Methane emissions from sheep fed *Eragrostis curvula* hay substituted with *Lespedeza cuneata*

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Summary text

The effects of climate change are predicted have adverse effects on crop and livestock production, particularly in developing countries. The study aimed to investigate practical mitigation strategies which could be implemented in extensive and semi-intensive sub-tropical ruminant production systems. The results suggest that *Lespedeza cuneata* has the potential to reduce CH_4 emissions and possibly increase production from sheep by improving the dry matter digestibility and through improved dry matter intake.

Abstract

Context

Reducing emissions of greenhouse gases (GHG) from livestock production systems is a global research priority.

Aims

The objective of this study was to investigate the effect of feeding condensed tannin-containing *Lespedeza cuneata* hay at different levels on the feed intake and enteric methane (CH_4) emissions of sheep fed a basal diet of sub-tropical *Eragrostis curvula* hay.

Methods

Four adult ruminally cannulated Dohne-Merino wethers with an initial body weight of 65.5 ± 3.5 kg were used in a 4 x 4 Latin square design. The experimental treatments were T1: 100% *E. curvula*: 0% *L. cuneata*; T2: 70% *E. curvula*: 30% *L. cuneata*; T3: 40% *E. curvula*: 60% *L. cuneata*; T4: 10% *E. curvula*: 90% *L. cuneata*. Each of the four experimental periods lasted for 27 days consisting of a 14 day adaptation period, a 7 day digestibility trial, and a 6 day methane measurement period. During the 6 day methane measurement period methane emissions were measured continuously over a 24 hour period using an open circuit respiration system.

Key results

The dry matter intake (g/kg $W^{0.75}$) was higher ($P<0.05$) for sheep receiving T3 and T4 compared to T1 and T2 (77.33 and 84.67 g/kg $W^{0.75}$ compared to 62.96 and 62.71 g/kg $W^{0.75}$, respectively). The increase in DMI corresponded with a linear increase in the dry matter digestibility of the experimental treatments from 38% to 45% as the level of *L. cuneata* substitution increased from T1 to T4. Methane emissions (g/ kg DMI) was not influenced

(P>0.05) by the 30% inclusion level of *L. cuneata* but decreased (P<0.05) as the level increased to 60% and 90% from 17.6 g CH_4/kg DMI to 13.8 g CH_4/kg DMI and 14.3 g CH_4/kg DMI respectively.

Conclusions

The inclusion of *L. cuneata* hay in a diet based on *E. curvula* hay improved diet digestibility, and led to increased concentrations of crude protein (CP), neutral detergent fibre (NDF) and non-fibre carbohydrates (NFC). Substituting *E. curvula* hay with 60% *L. cuneata* on a DM basis resulted in the highest CH_4 reduction of 21.4% compared to a 100% *E. curvula* diet.

Implications

The results suggest that *L. cuneata* has the potential to reduce CH_4 emissions and possibly increase production from sheep by improving the dry matter digestibility and through improved dry matter intake.

Key words: sericea lespedeza, rumen fermentation, respiration chamber, methane mitigation

Introduction

Reducing emissions of greenhouse gases (GHG) from livestock production systems is a global research priority. The effects of climate change are predicted to be highly dynamic and it can have adverse effects on crop and livestock production, particularly in developing countries (Scholtz *et al.* 2011). Numerous CH_4 mitigation strategies and technologies have been explored over the past decade, including interventions in livestock management, dietary composition, ruminal fermentation and altering the methanogen population in the rumen (Patra *et al.* 2017). Recent reviews on the mitigation of methane emission form livestock have showed that the viable options for mitigation have diminished over the past decade with many options showing inconsistent efficacy or impracticality for inclusion into livestock production systems (Hart *et al.* 2008; Patra *et al.* 2017). Most of the methane (CH_4) mitigation strategies has focussed on intensively managed ruminants fed high quality diets based on total mixed rations and animals grazing temperate pastures (Hristov *et al.* 2013). In contrast, the number of publications on mitigation strategies for sheep grazing low quality sub-tropical pastures is limited.

