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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 species tannins (using polyethylene glycol, PEG 6000) on *in vitro* gas production (GP), methane (CH_4), ammonia (NH_3), volatile fatty acids (VFA) and *in vitro* organic matter digestibility (IVOMD) were studied. Approximately 0.5 g of air-dried *A. etbaica*, *C. farinosa*, *C. tomentosa*, *D. angustifolia*, *D. cinerea*, *E. racemosa*, *M. angolensis*, *M. senegalensis*, *R. natalensis* and *S. singueana* leaves were used as substrates in an automated *in vitro* system. Proanthocyanidin (PA) contents were quantified using the modified HCl-butanol method and the PA composition analysed by ultra performance liquid chromatography using a diode array detector coupled with electrospray ionization mass spectrometry (UPLC-DAD-ESI–MS/MS). Substrates were inoculated for 72 h in buffered rumen fluid (2:1, v/v), pooled from three goats fed a grass silage and concentrate diet, on three separate occasions. Each substrate was incubated in triplicate with or without inclusion of PEG 6000 and GP measured. 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 after 72 h of incubation. Data from three runs were averaged for analysis. Addition of PEG increased ($P < 0.0001$) GP, CH_4, NH_3 and total VFA indicating that PA were mainly involved in reducing methanogenesis but also digestibility. Prodelphinidins were found to be the major PA affecting fermentation. Also the contribution of quercetin, myricetin and kaempferol derivatives 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 the possible minor effects of other phenolic and non-phenolic compounds.

Abbreviations: ADF, acid detergent fibre; ADL, acid detergent lignin; BCVFA, branched chain volatile fatty acids; CH_4, methane; CO_2, carbondioxide; CP, crude protein; DM, dry matter; GP, gas production; HAc, molar percent acetic acid; HB, molar percent butyric acid; HP, molar percent propionic acid; HV, molar percent valeric acid; IVOMD, *in vitro* organic matter digestibility; NDF, neutral detergent fibre; NGR, non-glucogenic ratio; NH_3, ammonia; OM, organic matter; PA, proanthocyanidin; PC, procyanidins; PD, prodelphinidins; PEG, polyethylene glycol; UPLC-DAD-ESI–MS/MS, ultra performance liquid chromatography with diode array detector coupled with electrospray ionization mass spectrometry; VFA, volatile fatty acids

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1. Introduction

Ruminants convert 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, on average, 10–16 while cattle produce 16–60 kg CH_4/head per year (Hristov et al., 2013). 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 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 feasible option to mitigate CH_4 (Buddle et al., 2011). Browse species, of which many contain substantial amounts of tannins, are predominantly found in arid and semi-arid areas where they form an important source of nutrition for ruminant production animals, such as goats. Regardless of previous exposure to tannins, the saliva of goats is high in proline-rich proteins (Ventura-Cordero et al., 2015). This adaptation enables goats to voluntarily consume substantial quantities of tannin-containing browse species (Yayneshet et al., 2008; Mengistu et al., 2016) commonly found in semi-arid areas.

In browse species, polyphenol contents may account up to 50% of the total organic matter (Reed, 1986). Although the knowledge on the ruminal CH_4 reducing property of polyphenols from forages has long been established (Bhatta et al., 2009; Oskoueian et al., 2015), the relationship between the phenolic composition and biological activities is only marginally studied. In the present study, the hypothesis was tested that PA content, composition and the presence of other polyphenols in browse species from a semi-arid region in Ethiopia modulate CH_4 production, 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, ammonia (NH_3) 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 polyphenolic components.

2. Materials and methods

2.1. Browse species collection and preparation

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. The browse species are voluntarily consumed by goats from the same region (Mengistu et al., 2016). 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. In order to minimize the loss of polyphenols, air drying was started immediately after the collection of the leaves. After the air drying, leaves were ground to pass through a 1-mm sieve and used for the determination of their chemical composition and further ground into a fine powder for the determination of their phenolic composition. The same samples were also used as substrates for *in vitro* incubation with rumen fluid. 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.

