general reluctant towards novel feeds and rely more on preingestive feedback mechanisms to make decisions on preference. In experienced animals, however, preference is based on the non-cognitive taste feedback interaction. For instance, previous postingestive consequences (positive or negative) after consumption of tannin-containing browse species determine whether phenolics are the first determinant factor for preference of these forages. Under practical conditions, browse species supply nutrients to browsing domestic ruminants particularly during a long dry season when herbaceous species become scarce. Browse intake, therefore, is primarily related to nutrient needs. Whether there is an increased intake of browse for self-medication by goats requires further investigation. The behavioural responses related to preference could be different depending on the association between gastrointestinal nematodes (GINs) infestation and the relief from consuming plants with anthelmintic properties. It is also important to note that animals have limited capacity to detoxify phenolic compounds and, therefore, a threshold level exists after which toxicity might occur. There are differences among domestic ruminants, and goats are one of the most tolerant species when it comes to the ingestion of phenolic compounds (Silanikove et al., 1996).

The results of the preference study (Chapter 2) can potentially be used for the pattern of browse disappearance through browsing when goats are in a natural environment (e.g. exclosures). The order of browse preference after PEG inclusion indicates the capacity of goats for substantial intake of tannin-containing browse due to their previous experience. It also demonstrates their adaptive behaviour to browse species. It is widely accepted that CT levels of >50 g/kg DM reduce voluntary feed intake, digestibility and productivity in ruminants (Min et al., 2003). Such a recommendation, however, was based on temperate forages (Mueller-Harvey, 2006) with sheep and may not be applicable to tropical forages for goats. Tropical browse species contain a diverse group of plant secondary metabolites (PSM) with phenolic compounds accounting up to 50% of the organic matter (Reed, 1986). Condensed tannins were also detected in all browse species in the present work, except two (Chapter 3, Table 3.1). Although the setup of the preference experiment did not allow determination of threshold PA levels, the voluntary intake of browse with higher tannin/phenolics by the goats, provides an indication that goats can consume in excess of 5% PA without adverse effects. A recent study by Yisehak et al. (2016) with tropical goats fed hay supplemented with tannin-containing forages (CT>10%) showed improved DM digestibility compared to goats on a control diet (hay). Being an intermediate browser, goats have developed adaptive mechanisms to overcome tannin intake levels which could be toxic to other production domestic ruminants. For example, their saliva proteins, rich in proline and histidine, have a high affinity towards tannins compared to sheep kept under the same browsing/grazing condition (Alonso-Díaz et al., 2012). Such a physiological adaptation also reduces the need to use PEG to inactivate browse tannins as a strategy to improve production of low input livestock production systems. Following the results of the preference study, further experiments were conducted and the next sections are devoted exclusively to browse forage value (e.g. role as anthelmintic and as rumen fermentation modulator).

6.3. Anthelmintic properties of browse differ in preference by goats

During the past twenty five years, there has been a growing interest in research on the use of forages as nutraceuticals (Hoste and Torres-Acosta, 2011). The browse species extracts exhibited anthelmintic activity against the common sheep and goat nematode, *H. contortus* (Brunet et al., 2008; Hounzangbe-Adote et al., 2005; Moreno-Gonzalo et al., 2013; Paloni et al., 2004). These anthelmintic properties are attributable to the presence of plant secondary metabolites (PSM) (Hoste and Torres-Acosta, 2011). Although goats used in the preference study (Chapter 2) were dewormed, they preferred browse species with a higher tannin/phenolic concentration and this can be expected to be beneficial to control GINs infestations (Chapter 3). All browse extracts exhibited anthelmintic properties and the doses to inhibit 50% of *H. contortus* L_3

exsheathment corresponded in the same direction of the PA concentrations (Chapter 3; Table 3.3). Based on the level of preference, browse species could be divided into three groups: *D. cinerea*, *R. natalensis*, *A. etbaica* (highly preferred), *M. senegalensis*, *C. tomentosa*, *M. angolensis*, *S. singueana* (moderately preferred) and *E. racemosa*, *D. angustifolia* (lowly preferred). On average, each group had an EC_{50} value of < 193.8, < 339.6 and < 275.9 μg/ml, respectively. These results indicate that at the lowest concentration of extracts, the preferred browse species can have a significant anthelmintic effect against 50% of *H. contortus* L_3 exsheathment. However, these results should be corroborated by *in vivo* trials. Higher EC_{50} of 213.2-772.1 μg/ml of heather species extracts was reported for *H. contortus* and *T. circumcincta* L_3 (Moreno-Gonzalo et al., 2013).