Ruminal micro-organisms digest plant fibre fractions into forms usable by livestock. During the process of ruminal fermentation enteric CH_4 is produced by methanogenic micro-organisms from the disposal of metabolic hydrogen (H_2) not utilized during the formation of volatile fatty acids (VFA) (Newbold *et al.* 2005). This process represents a loss of gross energy (GE) to livestock (Patra and Saxena 2010). Several researchers have reported reduced CH_4 emissions from ruminants consuming forages containing condensed tannins (CT), determined *in vitro* and *in vivo* from cattle and goats (Woodward *et al.* 2002; Min *et al.* 2003; Animut *et al.* 2008). Puchala *et al*. (2005) reported that the effect of CT in ruminants varies with the type of tannin or plant source and that ruminant species vary in their response to consuming CT containing forages. Tannins are compounds of high molecular weight containing reactive phenolic hydroxyl or carboxyl groups that enables it to complex with protein, minerals, and other macromolecules (Reed 1995). Jones and Mangan (1977) reported that tannin-protein complexes are pH dependent and stable between pH 3.7 and 7.0 but dissociates below a pH 3.5 (Sinclair *et al.* 2009). Forage sources containing tannins have

lower ruminal degradability and might, in addition to reducing CH_4, offer the potential of increasing the flow of undegradable protein to the small intestine, improving animal performance (Sinclair *et al.* 2009).

Lespedeza cuneata has been identified as a perennial legume high in condensed tannins (Puchala *et al.* 2012) and it is well adapted to low pH marginal agricultural soils in South Africa (Wasserman 1981). The objective of this study was to investigate the effect of substituting an *E. curvula* hay diet with different levels of *L. cuneata* containing CT, on dry matter intake, digestibility, and enteric methane emissions by sheep.

Material and Methods

The study was conducted at the Hatfield experimental farm of the University of Pretoria, South Africa. The Animal Ethics Committee of the University of Pretoria approved all experimental protocols (ECO18-14) before commencement of the study.

Animals and treatments

Four adult ruminally cannulated Dohne-Merino wethers with an initial body weight (BW) of 65.5 ± 3.5 kg were used in a 4 x 4 Latin square design. All animals were accustomed to experimental procedures and treated for internal and external parasites and each received an injectable vitamin A, D, and E supplement prior to the start of the study. The experimental treatments entailed the feeding of commercially sourced *E. curvula* hay substituted with 0%, 30%, 60% and 90% *L. cuneata* hay on a dry matter basis as treatments 1 to 4, respectively. The *L. cuneata* hay contained 17.7 mg CT/ g DM. All diets were offered as hammer milled hay to a particle length of 2 to 3 cm to ensure thorough mixing and prevent separation of particles when fed. The trial ran across four experimental periods. Each period lasted for 27 days consisting of a 14 day adaptation period, a 7 day digestibility trial, and a 6 day methane measurement period. After each experimental period sheep were penned as a group and fed a 50:50 high quality forage/ legume diet (consisting of E. curvula and M. sativa hay) for two weeks prior to the start of the next experimental period to improve nutritional status and minimise possible carryover effect of experimental diets.

Digestibility trial

At the start of each experimental period the sheep were weighed and housed in individual metabolic crates for the duration of the digestibility study. They were offered the experimental diets *ad libitum* at 08h00 and 15h30 daily and had free access to water and a commercial mineral supplement. After the adaptation period they were fitted with faecal collection bags and total daily feed intake and faecal output were recorded according to Kennedy and Charmley (2012). Subsamples of feed offered were collected daily and dried at 55°C for 48 hours. These subsamples were pooled at the end of the collection period and stored for analysis. Total daily feed refusals and faecal output were collected daily before the morning feeding, weighed and subsamples of feed refusals and faeces were taken. Feed refusals were dried at 55°C for 48 hours and stored. Feed refusals were analysed to determine the nutrient intake of animals on specific experimental diets according to Osuji *et al*. (1993). Faecal material was sampled and stored at -20°C. At the end of the collection period representative samples were taken and dried at 55°C in a forced air oven for analysis.

After the digestibility collection period 12 representative rumen fluid samples were taken from various parts of the rumen through the rumen cannula over a 72-hour period at 08h00, 12h00, 16h00, 20h00, 00h00, 04h00, 10h00, 14h00, 18h00, 22h00, 02h00, 06h00. Rumen samples were filtered through 4-layers of cheesecloth and the remaining material returned to the rumen. The pH of the rumen fluid samples was taken prior to being preserved with 4ml of a 25% H_3PO_4 solution per 20ml of rumen fluid for volatile fatty acid (VFA) determination, as described by Webb (1994) and 5ml of a 50% H_2SO_4 solution per 30ml of rumen fluid for NH_3–N determination, as described by Broderick and Kang (1980). All rumen fluid samples were stored at -20°C before analysis of ruminal NH_3-N and ruminal VFA.