2.2. Browse species chemical composition

Browse samples were analysed according to AOAC (1990) for dry matter (DM) (ID 930.15), ash (ID 942.05) and crude protein (CP) (ID 955.04). Neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) were analysed according to Van Soest and Robertson (1985). Extraction and preparation of samples for PA analysis was done as described by Mengistu et al. (2017). Proanthocyanidin analysis was done according to Engström et al. (2014).

Total proanthocyanidin contents were analysed as previously described by Grabber et al. (2013). Total PA were analysed directly from the browse plant materials. Soluble PA were analysed from the browse plant extracts and the insoluble, *i.e.* bound, PA from the extraction residues. Proanthocyanidin were quantified against an external PA standard (extracted from *Calluna vulgaris*) which had a procyanidin:prodelphinidin (PC:PD) ratio of 99:1 and a mean degree of polymerization of 4.9 (Mengistu et al., 2017). Other polyphenols were analysed by UPLC-DAD-ESI–MS/MS as described by Engström et al. (2015).

2.3. Animals and diet

Nine dry female Saanen goats (51.7 ± 6.7 kg) were ranked by body weight (BW) and then divided based on similar BW into three groups (heavier, mid and lighter BW) with three goats per pen (4.0 m × 2.5 m) 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 (568 g DM/kg) and concentrate diet, offered in two equal portions at 9:00 h and 15:00 h. The total adaptation period to the diet and pen conditions lasted for eight weeks. The mean (± SEM) daily intake per goat over the 8 weeks was 834.1 ± 21.4 g DM grass silage and 200 ± 0.0 g concentrate with grass silage concentrate ratio of approximately 80:20. The total DM intake (2% body weight) was within the recommendations of the 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 Committee at Wageningen

University & Research (Wageningen, the Netherlands). Prior to the *in vitro* incubations, goats were re-divided into three groups, but now with a similar average BW (51.4 ± 0.3 kg) between groups and each group was used in successive incubation runs.

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 1.0 l 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 of rumen fluid per goat was pooled and 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).

Browse substrates of approximately 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 6000 to counteract the effect of tannins (Getachew et al., 2002). Pre-warmed bottles containing substrate (and PEG) were inoculated with 60 ml buffered rumen fluid under flushing with CO_2 and were connected to an automated pressure evaluation system in a shaking water bath at 39 °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. In each run, blank incubations (buffered rumen fluid without substrate) were included.

A fully automated *in vitro* system (Cone et al., 1996) was used to measure cumulative GP and CH_4 production at fixed time points based on the procedure as described by Pellikaan et al. (2011a). In brief, 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, 10 µ, 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).

2.5. Analysis and calculation 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 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 for NH_3 and VFA analysis were stored at −20 °C and analysed as described by Pellikaan et al. (2011b). 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.

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

$$NGR = (HAc + 2 \times HB + HV)/(HP + HV)$$

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:

$$BCVFA(\%) = [(isobutyric\ acid + isovaleric\ acid)/total\ VFA] \times 100$$

with isobutyric acid, isovaleric acid and total VFA expressed as mmol/g organic matter (OM).

2.6. 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, incubation 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 i^{th} effect of browse species (i = 1 to 10), P_j the j^{th} effect of PEG (j = − or +), T_k the k^{th} effect of time (k = 5; 6 h, 12 h, 24 h, 48 h and 72 h), $(B \times P)_{ij}$ 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 and NGR were subjected to ANOVA using the PROC GLM procedure of SAS.

Table 1
Chemical composition of browse species used in the *in vitro* experiment.

Browse species	DM	Ash	CP	NDF	ADF	ADL	Polyphenol contents								
	(g/kg)	(g/kg DM)					Proanthocyanidin				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
							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, 0–4 mg/g; +, 5–50 mg/g; ++, 51–100 mg/g; +++, 101–150 mg/g; ++++, 151–200 mg/g; +++++, > 200 mg/g.