The possibility to use browse species as anthelmintic agents for local goats has the advantage that they are not novel feeds. Rejection of novel feeds is one behavioural strategy of animals to avoid the consequences of ingesting toxins (Provenza et al., 2003). Although PA are absent in *M. angolensis* and only traces are found in *C. tomentosa*, both exhibited anthelmintic properties. Behaviourally, goats are adapted to forage on a diverse group of browse species and such adaptive behaviour can be advantageous in terms of benefiting from the anthelmintic properties of the less preferred species such as *M. angolensis* and *C. tomentosa*. Feeding a mixture of tannin-containing browse species minimizes the energy costs associated with detoxification in the small intestine, dilutes deleterious effects of tannins and was suggested as a useful strategy for parasite control (Makkar, 2003; Mueller-Harvey, 2006). *Maerua angolensis* and *C. tomentosa* appear to contain non-phenolic compounds responsible for direct anthelmintic properties against *H. contortus* L_3 and this warrants further study to identify these compounds. Other attributes of browse species such as protein, fibre content and digestibility of nutrients are also important in evaluating anthelmintic effects *in vivo*. These parameters are important to maintain optimal microbial fermentation, ultimately benefiting the host animal. *Maerua angolensis* and *C. tomentosa* contained 220-246 g/kg DM crude protein (CP) which increases the flow of microbial protein into the small intestine and enhances host immunity with indirect effects against parasite establishment. In PA-containing browse, the formation of CT-protein complexes in the gastrointestinal tract is dependent on the characteristics of the tannin (molecular weight, composition) and protein (molecular weight, amino acid composition) as well as pH (Hoste et al., 2006). The dissociation of this complex in abomasum (low pH) and the subsequent availability for absorption in the small intestine enhances the host immune response (Coop and Kyriazakis, 2001).

The PA-containing browse species used in this thesis had a CP ranging from 69-220 g/kg DM (Chapter 2; Table 2.1). The preference of goats for the browse with a high PA content, therefore, contributes to the availability of rumen escape protein to the small intestine for absorption to improve host immunity, in addition to direct anthelmintic effects. For a direct anthelmintic effect on larval exsheathment to occur in the rumen, availability of free protein (not complexed with tannins) is important, since L_3 exsheathment occurs in the rumen. It is worthy to note that phenolic and non-phenolic compounds appeared to contribute to the anthelmintic properties of browse extracts (Chapter 3; Fig. 3.1). The EC_{50} values seem to correlate more to the PA concentration than other phenols. Anthelmintic properties of non-phenolic compounds was observed at high concentrations and substantial intake of browse species containing these compounds may need to occur to ensure the desired anthelmintic effects. Strategies to encourage optimal intake levels may be required for the less preferred browse species. For instance, exposing goats to browse species with anthelmintic properties at the onset of rainy season (where the risk of infection is high) would allow goats to express self-medicative behaviour which can also be transmitted from mother to offspring (Villalba and Landau, 2012). Hai et al. (2014) showed that the consumption of goats with *Chromolaena odorata* during late pregnancy (100 to 145 days of gestation), resulted in an increased intake of the kids after post-weaning. Such strategies could be adopted to stimulate the intake of browse species with high anthelmintic properties but low in preference.

Results of the present experiments are useful in exploiting the studied browse species for anthelmintic properties, in addition to their nutritional significance to local goats. However, confirmation of the observed effects *in vivo* is required. Comparison or extrapolation of results of such experiments could be limited because of the variability in characteristics, concentration and method of tannin/other phenolic compound analysis. Differences could also result from the strain of parasite and the stage of development evaluated. Table 6.1 presents anthelmintic properties of some tropical browse species against *H. contortus.*

Table 6.1. Anthelmintic properties of tropical browse species against *Haemonchus contortus in vivo* and *in vitro*.