Methane measurement

After completion of the digestibility study the sheep were moved to open circuit respiration chambers for 6 days. Methane was measured using four chambers arranged in two rows of two with a 2m corridor between the two rows. The sheep were allowed to acclimatize for the first 3 days in the chambers, thereafter CH_4 was recorded over a 24 hour period. The chamber construction and operation was based on respiration chambers at Aberystwyth University (Hart *et al.* 2012) as described by Gemeda (2014). Methane concentration was measured per second continuously over a 5 min period per chamber using an multigas analyser with a solid state non dispersive infrared aborption detector (ADC MGA3000, Spurling works, Herts, UK). It took 20 min to sequentially sample the airflow in all chambers where after the system was calibrated using a zero gas (100% nitrogen gas) and a span gas (150ppm CH_4 standard gas). All animals received the experimental diets twice daily at 08h00 and 15h30 *ad libitum* and had free access to water and a commercial mineral supplement. Daily feed intake was determined as described above. Chamber floors were cleaned during the morning feed in between the measuring periods of each chamber to minimize interruptions. The methane flux for each chamber was calculated as the average flux over each of the 48 sampling times in the 24 hour sampling period. Gas recovery tests were conducted on individual chambers at the start of each sampling period, according to the method described by Hart *et al*. (2012). The average recovery rate was 98.7%, 100%, 97.9% and 101.9% for the four chambers used respectively. All chamber data was corrected for gas recovery rates.

Sample analysis

Samples of experimental diets, feed refusals, and faeces were ground to pass a 1mm screen after drying in a forced air oven at 55°C for 48 hours. Samples were analysed for dry matter (DM), ash, nitrogen (N), ether extract (EE), calcium (Ca) and phosphorous (P) according to procedures of the AOAC (2000). Samples were also analysed for neutral detergent fibre (NDF) and acid detergent fibre (ADF) concentration using an ANKOM 200/220 Fibre Analyser (ANKOM Technology, Fairport, NY, USA) based on the methods described by Van Soest *et al*. (1991). Sodium sulphite and heat stable amylase were used in the analysis of NDF. The NDF and ADF were expressed inclusive of residual ash. The lignin concentration (ADL) was determined according to Van Soest *et al*. (1991) through the solubilisation cellulose with sulfuric acid in the ADF residue. Acid detergent insoluble nitrogen (ADIN) was determined from the ADF analysis followed by N analysis. Samples were analysed for gross energy (GE) using a bomb calorimeter (Parr 3600, Parr Instrument Co. Inc., Moline, IL, USA) according to the AOAC (2000). Samples of the *E. curvula* and *L. cuneata* hay were mixed with diatomaceous earth and extracted with 70% methanol in steel extraction cells of an accelerated solvent extraction system (ASE 200, Dionex, Sunnyvale, CA) for tannin

concentration analysis. Tannins were analysed by means of the vanillin-HCL method of Broadhurst and Jones (1978). The metabolizable energy (ME) concentrations of the experimental treatments were estimated according to AFRC (1993) from diet digestible organic matter (DOM). Sample non-fibre carbohydrates (NFC) were estimated according to Fox *et al*. (2004). Rumen fluid samples were analysed for ammonia-N and volatile fatty acids (VFA) according to procedures described by Broderick and Kang (1980) and Webb (1994).

Statistical analysis

An analysis of variance using the GLM model of SAS (SAS 2015) for a Latin square design was used for all the variables to determine differences between periods, treatments, and sheep. The means and the standard error of the means (SEM) were calculated, while the significance of differences ($P < 0.05$) and tendencies ($P \leq 0.10$) between means were determined using Fischer's test (Samuels and Wittmer 2003).