3. Results

3.1. Chemical composition of browse species

Chemical composition of browse species is shown in Table 1. Among the browse with a moderate to high PA contents (> 5 mg/g), CP ranged from 69 g/kg DM in *E. racemosa* to 145 g/kg DM in *D. cinerea*. Neutral detergent fibre, ADF and ADL were the highest in *E. racemosa* (561, 501, 244 g/kg DM, respectively). *Senna singueana* had the lowest NDF (295 g/kg DM) and ADL (70 g/kg DM) content; while ADF was lowest in *D. angustifolia* (216 g/kg DM). Proanthocyanidin concentration was the highest (> 200 mg/g DM) in *E. racemosa*, *M. senegalensis* and *R. natalensis*. Moderate to low PA concentrations were found in *D. cinerea* (101–150 mg/g) and *D. angustifolia* (51–100 mg/g). *Acacia etbaica* and *S. singueana* had the lowest (5–50 mg/g) PA concentrations, while only traces (0–4 mg/g) of PA were found in *C. tomentosa*. *Acacia etbaica* and *S. singueana* contained PC, PD and oligomeric propelargonidins (PP) or profisetinidins (PF). *Dichrostachys cinerea*, *E. racemosa* and *R. natalensis* contained PC and PD. *Dodonaea angustifolia* contained mainly PC. *Maytenus senegalensis* contains both PC and PD with the latter found in higher proportions. Galloyl derivatives were detected in six of the 10 browse species and varied considerably in their concentrations with the highest concentration found in *D. cinerea* (6.9 mg/g DM). The contents of kaempferol based polyphenols were the highest (9.0 mg/g DM) in *S. singueana*. The contents of quercetin based polyphenols were the highest (7.7 mg/g DM) in *D. cinerea* while myricetin based polyphenols were the highest (7.1 mg/g DM) in *E. racemosa*.

3.2. In vitro gas and CH_4 production of browse species

Gas production differed ($P < 0.0001$) among browse species, with PEG and time of incubation (Table 2). The addition of PEG had significant effect on the GP of *D. angustifolia* from 12 h, but from 6 h onwards for the other browse species where PEG affected GP. The percent increases in total GP at 72 h by the addition of PEG were 183%, 83%, 54%, 50%, 42%, 41% and 37% for *A. etbaica*, *D. angustifolia*, *M. senegalensis*, *D. cinerea*, *S. singueana*, *R. natalensis* and *E. racemose*, respectively. Gas production of *C. tomentosa*, *C. farinosa* and *M. angolensis* was not affect regardless of PEG addition.

Methane production differed ($P < 0.0001$) among browse species with PEG and time of incubation (Table 2). The addition of PEG significantly increased CH_4 production except for *C. tomentosa, C. farinosa* and *M. angolensis*. The increase in CH_4 with PEG was, however, observed for *E. racemosa*, *R. natalensis*, *D. cinerea* and *A. etbaica* after 6 h while after 12 h in *D. angustifolia*. Graphical representations of total GP (Fig. 1A,B) and CH_4 as a percentage of total GP (Fig. 1C, D) are shown for *M. senegalensis*, *D. cinerea* and *S. singueana* with high, moderate and low PA concentration, respectively and representing different fermentation patterns. A rapid increase in GP during the first 3 h was observed in these browse species (Fig. 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. The shape of the GP profile differed (Fig. 1B) after PEG addition. Methane production as a percentage of total GP for the three browses increased in the presence of PEG (Fig. 1C, D). A rapid increase in CH_4 percentage was observed in *M. senegalensis* during the first few hours of fermentation in the absence and presence of PEG (Fig. 1C, D). *Dichrostachys cinerea* showed a similar rapid increase but only in the absence of PEG (Fig. 1C). *Maytenus senegalensis* had similar CH_4 percentage profile in the absence and presence of PEG (Fig. 1C, D). There was a continuous curvilinear increase in CH_4 concentration for *S. singueana*.