Browse	Assay	Dose	Anthelmintic effect	Polyphenols			Active component	References
				Condensed tannin (CT)	Total tannin	Total phenols		
Acacia karoo *Acacia nilotica*	*in vivo*	40% (DM) of the basal diet	Decrease in faecal egg count	1.30±0.105 A_{550}/g 0.07±0.002 A_{550}/g	-	0.56 A_{675}/g 2.21 A_{675}/g	CT & other polyphenols	Kahiya et al. (2003)
Acacia polyacantha	*in vivo*	100-130 g/day	Reduced faecal egg count and worm burden	283 g/kg DM	-	Not Provided	CT	Max et al. (2007)
32 shrubs	*in vitro*	700-1400 µg/ml	Inhibition of development from egg to third stage larvae (L_3)	not provided	-	not provided	Tannin and non-tannin compounds	Kotze et al. (2009)
Anadenanthera colubrina *Leucaena leucocephala* *Mimosa tenuiflora*	*in vitro*	300 µg/ml	Inhibition of L_3 exsheathment	-	48.2-99.6 mg gallic acid/g 58.8-37.7 mg gallic acid/g 78.7-147.9 mg gallic acid/g	(mg gallic acid/g) 40.7-78.4 45.42-24.1 30.1-138.9	Tannins	Oliveira et al. (2011)
10 trees/shrubs	*in vitro*	150-1200 µg/ml	Inhibition of L_3 exsheathment	0 to ≥200 mg/g DM	-	-	CT non-phenols	Chapter 3 (this thesis)

6.4. Methane reduction and optimal fermentation in browse species

Various nutritional strategies have been devised including the use of PSM to reduce ruminal CH_4 production by ruminants (Knapp et al., 2014), as CH_4 is responsible for feed energy loss and contributes to greenhouse gas emissions. Browse species are, in general, rich in PSM (mainly phenolics) and evaluation and selection of promising species may contribute to improving energy utilization in the rumen. The fermentation characteristics of the 10 selected browse species and their CH_4 reducing properties were evaluated *in vitro* (Chapter 4) using PEG as a model to study the effect of tannins. Results showed that browse tannins containing PA significantly reduced CH_4 with a concomitant significant reduction in GP, although with a different magnitude. Tannins reduce CH_4 through decreasing the fibre digesting bacteria, methanogens and protozoa (Bhatta et al., 2009; Jayanegara et al., 2015; Tavendale et al., 2005). However, a suitable substrate for CH_4 mitigation would reduce CH_4 with little or no effect on GP (Pellikaan et al., 2011) while increasing propionate production (Tavendale et al., 2005). Although GP decreased, the increase in propionate with decreasing CH_4 concentrations were the positive attributes of the studied PA-containing browse species. The decrease in GP in the browse species was due to a reduction in digestibility caused by the PA, which agrees with other studies reported in the literature (Jayanegara et al., 2015; Makkar, 2003; Tavendale et al., 2005). A comparison between hydrolysable and CT on *in vitro* fermentation characteristics showed that *in vitro* organic matter digestibility was more reduced by PA (Jayanegara et al., 2015). Although all PA-containing browse species (n=7) in the current study decreased GP, the magnitude of reduction is different and comparisons can be made based on the decrease in CH_4 and GP as evaluated by the addition of PEG (Fig. 6.1).

When CH_4 is expressed as a % of GP at 72 h, for only 7 browse containing PA, the ranking could be as *A. etbaica* < *D. angustifolia* < *D. cinerea* < *S. singueana* < *R. natalensis* < *M. senegalensis* < *E. racemosa*. Jayanegara et al. (2015) indicated that the biological activity of tannins expressed as a decrease or increase in CH_4 using PEG is a more accurate indicator than expressing its mere change in concentration (CH_4 as a % of GP), when evaluating different tannin sources for CH_4 mitigation. This is important due to the fact that the presence of PA in the browse species reduced both CH_4 and GP. The ratio of the decrease in CH_4 and the decrease in GP can be used to compare among the browse species. As shown in Fig 6.1, the magnitude of the ratio in all browse species is greater than 1. This indicates more CH_4 reduction than GP when compared based on a per gram organic matter incubated. A higher ratio represents a relatively more suitable browse species to reduce CH_4 with a lesser effect on GP. Accordingly, browse ranking using this criterion would be: *E. racemosa* (1.29) < *A. etbaica* (1.33) <

M. senegalensis (1.37) < *D. angustifolia* (1.45) < *R. natalensis* (1.47) < *S. singueana* (1.68) < *D. cinerea* (1.72). In the practical feeding of goats, browse species either make a large proportion of the diet or are used as a supplement. Therefore, the CH_4 reduction *in vivo* would also be determined by their relative contribution to the goat's diet and also the characteristics of the basal diet.