Results

Roughage composition

The chemical composition (DM basis) of the experimental diets offered is presented in Table 1. Substituting *E. curvula* hay with *L. cuneata* hay increased ($P<0.05$) the CP concentration of the diets from 9.3% to 13.3% from the control (T 1) to the 90% substitution level (T 4). Neutral detergent fibre and ADF concentrations decreased ($P<0.05$) with increased levels of *L. cuneata* substitution. The lignin (ADL) concentration of diets increased with increased levels of *L. cuneata* in experimental diets ranging from 8.32% to 14.4% from the control (T 1) to the 90% substitution level (T 4). The EE concentration of diets was not affected by experimental treatments but the NFC increased ($P<0.05$) from 4.07% to 30.3% from pure *E. curvula* hay to the 90% *L. cuneata* substituted diet (T 4).

Table 1 Nutrient composition of experimental diets fed to sheep (% of DM)

	Experimental treatment				
Item (% of DM)	**T1**	**T2**	**T3**	**T4**	**SEM**
OM	94.9	94.8	94.7	94.2	0.21
CP	9.3^{c}	11.7^{b}	13.2^{a}	13.3^{a}	0.27
NDF	79.9^{a}	68.0^{b}	58.4^{c}	49.08^{d}	1.10
ADF	45.9^{a}	45.1^{a}	44.3^{a}	41.8^{b}	0.50
ADL	8.3^{d}	11.5^{c}	12.6^{b}	14.4^{a}	0.26
ADIN	2.4^{c}	3.3^{b}	3.6^{b}	4.9^{a}	0.12
EE	1.5	1.6	1.6	1.6	0.47
NFC	4.1^{d}	13.5^{c}	21.5^{b}	30.3^{a}	0.01
GE MJ/ kg DM	16.9	18.0	17.2	17.4	0.01
ME MJ/ kg DM	5.9	6.4	6.6	5.8	0.32
Ca	0.3^{d}	0.4^{c}	0.6^{b}	0.7^{a}	1.04
P	0.2	0.2	0.2	0.2	0.31
CT mg/ g DM	0^{d}	0.5^{c}	1.1^{b}	1.5^{a}	0.01

T1: 100% *E. curvula*: 0% *L. cuneata*; T2: 70% *E. curvula*: 30% *L. cuneata*; T3: 40% *E. curvula*: 60% *L. cuneata*; T4: 10% *E. curvula*: 90% *L. cuneata*; OM: Organic matter; CP: Crude protein; NDF: Neutral detergent fibre; ADF: Acid detergent fibre; ADL: Acid detergent lignin; ADIN: Acid detergent insoluble nitrogen; EE: Ether extract; NFC: Non-fibre carbohydrate; GE: Gross energy; Ca: Calcium; P: Phosphorous; ME: Metabolizable energy; CT: Condensed tannins.

^{a,b,c,d} Means with different superscripts in the same row differ ($P<0.05$)

SEM: Standard error of means

There were no differences in GE and ME concentrations across the experimental treatments. The P concentrations were not affected by experimental treatments but Ca concentrations increased ($P<0.05$) with increased levels of *L. cuneata* substitution. The *L. cuneata* hay utilized in the study contained a CT concentration of 17.7 mg/ g DM. Increasing the *L. cuneata* content of the experimental diets increased the CT ($P<0.05$) concentration from 0 for T 1 to respectively 0.53, 1.06, and 1.53mg/g DM for Treatments 2, 3, and 4.

Body weight, intake, digestion, and methane production

The results in Table 2 show that the average live weights of the sheep were 65.4, 66, 65.3 and 64 kg for Treatments 1, 2, 3 and 4 respectively. Dry matter intake (DMI), diet digestibility and methane (CH_4) production are also reported in Table 2. Substituting *E. curvula* hay with *L. cuneata* hay increased the apparent diet dry matter digestibility (aDMD) from 38% (T 1) to 45% of DM at the 90% substitution level (T 4). Daily dry matter intake (DMI) was similar for T 1 and T 2 but increased ($P<0.05$) in sheep receiving T 3 and T 4. The gross energy intake (GEI), digestible dry matter intake (DDMI) and digestible organic matter intake (DOMI) all showed a similar pattern to the DMI across all experimental treatments. Daily CH_4 emissions (g/day) were not affected by the experimental treatments, but CH_4 emissions per kg DMI

decreased (P<0.05) with increased levels of *L. cuneata* substitution. Methane emissions expressed as g CH_4/ g mDDMI.$W^{-0.75}$ decreased (P<0.05) as the level of substitution was increased from 30% (T2) to 60% (T3) and from 30% (T2) to 90% (T4). The energy expenditure as CH_4 (MJ/day) was unaffected by the treatments but the ratio of CH_4 energy as a percentage of gross GEI decreased (P<0.05) from T 1 to T 3 and from T 1 to T 4.