3.3. Volatile fatty acids, ammonia and organic matter digestibility

Total and individual VFA concentrations in the 72 h fermentation liquid significantly ($P \leq 0.002$) differed between browse species, the PEG addition and the interaction between browse and PEG addition (Table 3). 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 species. The non-glucogenic ratio differed ($P \leq 0.006$) with browse species and increased in the presence of PEG (Table 3). Ammonia concentration differed among browse and increased significantly with PEG ($P < 0.0001$) in PA-containing browse as shown in Table 3. The highest increase in NH_3 concentration was observed in *A. etbaica* (157%) and the lowest in *D. angustifolia* (36%), among the PA-containing browse in the presence of PEG. There was an overall effect of browse ($P < 0.0001$, Table 3) on IVOMD and *A. etbaica* had the lowest increase (4.4%) in the presence of PEG. A decrease in IVOMD was observed in *E. racemosa* ($P < 0.0001$), *M. senegalensis* ($P < 0.0001$) and *R. natalensis* ($P = 0.001$).

4. Discussion

4.1. Browse polyphenol composition and in vitro gas and CH_4 production

Total PA concentrations in most PA-containing browse species were comparable and/or higher than the values (52.8–98.3 mg/g DM) previously reported for five Acacia species (Rubanza et al., 2005), a dominant browse species in the semi-arid tropics.

Table 2
In vitro gas production (GP, ml/g OM incubated) and methane (CH_4, ml/g OM incubated) production of browse species in the absence (−) and presence (+) of polyethylene glycol (PEG).

Browse species		6 h		12 h		24 h		48 h		72 h	
		GP	CH_4	GP	CH_4	GP	CH_4	GP	CH_4	GP	CH_4
E. racemosa	(−)	49.4	4.6	64.2	8.1	113.5	14.2	147.4	22.6	160.0	28.4
	(+)	83.7*	9.5	126.0***	18.0***	181.2***	29.9***	213.1***	39.8***	219.2***	43.6***
R. natalensis	(−)	60.4	4.4	79.0	7.0	111.7	11.5	148.8	18.5	164.0	24.3
	(+)	101.3**	9.1	152.9***	16.0***	190.7***	26.2***	224.2***	37.1***	230.7***	42.3***
M. senegalensis	(−)	53.9	6.5	70.8	9.4	105.5	13.8	149.2	20.3	169.3	25.1
	(+)	111.5***	15.0	171.9***	25.0***	216.6***	35.4***	250.8***	44.6***	260.3***	48.3***
D. cinerea	(−)	54.9	3.4	77.5	5.4	102.6	8.6	138.3	14.0	150.6	18.1
	(+)	96.8***	8.7	138.0***	17.0***	187.4***	27.6***	220.4***	38.1***	226.2***	42.5***
D. angustifolia	(−)	64.8	3.3	73.8	4.6	89.7	6.5	112.3	9.2	116.8	11.1
	(+)	81.6	5.4	106.2*	9.6	157.7***	16.7***	206.5***	26.5***	213.5***	32.5***
A. etbaica	(−)	39.0	1.9	47.4	2.8	64.9	3.9	71.8	5.0	76.4	5.6
	(+)	101.9***	8.9	149.1***	18.0***	189.3***	28.0***	211.4***	36.2***	216.1***	39.3***
S. singueana	(−)	66.9	3.5	106.9	7.6	147.1	14.1	174.0	21.0	179.1	24.2
	(+)	131.3***	13.0***	176.3***	23.0***	219.9***	34.3***	249.8***	44.5***	255.1***	48.5***
C. tomentosa	(−)	129.3	14.8	181.8	28.0	224.6	43.2	259.5	59.3	272.5	67.2
	(+)	126.9	14.8	185.3	28.0	232.3	45.3	269.6	63.6	284.7	72.6
C. farinosa	(−)	106.9	8.9	171.0	21.0	233.7	41.7	279.8	61.5	290.6	69.0
	(+)	108.6	8.4	170.6	21.0	231.8	40.9	274.0	60.1	284.4	67.4
M. angolensis	(−)	145.7	13.3	213.5	27.0	265.7	46.0	311.0	65.0	317.0	72.4
	(+)	141.9	12.8	210.5	26.0	259.2	44.7	304.5	63.3	311.1	70.7