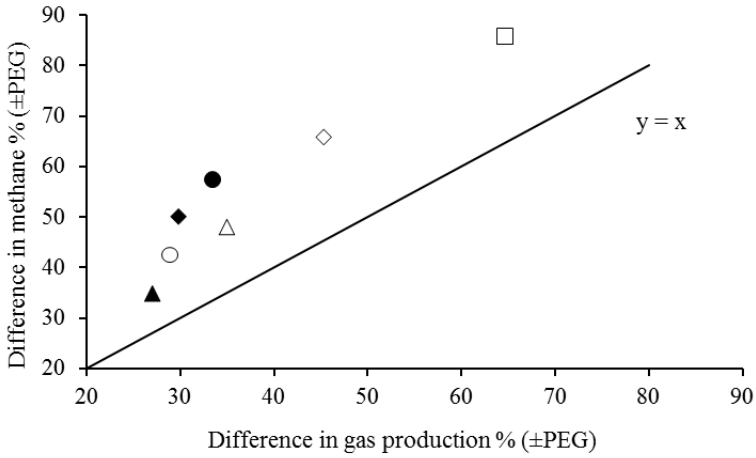

Fig. 6.1 The ratio of percent difference in gas production with or without (±) polyethylene glycol (PEG) and percent difference in CH_4 with or without (±) PEG after 72 h of *in vitro* incubation of proanthocyanidins- containing browse species. *E. racemosa* (▲), *R. natalensis* (○), *S. singueana* (◆), *M. senegalensis* (△), *D. cinerea* (●), *D. angustifolia* (◇), *A. etbaica* (□).

For nutritive evaluation of tannin-containing browse, it is imperative to consider attributes other than CH_4 reduction. Total volatile fatty acid (VFA) production was lowered when browse species were incubated without PEG, predominantly attributable to the presence of PA. A large portion (75%) of the carbohydrates fermented in the rumen is converted to VFA and serves as the main energy source for the host animal. The lowered VFA production in the presence of PA is due to lowered fibre degradation as observed in Chapter 4. The normal range of VFA in the rumen of goats is between 60 and 150 mmol/L, but also higher values of 200 mmol/L are recorded with highly digestible fresh grass or starch rich diets (Bergman, 1990). The order of VFA concentrations in mmol/L for the 7 browse species were: *A. etbaica* (47.6) < *D. angustifolia* (58.0) < *D. cinerea* (64.1) < *E. racemosa* (64.8), *M. senegalensis* (64.9) < *R. natalensis* (64.5) < *S. singueana* (69.0). The values can be considered to be on the low side of the optimum levels and indicates lower degradability of the browse species due to PA but also due to their inherent high lignin content. This suggests that digestible energy sources are required to increase availability of ruminal VFA in browse species feeding. All PA-containing browse stimulated propionate and in this way could be energy conserving and contributing to the productivity of animals.

Dietary components that favour propionate production are accompanied by CH_4 reduction (Knapp et al., 2014). A reduction in ammonia (NH_3) concentration and the proportion of branched chain VFA indicate the effects of PA on protein digestion. Protein degradation in the rumen results in the production of NH_3 and tannins have a high affinity towards protein, rendering them less accessible to microbial degradation and hence reduce NH_3 production. Ensuring adequate NH_3-N (200 mg/L) in the rumen is the first priority in optimizing fermentative digestion of forage in order to supply the majority of N for microbial growth (Leng, 1990). In the present study, NH_3-N concentrations in PA-containing browse ranged from 162.5 in *A. etbaica* to 270.9 mg/L in *S. singueana* indicating the importance of the browse species in supplying a nitrogen source to allow microbial growth. As such, production efficiency in goats consuming predominantly browse can be enhanced by supplementing fermentable substrates rather than N.

6.5. Goats superior performance in digesting fibrous and phenolic- containing forages

A clear difference in *in vitro* fermentative activity of goat and cow inocula was observed in Chapter 5. To avoid differences in dietary background, goats and cows were fed identical diet in equal proportions. Previous studies showed higher feed particle size reduction in goats compared to sheep (Domingue et al., 1991) and to cattle (Jalali et al., 2015). The former authors reported the superior ability of goats to digest ligneous forages. The particle size reduction could be attributable to the presence of a specialized group of microbes, which may also explain the higher GP ($P<0.0001$) in Chapter 5 regardless of substrate type (Fig. 6.2A). It also indicates the ability of goats to degraded the relatively ligneous browse substrates better, which was also reported by Domingue et al. (1991). In both goat and cow inocula, PA affects GP during the early hours with more sever effects in cow inoculum. Regardless of PA concentration, PA reduced GP in *A. etbaica*, *D. cinerea* and *R. natalensis* similarly. But the order of browse fermentation with cow inoculum was inversely related to PA concentration (Fig. 6.2B) and it more closely followed the digestible fibre content of the browse species (NDF-ADF). Similarly, in goat inoculum (Fig. 6.2A), fermentation pattern and GP was similar with moderate to high PA-containing browse but different from *A. etbaica*. Under identical dietary regimes, cow inoculum can be used instead of goat inoculum and *vice versa* to rank conventional feedstuffs (maize and grass silage), but for browse species, the same cannot be said as there is a difference in the response of the different inocula due to the variable effect of PA.