Table 2 Body weight, dry matter intake, diet digestibility and methane emissions of sheep consuming *Eragrostis curvula* hay substituted with *Lezpedeza cuneata* hay

	Experimental treatments				SEM
Item	**T1**	**T2**	**T3**	**T4**	
LW(kg)	65.4	66	65.3	64	1.94
aDMD (%)	38^{b}	41^{ab}	42^{ab}	45^{a}	2.01
Intake					
DMI (kg/d)	1.4^{b}	1.3^{b}	1.6^{a}	1.8^{a}	0.07
DMI (g/kg $W^{0.75}$)	63.0^{b}	62.71	77.3^{a}	84.7^{a}	2.65
GEI (MJ/ d)	23.1^{b}	23.0^{b}	28.3^{a}	31.2^{a}	1.23
DDMI (g/kg $W^{0.75}$)	24.2^{b}	25.1^{b}	32.8^{a}	38.2^{a}	2.16
DOMI (g/kg $W^{0.75}$)	17.1^{c}	20.8^{bc}	28.0^{ab}	32.0^{a}	2.55
Methane emissions					
CH_4 (g/d)	24.1	22.5	22.5	25.7	1.40
CH_4 (g/ kg DMI)	17.6^{a}	16.8^{ac}	13.8^{b}	14.3^{bc}	0.80
CH_4 (g/g mDDMI)	1.1^{a}	0.9^{a}	0.7^{b}	0.7^{b}	0.06
CH_4 (g/g mDOMI)	1.7^{a}	1.1^{ac}	0.8^{bc}	0.8^{bc}	0.25
CH_4 (% GEI)	6.3^{a}	5.9^{ac}	4.8^{bc}	4.9^{bc}	0.30

T1: 100% *E. curvula*: 0% *L. cuneata*; T2: 70% *E. curvula*: 30% *L. cuneata*; T3: 40% *E. curvula*: 60% *L. cuneata*; T4: 10% *E. curvula*: 90% *L. cuneata*; LW: Live weight; DMI: Dry matter intake; aDMD: Apparent dry matter digestibility; DDMI: Digestible dry matter intake; DOMI: Digestible organic matter intake; GEI: Gross energy intake; CH_4: Methane; mDDMI: Digestible dry matter intake per kg $W^{0.75}$; mDOMI: Digestible organic matter intake per kg $W^{0.75}$

^{a,b,c,d} Means with different superscripts in the same row differ (P<0.05)

SEM: Standard error of means

Ruminal fermentation

The results for rumen pH, rumen ammonia-N (NH_3-N) and volatile fatty acid (VFA) production are presented in Table 3. There were no differences (P>0.05) in ruminal pH and rumen ammonia-N across the experimental treatments (Table 3). The total VFA concentrations did not differ between the experimental treatments, except at T 3 which resulted in a lower (P<0.05) total VFA production compared to T 1 and T 4. Differences (P<0.05) were observed for the individual VFA ratios (Table 3). Acetate as a molar proportion of the total VFA concentration decreased (P<0.05) with increased levels of *L. cuneata* substitution ranging from 71.4% in T 1 to 59.7% in T 4. No differences were found in propionate proportion between T 1 and T 2 but an increase (P<0.05) in propionate proportion resulted from T 1 to T 3 and from T 1 to T 4 (22.3% to 25.1% and 22.3% to 24.3%), respectively. The molar proportion of butyrate increased across all treatments from 6.35% (T 1) to 16% (T 4) whereas a decrease in the acetate: propionate ratio (A: P) resulted when the level of *L. cuneata* substitution was increased from 30% (T2) to 60% (T3) and from 30% (T2) to 90% (T4).