	GP		CH_4	
	SEM	P-value	SEM	P-value
Browse	4.95	< 0.0001	0.99	< 0.0001
PEG	4.73	< 0.0001	0.93	< 0.0001
Time	4.81	< 0.0001	0.95	< 0.0001
Browse × PEG	5.20	< 0.0001	1.05	< 0.0001
PEG × Time	4.95	< 0.0001	0.99	< 0.0001
Browse × PEG × Time	6.89	< 0.0001	1.45	< 0.0001

OM, organic matter; SEM, standard error of the mean.
$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ from corresponding values without (−) PEG.

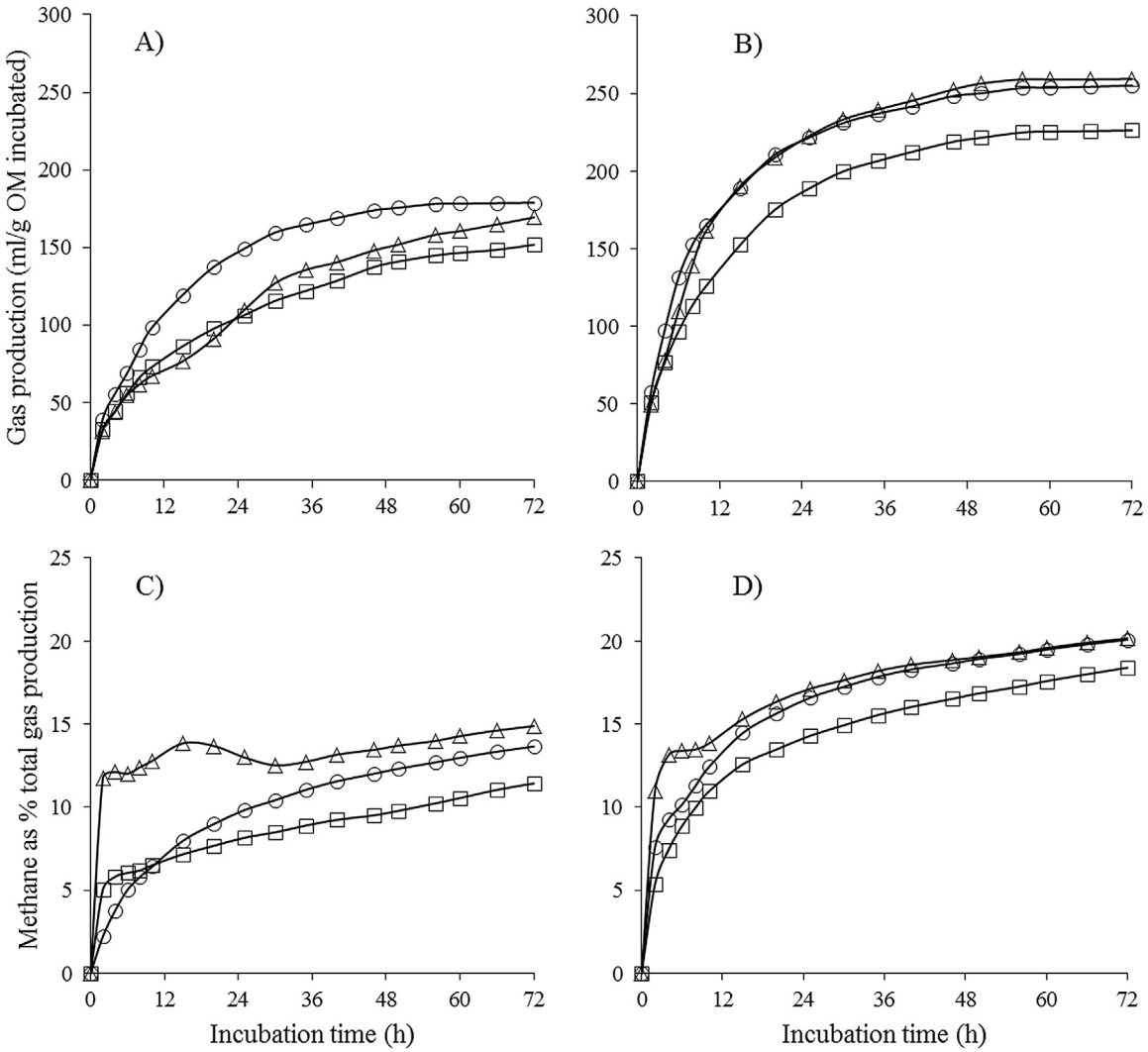

Fig. 1. *In vitro* cumulative gas production (GP) (A, B) and methane production as a percentage of total GP (C, D) in the absence (A, C) and presence (B, D) of polyethylene glycol. *M. senegalensis* (△), *D. cinerea* (□), *S. singueana* (○).

Proanthocyanidin in Acacia species have the typical characteristics of the resorcinol-type hydroxylation pattern in the A-ring rather unique to this genus, and consist of PF and prorobinetinidins (Foo, 1984). These PA types were not common to all the browse species and only *A. etbaica* and *S. singueana* contained PF or PP. The presence of PC and PD in PA-containing browse was expected based on their wide distribution in plants (Foo, 1984). Mueller-Harvey et al. (1987) detected myricetin, quercetin and kaempferol in *C. farinosa* and *R. natalensis*. However, the content of quercetin based flavonols was lower and kaempferol or its derivatives were not detected in *C. farinosa*, while *R. natalensis* had higher content of quercetin, myricetin and kaempferol based flavonols 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 sample preparation. Among the PA-containing browse, the high