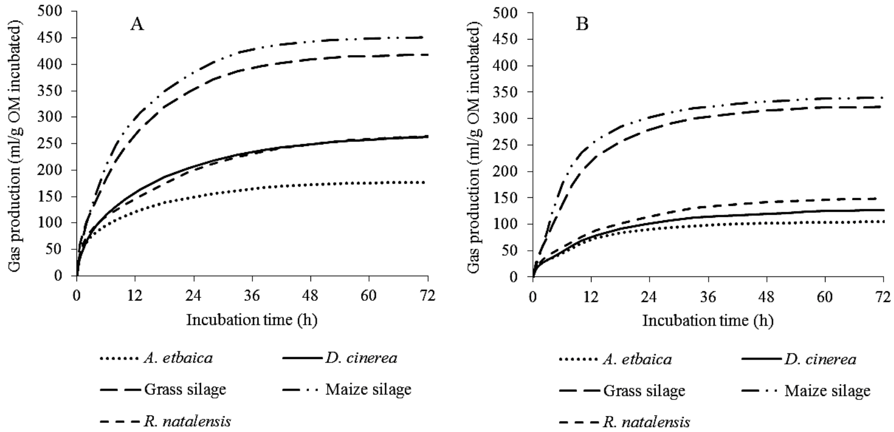

Fig. 6.2. *In vitro* gas production of proanthocyanidins-containing browse species and temperate forages incubated in goat (A) and cow (B) rumen fluid.

The fermentative activity of goat inoculum was higher in the *in vitro* incubations in Chapter 5 (*in vitro* 2) compared to results of Chapter 4 (*in vitro* 1). The same breed of goats (Saanen) and substrates (*A. etbaica*, *D. cinerea* and *M. senegalensis*) with different concentration of PA were compared. Gas production in these species in *in vitro* 1 and 2 were, 76.4 vs 181, 150.6 vs 265, 164.0 vs 264.0 ml/g OM, respectively. The differences are attributable mainly to the dietary background that goats in *in vitro* 2 had a 10% more concentrate and better quality roughage than goats in *in vitro* 1. Concentrate diets are highly fermentable and promote the growth of rumen microbes and also increase protozoal population. Therefore, increased microbial population is responsible for increased GP in *in vitro* 2. For the three browse species, percent CH_4 expressed as total GP was also increased: 7.4 vs 13.9, 12.0 vs 19.0, 14.8 vs 19, respectively. These observations indicate that availability of digestible protein and carbohydrate source determines the extent of CH_4 reduction by PA.

6.6. Simultaneous effects of browse PSM on rumen efficiency and parasite control

Nutritional constraints related to both the quantity and quality of forages and parasitism, impose major constraints on low input sheep and goat production systems. Different approaches aiming to increase feed availability and increase the efficiency of feed utilization, through participatory approaches with livestock keepers have been suggested as key tools in improving small ruminant productivity in arid and semi-arid areas (Salem, 2010). Improved feeding through supplementation of urea-molasses blocks in Small East African goat kids reduced fecal egg count, worm count and resulted in weight gain (Waruiru et al., 2004). However, feed supplementation to improve livestock productivity is not a feasible option in low input production systems and, therefore, alternative affordable strategies are required for practical application. This section focuses on making comparisons among browse used in the present study based on the combined results obtained on preference, anthelmintic and CH_4 reduction properties of browse species (Chapters 2-4).

The studied browse species (Table 6.2) are voluntarily consumed by local goats and also demonstrated to have contain anthelmintic activity against *H. contortus* larval establishment and, therefore, can be considered as nutraceuticals. However, significant CH_4 reduction was observed only in PA containing browse species. Exsheahtment of the L_3 and CH_4 production occurs in the rumen. It can be stated that in parasitized goats, a simultaneous effect of PA can occur in the rumen against CH_4 production and *H. contortus* establishment. It is known that goats can increase their intake of PA-rich forages due to secretion of high levels of proline-rich saliva. However, the preferential binding of tannins to proline-rich salivary proteins also reduces the availability of active tannins in the rumen to bind proteins (Mcmahon et al., 2000; Muir (2011). Tannin-protein complexes are stable in the rumen pH (5.5-7) but dissociate in the low pH (2-3) of abomasum (Perez-Maldonado et al., 1995). The latter author reported the formation of soluble salivary protein-tannin complex in sheep and goat. This phenomenon allows browse tannins to act against *H. contortus* exsheathment and reduce methanogenesis. The tannin-protein complexes escaping the rumen and the subsequent dissociation enable in the abomasum also to have enhanced activity of tannins against the adult *H. contortus*. The latter, however, was not studied in the present work.