Table 3 Rumen pH, ammonia-nitrogen and volatile fatty acid concentration in sheep fed *Eragrostis curvula* hay substituted with *Lespedeza cuneata* hay

	Experimental treatments				
Item	**T1**	**T2**	**T3**	**T4**	**SEM**
Ruminal pH	6.4	6.5	6.5	6.5	0.08
NH3-N (mg/ 100ml)	5.2	4.8	4.5	3.9	0.55
Total VFA (mmol/L)	49.9^{a}	48.9^{ac}	46.8^{bc}	49.6^{a}	0.56
Acetate (%)	71.4^{a}	65.8^{b}	61.6^{c}	59.7^{d}	0.52
Propionate (%)	22.3^{b}	22.5^{bc}	25.1^{a}	24.3^{ac}	0.46
Butyrate (%)	6.4^{d}	11.7^{c}	13.3^{b}	16.0^{a}	0.33
A:P ratio	3.9^{a}	3.0^{a}	2.5^{b}	2.5^{b}	0.11

T1: 100% *E. curvula*: 0% *L. cuneata*; T2: 70% *E. curvula*: 30% *L. cuneata*; T3: 40% *E. curvula*: 60% *L. cuneata*; T4: 10% *E. curvula*: 90% *L. cuneata*; NH3-N: Rumen ammonia-N; VFA: Volatile fatty acid; A:P acetate: propionate ratio.

^{a,b,c,d} Means with different superscripts in the same row differ (P<0.05)

SEM: Standard error of means

Discussion

Roughage composition

The increase (P<0.05) in CP, ADIN, ADL, NFC, Ca and the decrease in diet NDF concentration (P<0.05) as the level of *L. cuneata* increased in the experimental diets reflects the higher quality of the *L. cuneata* substitution diets compared to the pure *E. curvula* basal diet (T1). The CT concentration in *L. cuneata* in the current study (17.7 g/kg DM) was lower

than CT concentrations reported for *Lespedeza* spp. in previous studies (Terrill *et al.* 1989; Puchala *et al.* 2005; Animut *et al.* 2008; Puchala *et al.* 2012) ranging from 34 g/kg DM to 199 g/kg DM. These differences could be due to climatologically variations during the growth period of the forage, cultivar variations and Terrill *et al*. (1989) reported that the drying method employed to preserve *L. cuneata* affected the CT concentration of the forage with a decrease in CT concentration when *L. cuneata* was dried as hay. The CP concentration of all the experimental diets was above the minimum level of between 7 and 8% required for optimal microbial function in the rumen (Norton 2003).

Dry matter intake, digestion, and methane production

The DMI reported in Table 2 is similar and higher than values reported by Reid *et al*. (1990) for sheep receiving C4 grass diets of 65.8 g/kg $W^{0.75}$ and by Animut *et al*. (2008) for goats receiving *L. cuneata* diets of 66.1 g/kg $W^{0.75}$ respectively. In the present study DMI increased with higher levels of *L. cuneata* substitution in the diets from T 1 and T 2 to T 3 and T 4 ($P<0.05$). Treatments 3 and 4 had higher CP concentrations ($P<0.05$), and lower NDF concentrations ($P<0.05$) compared to T 1 and T 2 (Table 1). McDonald *et al*. (2011) stated that voluntary feed intake is closely related to the rate of digestion of feed and that the NDF concentration of feedstuffs played a major role in the rate of digestion of forages. This is supported by the aDMD of the experimental treatments reported in Table 2 of 38%, 41%, 42% and 45% for Treatments 1, 2, 3, and 4 respectively. Aitchison *et al*. (1986) also reported that DM and NDF digestion are higher for legumes compared to grasses. The aDMD reported in Table 2 is lower than expected for T 3 and T 4 with NDF concentrations of 58.4% and, 49.0% and NFC concentrations of 21.5% and 30.3%, respectively. This could partially be explained by the lignin and the tannin concentrations that increased ($P<0.05$) with increasing levels of *L. cuneata* in the experimental diets ranging from 32% to 14.4% and 0% to 1.53% for lignin and tannin respectively. The ratio of NDF to ADF concentration in the present study became smaller as the level of *L. cuneata* in the diets increased. Puchala *et al*. (2005) reported similar results for *L. cuneata* and these authors related the results to the presence of CT in *L. cuneata*. It was not clear if the presence of CT affected the DMI in the present study probably due to the relative low concentrations of CT in the experimental diets. Bhatta *et al*. (2002) reported that CT in forages negatively affected DMI when present in concentrations greater than of 6% of DM. The extent to which lignin in the diets containing *L. cuneata* influenced the digestibility is also unclear as the lignin in legumes influences the digestibility of other cell wall constituents less adversely than lignin in grasses (Puchala *et al.* 2005) possibly due to differences in the differential partitioning of lignin among plant tissues between legumes and tropical grasses (Moore and Jung 2001). The DDMI and DOMI reported in Table 2 is higher than values reported by Animut *et al*. (2008) for diets containing *L. cuneata*. This might have been due to a lower CT concentration of *L. cuneata* in the present study.