PA content in *E. racemosa*, *R. natalensis* and *M. senegalensis* presents immense potential in reducing the ruminal CH_4 whereby improving feed energy utilization and protecting the environment at large. In contrast, *C. tomentosa*, *C. farinosa* and *M. angolensis* have only traces or no PA but possess higher CP level and lower NDF. These browse species with contrasting PA and macronutrients can be considered as complimentary species in terms of reducing methane while increasing the availability of nutrients for ruminants.

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 (Getachew et al., 2002; Rubanza et al., 2005; Bhatta et al., 2012). One of the effects of tannins on GP is considered to be through inhibition of bacteria by forming complexes with bacteria cell wall, membrane and extracellular enzymes (Patra and Saxena, 2009). Methane reducing activity of PA on the other hand may be mediated through direct inhibition of methanogens and depends on the PA structures (Patra and Saxena, 2009; Huyen et al., 2016). Proanthocyanidin may also have an indirect effect on methanogenesis through the inhibition of fermentation which is more pronounced for PA with more PD units and higher mean degree of polymerization (Lowry et al., 1996; Huyen et al., 2016). All PA-containing browse with the

Table 3
Total and molar proportions of volatile fatty acids (VFA, mmol/g OM), non-glucogenic ratio (NGR), ammonia (NH_3, mmol/g OM) and *in vitro* organic matter digestibility (IVOMD) of browse species *in vitro* fermentation in the absence (−) and presence (+) of polyethylene glycol (PEG).