The use of PEG in the *in vitro* gas and PVPP in the larval exsheathment assay clearly indicated the biological activity of PSM (tannin, other phenols and non-phenols) and their possible interaction (Table 6.2). The absence of differences between PVPP treated *C. tomentosa* and *M. angolensis*, and that both exhibit anthelmintic properties suggest activity of non-phenolic compounds. However, the anthelmintic properties of the non-phenolic compounds in these species occur at higher concentrations. On the other hand, there was no CH_4 reducing activity in *C. tomentosa* and *M. angolensis*. From these results, it can be deduced that non-phenolic PSM play little or no role in CH_4 reduction but at optimal levels, anthelmintic properties can exist. Comparatively, these species have a higher protein content and indirect anthelmintic effects through stimulating host immunity could be additional beneficial effects. Non-phenolic compounds had significant contribution in inhibition of L_3 exsheathment in *D. cinerea*, *R. natalensis* and *A. etbaica*. Whereas, *M. senegalensis*, *S. singueana*, *E. racemosa* and *D. angustifolia* exert anthelmintic properties mainly due to their phenolic composition. Comparatively, the latter group of browse species are more potent at low concentrations. The relatively high concentration in the former group also supports the earlier statement that non-phenols exhibit anthelmintic properties at higher concentrations.

Comparing anthelmintic properties of the different browse species with the corresponding CH_4 reducing properties, it is not possible to establish a direct relationship based on EC_{50} values and CH_4%. As mentioned earlier, co-occurring non-phenolic components of browse species plays a role in L_3 exsheathment inhibition. This was the case in *D. cinerea*, *R. natalensis* and *A. etbaica*, and the higher preference of these species provides a possibility for ingestion of substantial quantities of non-phenols for anthelmintic effect. This in turn increases the availability of active tannins in the rumen for antimethanogenic effects. It is, thus, important to consider PSM other than phenols in evaluating the anthelmintic properties of tannin-containing browse. It can be hypothesized that, anthelmintic activity is more correlated to the total phenol contents than PA concentration; whereas CH_4 reduction activity is more related to phenolic contents but also with PA concentration except *A. etbaica*.

Table 6.2. Relationship between browse proanthocyanidins (PA) content, preference ranking anthelmintic properties and methane (CH_4) reducing properties.

Browse species	Preference ranking*	PA (mg/g DM)	EC_{50} (µg/ml)	Bioactives against larval exsheathment†	%GP§	%CH_4§	Ratio‡
Dichrostachys cinerea	1	101-150	127.4	mostly phenolic	33.4	57.4	1.72
Rhus natalensis	2	≥200	168.6	mostly non-phenolic	28.9	42.6	1.47
Acacia etbaica	3	0-50	285.5	mostly non-phenolic	64.6	85.8	1.33
Maytenus senegalensis	4	≥200	UD	tannin and phenolic	35.0	48.0	1.37
Capparis tomentosa	5	0-4	333	entirely non-phenolic	NS	NS	1.74
Maerua angolensis	6	-	346.1	entirely non-phenolic	NS	NS	1.27
Senna singueana	7	0-50	179.1	tannin and phenolic	29.8	50.1	1.68
Cadaba farinosa	8	-	2036.6	-	NS	NS	1.09
Euclea racemosa	9	≥200	UD	tannin and phenolic	27.0	34.9	1.29
Dodonaea angustifolia	10	51-100	275.9	tannin and phenolic	45.3	65.8	1.45

*Determined using 10 min intake as proxy (Chapter 2).

†see Fig. 3.1 (Chapter 3).

§The decrease in gas production (GP) or CH_4 measured using polyethylene glycol (Chapter 3).

‡The ratio between %GP and %CH_4

UD, unable to determine; EC_{50}, extract concentration required to inhibit 50% of *Haemnochus contortus* L_3 exsheathment calculated at 60 min; NS, non-significant.