Daily CH_4 emissions were similar ($P>0.05$) across all experimental diets (Table 2) ranging from 22.5 g/day to 25.7 g/day. These values are similar to values reported by Pelchen and Peters (1998) in a review of sheep CH_4 emissions ranging from 20.5 g/d to 23.2 g/d across a variety of diets. Animut *et al*. (2008) and Hammond *et al*. (2013) reported higher daily CH_4 emission from sheep fed a diet of 100% *L. cuneata* (33.3 g/d) and sheep receiving a fresh ryegrass/ white clover diet (24.0 to 31.8 g/d) respectively. The daily CH_4 emissions in the present study are comparable to predicted daily CH_4 values for South African sheep in commercial operations ranging from 22.6g/d to 29.0 g/d (Du Toit *et al.* 2013).

The CH_4 emissions expressed as g/kg DMI decreased ($P<0.05$) from T 1 to T 3 and from T 1 to T 4 with the lowest CH_4 emissions found for T 3 (60% *L. cuneata*) of 13.8 g/kg DMI. The higher CH_4 production (g/kg DMI) reported in Table 2 for Treatments 1 and 2 could be due to the associated higher concentrations of cell wall components (NDF and ADF) and lower concentrations of CP and NFC compared to T 3 and T 4. Gemeda and Hassen (2015) reported a negative correlation between *in vitro* CH_4 production and the NFC, CP and ADIN concentrations in feed samples. These authors also reported a positive correlation between forage fibre concentration and *in vitro* CH_4 production. Similarly, Eun *et al*. (2004) reported a positive relationship between CH_4 production and the fibre concentration in livestock diets. Although not measured in the current study, the increased intake of sheep fed T 3 and T 4 suggests an increased rate of passage of feed particles in the rumen. An increased rate of passage is associated with a reduction in ruminal CH_4 production in sheep (Reid *et al.*1990; Muetzel and Clark 2015).

Carulla *et al*. (2005) reported CH_4 emissions of 4.9 to 5.3% of GEI by growing wethers with *ad libitum* consumption of ryegrass fed alone or mixed with red clover or lucerne. Similarly, Ominski *et al*. (2006) and Chaves *et al*. (2006) reported CH_4 emissions from cattle receiving diets ranging from 46 to 61% NDF of 5.1 to 5.9% and 4.6 to 6.6% of GEI, respectively. Methane emissions relative to GEI in the present study were similar to these values reported in the literature and decreased ($P<0.05$) as the level of *L. cuneata* substitution in the experimental treatments increased. This decrease in CH_4 (% GEI) could be explained by the higher digestibility and higher DMI of sheep fed diets containing 60% (T3) and 90% (T4) *L. cuneata* in the present study (Johnson and Johnson 1995; Benchaar *et al*. 2001). Tannins decrease CH_4 production by directly inhibiting methanogens and indirectly decreasing H_2 production as a result of decreased fibre digestion and protozoal population in the rumen (Patra *et al*. 2017). Previous researchers have shown that supplementing diets with CT (from various sources) decreased *in vitro* and *in vivo* CH_4 production (Tan *et al.* 2011; Hassanat and Benchaar 2013; Yang *et al.* 2016). These earlier results indicated that either CT or hydrolysable tannins (HT) at certain levels inhibit rumen CH_4 production, but that the extent of the reduction depends on the tannin source and possibly the composition of the diet (Yang *et al.* 2016). Methane production (g/kg $W^{0.75}$ and as % GEI) in the present study decreased as the concentration of the CT in the experimental diets increased. Although the data suggest a mitigation effect of CT in the present study the authors were unable to confirm the effect of tannins as the experimental treatments were not replicated with the inclusion of polyethylene glycol (PEG) to inhibit the effect of CT on dietary parameters, DMI and digestibility in the present study.

Rumen pH and fermentation

Diet composition can influence rumen fermentation and ruminal CH_4 production as a result of altered VFA production or a reduced degradation of feed consumed in the rumen (Bell *et al.* 2016). Both ruminal pH and ruminal ammonia nitrogen (NH_3-N) was not affected ($P>0.05$) by the experimental treatments (Table 3) in the present study. Although a numerical decrease in ruminal NH_3-N was observed in Table 3, the increase ($P<0.05$) in diet CP concentration and DMI reported in Table 1 and Table 2 did not affect the rumen NH_3-N concentration in sheep fed diets containing *L. cuneata*. Kanjanapruthipong and Leng (1998) stated that the effective degradability of tropical roughages fed to sheep was maximised at 8 mg NH_3-N / dL rumen fluid. This is higher than the concentration reported in Table 3 ranging from 3.86 (T4) to 5.15 mg/ 100 ml rumen fluid (T1). The lack of increase in the ruminal NH_3-N with increased CP intake could indicate the protein binding effect of *L. cuneata* CT

reported by Waghorn (1996) and Puchala *et al*. (2005). These authors reported an increased intestinal amino acid (AA) absorption in sheep receiving diets consisting of *L. cuneata*. The increase in intestinal AA absorption could enhance the growth rate and wool production of sheep receiving diets containing CT (Shewangzaw 2016).

Total volatile fatty acid concentration in sheep was not affected (P>0.05) by the experimental treatments. However, increasing the level of *L. cuneata* in experimental treatments decreased (P<0.05) the molar proportion of acetic acid with a simultaneous increase in the propionic acid and butyric acid concentrations. Hammond *et al*. (2013) stated that higher feed intakes resulted in shorter mean rumen retention times, consequently decreasing the extent of rumen fermentation compared to lower feed intakes. Decreased ruminal retention times could decrease CH_4 yield due to a shift in fermentation pathways towards more propionate production and thus less CH_4 production per unit of DMI (Janssen 2010). Dumeric *et al*. (2017) reported that feeding diets containing CT to sheep affected ruminal fermentation and resulted in a reduction (P<0.05) of ruminal fibrolytic bacteria. This supported the reduction in the molar proportion of acetate with increased levels *L. cuneata* in the experimental treatments (Table 3). The decreased NDF and increased NFC concentration in the experimental diets (Table 1) with increased levels of *L. cuneata* favoured the formation of propionate and butyrate, proportionally as a percentage of total VFA, in the ruminal fluid (Table 3). This data supports data reported by Hindrichsen *et al*. (2004) for diets high in NFC concentrations. Friggens *et al*. (1998) stated that the sugar and pectin content in the NFC concentration of feedstuffs are preferential to the formation of butyrate in the rumen at the expense of propionate. This could explain the relative high molar proportions of butyrate in the current study (Table 3). The decrease (P<0.05) in the A:P ratio from T 1 to T 3 and T 1 to T 4 is consistent with the reduction in CH_4 production reported in Table 2. The formation of propionate in the rumen serves as a competitive pathway for metabolic H_2 to CH_4 production (Moss *et al.* 2000). Yang *et al*. (2016) reported that supplementing diets with tannic acid increased the propionate concentration and decreased the ruminal A:P ratio in rumen fluid. These results are consistent with results for the present study reported in Table 3.

Conclusion

Results from this study suggest that *L. cuneata* has the potential to reduce CH_4 emissions from sheep fed a sub-tropical hay in addition to possible benefits of improved production. Substituting *E. curvula* hay with *L. cuneata* hay improved diet digestibility, and led to increased concentrations of CP, NDF and NFC. The increased intake of diets containing *L. cuneata* compared to *E. curvula* indicated that the potential adverse effects of CT in the *L. cuneata* used in the study were relatively low. Substituting *E. curvula* hay with 60% *L. cuneata* on a DM basis resulted in the highest CH_4 reduction of 21.4% compared to a 100% *E. curvula* diet. Further research is necessary to identify the optimal inclusion level of *L. cuneata* in a sub-tropical hay based diet and to explore the possible long term feeding effects on the production potential of ruminants.

Conflicts of interest

The authors declare no conflicts of interest.

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