6.7. The benefits of browse species and implication for their management as forages for goats in Ethiopia

Goats can adapt to a wide range of browse species compared to sheep and cattle (Sanon et al., 2007). In open grazing systems, intensive selection for the most preferred species inevitably results in the gradual disappearance of these species. Preference studies in confinement provide an indication of the selection pattern of preference of animals under a free grazing setup. Availability of browse species determines preference under free grazing. The most abundant species is not necessarily the most preferred species and that accessibility determines preference (Odo et al., 2001). Pattern of browse preference can also be different in dry and wet seasons as reported for naturally browsing goats (Yayneshet et al., 2008). Seasonal differences are related to both the quality and quantity of browse, which in turn influences preference. Management scheme for the use of browse species as forages need to consider both preference data and abundance of browse species in a given area. Preference data can be used as a basis for formulating a diet mixture for efficient utilization of available browse species.

The presence of phenolic compounds in browse species should not be generalized as antinutritonal factor and emphasis should be given to its beneficial effects. In particular this is important in goat production in Ethiopia where the production system relies on the use of natural browse species. In addition, the characteristics of the local goats to voluntarily ingest high tannin/phenolic-containing browse broadens the use of these resources for extra-nutritional properties (e.g. GIN control). For full exploitation of tannin-containing browse species for nutritional and extra-nutritional properties, proper feeding strategies should be in place. Direct browsing in rangelands, fodder banks (concentrated plantation in a given area) and cut-and-carry systems are proposed for the use of tropical tannin-containing browse (Alonso-Díaz et al., 2010). Although the practical implementation of browse management strategies depends on the local conditions, the following strategies are proposed for browse species utilization for goats in Ethiopia:

- Increasing the stocking density during the favourable time of the year such as at the end of the long rainy season. Short duration intensive browsing favours minimization of browse selection.
- Implementation of the cut-and-carry system: foliage removal when abundant browse is available and proper storage could enable efficient utilization of materials to fill the gap in feed availability during the dry season.

6.8. Conclusions

Based on the findings described in this thesis, the following conclusions can be drawn:

- Browse preference in local goats with browsing experience is not primarily dependent on tannin content, but on increasing digestible energy intake.
- Browse species with the exception of *Cadaba farinosa* are potent inhibitors of *Haemonchus contortus* L_3 exsheathment.
- Phenolic and/or non-phenolic components of browse species are responsible for anthelmintic activity against *Haemonchus contortus*.
- Proanthocyanidins in browse species decrease *in vitro* fermentation, but with a higher magnitude of decrease in methane than gas production.
- Proanthocyanidins contents do not always result in the corresponding level of methane reduction, therefore, information on proanthocyanidins composition and structure is required.
- Regardless of the presence of phenolic compounds, goats can better ferment forages compared to cows kept on an identical dietary regime.

The studied browse species in the present work can be strategically integrated in goat diets to reduce establishment of *Haemonchus contortus* and also to improve feed energy utilization in the rumen by reducing methane formation.

6.9. References

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Genet

ABOUT THE AUTHOR

Curriculum vitae

List of publications

Training and supervision plan

Curriculum vitae

Genet Feseha Mengistu was born on April 06, 1982 in Harar, Ethiopia where she attended elementary and high school education. She obtained a BSc degree in Animal and Range Sciences from Mekelle University, Ethiopia in 2003. Immediately after graduation she joined the same department as a graduate assistant. In 2005, she was granted with a two-year scholarship by the Netherlands Organization for International Cooperation in Higher Education (NUFFIC) and obtained MSc degree specializing in Animal Nutrition from Wageningen University in 2007. Her MSc thesis research was about the effect of DHA-edible algae on rumen biohydrogenation of poly-unsaturated fatty acids and milk fatty acids in dairy cows. Upon completion of her MSc, she has returned back to her home institution and engaged in teaching, research and supervising undergraduate students. In 2009, she joined a nine month training and obtained international diploma in poultry husbandry and animal feed from PTC+, the Netherlands which was financed by NUFFIC. She resumed work at her home institution after the training and in 2012 she pursued a PhD study in the Animal Nutrition Group, Wageningen University by the financial support of NUFFIC. In 2015, she received a WIAS fellowship which allowed her to collaborate with INRA, Toulouse, France and University of Turku, Finland for part of her PhD work. During the PhD, Genet studied Ethiopian browse species of importance in goat production with a particular interest in browse polyphenol composition in relation to ruminal methane reducing and anthelmintic properties. The result of her PhD project is presented in this thesis.

List of publications

Peer reviewed scientific publications

Mengistu, G., Bezabih, M., Hendriks, W.H., Pellikaan, W.F. 2016. Preference of goats (*Capra hircus* L.) for tanniniferous browse species available in semi-arid areas in Ethiopia. Journal of Animal Physiology and Animal Nutrition, DOI: 10.1111/jpn. 12648.

Mengistu, G., Hoste, H., Karonen, M., Salminen, J.-P., Hendriks, W.H., Pellikaan, W.F. 2016. The *in vitro* anthelmintic properties of browse species against *Haemonchus contortus* is determined by the polyphenol content and composition. Journal of Veterinary Parasitology, DOI: 10.1016/j.vetpar.2016.12.020.

Mengistu, G., Karonen, M., Salminen, J.-P., Hendriks, W.H., Pellikaan, W.F. 2016. *In vitro* fermentation of browse species using goat rumen fluid in relation to browse polyphenol content and composition. Submitted to Journal of Animal Feed Science and Technology.

Mengistu, G., Hendriks, W.H., Pellikaan, W.F. 2016. *In vitro* methane and gas production with inocula from cows and goats fed an identical diet. Submitted to Journal of the Science of Food and Agriculture.

Conference and symposia proceedings

Mengistu, G., Pellikaan, W.F., Bezabih, M, Hendriks, W.H. 2015. Goats' browse preference in relations to chemical composition and tannin content of Ethiopian browse. In: proceedings of 66th Annual Meeting of the European Federation of Animal Sciences, Warsaw, Poland, pp 382.

Mengistu, G., Hoste, H., Pellikaan, W.F., Hendriks, W.H. 2016. Anthelmintic activity of tanniniferous browse against *Haemonchus contortus* infective stage *in vitro*. In: proceedings of WIAS Science Day, Wageningen, the Netherlands, pp 9.

Mengistu, G., Derseh, M., Hendriks, W.H., Pellikaan, W.F. 2016. The role of condensed tannins in browse species preference by goats. In: proceedings of the 41st Animal Nutrition Research Forum, Wageningen, the Netherlands, pp 39.

Mengistu, G., Hoste, H., Hendriks, W.H., Pellikaan, W.F. 2016. *In vitro* inhibitory activities of browse extracts on larval exsheathment of goat nematodes. In: proceedings of the 16th Internatinal Conference on Production Disease in Farm Animals, Wageningen, the Netherlands, pp 71.

Training and supervision plan[1]

The Basic Package (3 ECTS[2])

WIAS introduction course 2013

Course on philosophy of science and/or ethics 2013

Scientific Exposure (9 ECTS)

International conferences

7th Novel approaches meeting with a session of the CAPARA COST ACTION

Goat parasite interaction: from knowledge to control, Toulouse, France 2013

Innovation in livestock production: from ideas to practice, Warsaw, Poland 2015

16th International conference on production disease in farm animals, Wageningen, the Netherlands 2016

Seminars and workshops

International symposium on dairy cattle nutrition, Wageningen, the Netherlands 2012

WIAS science day, Wageningen, the Netherlands 2013

WIAS science day, Wageningen, the Netherlands 2014

WIAS science day, Wageningen, the Netherlands 2016

Animal nutrition research forum, Wageningen, the Netherlands 2016

Presentations

Goats' browse preference in relation to chemical composition and tannin content of Ethiopian browse, Warsaw, Poland, poster presentation 2015

Anthelmintic activity of tanniniferous browse against *Haemonchus contortus* infective stage *in vitro*, Wageningen, the Netherlands, oral presentation 2016

The role of condensed tannins in browse species preference by goats, Wageningen, the Netherlands, oral presentation 2016

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[1] Completed in the fulfilment of the requirements for the education certificate of the Graduate School Wageningen Institute of Animal Sciences (WIAS).

[2] One ECTS equals a study load of 28 hours.

In vitro inhibitory activities of two browse extracts on larval exsheathment of goat nematodes, Wageningen, the Netherlands, poster presentation	2016

In-Depth Studies (8 ECTS)

Disciplinary and interdisciplinary courses

Forage evaluation in ruminant nutrition	2015

Advanced statistic courses

Advanced statistics course: design of experiments	2012
Statistics of the life sciences	2013

Professional Skills Support Courses (4 ECTS)

Reviewing a scientific paper	2012
Project and time management	2012
Information literacy including EndNote introduction	2012
Data management	2012
Techniques for writing and presenting a scientific paper	2013
Voice and presentation skills	2013

Research Skills Training (10 ECTS)

Preparing own PhD research proposal	2013
External training period, INRA, Toulouse, France	2013
External training period two months, INRA, Toulouse, France and University of Turku, Finland	2015

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Colophon

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