Browse species	PEG	Total VFA	% of total VFA concentration					NGR	NH_3	IVOMD
			HAc	HP	HB	HV	BCVFA			
ER	(−)	9.0	69.7	18.0	7.9	1.4	3.1	4.5	42.3	40.8
	(+)	10.3***	67.5	17.3	8.7	1.8***	4.8***	4.6	64.4***	23.9***
RN	(−)	9.1	72.8	17.5	5.5	1.6	2.7	4.5	41.8	48.0
	(+)	10.7***	69.2*	17.0	7.2***	2.0***	4.6***	4.5	69.5***	39.5**
MS	(−)	9.3	71.8	19.0	5.7	1.2	2.5	4.2	34.6	47.9
	(+)	11.3***	68.5	17.7	7.5***	1.7***	4.6***	4.4	66.0***	31.6***
DC	(−)	8.9	70.9	20.7	4.8	1.3	2.3	3.7	37.5	51.2
	(+)	11.0***	66.7**	19.7	6.4***	2.1***	5.1***	3.8	72.1***	71.7***
DA	(−)	8.0	68.8	22.3	5.5	1.3	2.1	3.4	32.8	44.3
	(+)	10.1***	69.6	19.4†	6.6†	1.5	2.9***	4.0	44.5	51.7***
AE	(−)	6.7	70.1	21.9	5.4	0.8	1.8	3.7	27.5	48.1
	(+)	10.9***	66.1**	20.2	7.0***	1.9***	4.9***	3.7	70.6***	50.3
SS	(−)	9.7	64.4	25.3	6.0	1.1	3.2	2.9	45.8	66.1
	(+)	11.7***	65.8	20.4***	6.9	1.9***	4.9***	3.7†	73.8***	69.9
CT	(−)	11.8	65.4	18.9	9.2	1.9	4.7	4.1	84.5	77.3
	(+)	11.9	65.0	18.6	9.3	2.0	5.1	4.2	89.8	77.5
CF	(−)	12.7	67.4	16.4	9.5	1.9	4.9	4.9	94.6	79.2
	(+)	12.7	67.8	16.3	9.3	1.9	4.8	4.9	92.8	80.2
MA	(−)	13.7	66.1	18.0	9.1	1.8	5.0	4.4	102.2	87.0
	(+)	13.8	66.4	17.8	9.0	1.8	5.0	4.4	102.9	87.6
SEM										
	Browse	0.35	0.67	0.40	0.23	0.07	0.25	0.10	7.27	0.83
	PEG	0.33	0.56	0.18	0.19	0.07	0.25	0.05	7.08	0.37
	Browse × PEG	0.37	0.80	0.56	0.28	0.08	0.26	0.14	7.50	1.17
P-value										
	Browse	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
	PEG	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.006	< 0.0001	0.263
	Browse × PEG	< 0.0001	< 0.0001	0.002	< 0.0001	< 0.0001	< 0.0001	0.091	< 0.0001	< 0.0001

AE, *Acacia etbaica*; CF, *Cadaba farinosa*; CT, *Capparis tomentosa*; DA, *Dodonaea angustifolia*; DC, *Dichrostachys cinerea*; ER, *Euclea racemosa*; MA, *Maerua angolensis*; MS, *Maytenus senegalensis*; RN, *Rhus natalensis*; SS, *Senna singueana*.
BCVFA, branched chain volatile fatty acids; HAc, molar proportion of acetic acid; HB, molar proportion of butyric acid; HP, molar proportion of propionic acid; HV, molar proportion of valeric acid; 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.

exception of *D. angustifolia* contained PD, attributable to their GP and CH_4 depressing effect. Although *D. angustifolia* did not contain PD, it has CH_4 depressing effect as shown by PEG addition. The observed effect on GP, however, was not necessarily consistent with the browse PA concentration. The addition of PEG to *A. etbaica* and *D. angustifolia*, both containing low to moderate PA concentrations, markedly increased gas and CH_4 production. Such effects could be attributed mainly to the chemical properties of PA, *e.g.* the molecular weight and the degree of polymerization (Huyen et al., 2016) and the presence of other compounds with synergistic effects. Therefore, for nutritional significance it is crucial to combine quantitative and qualitative analysis of PA, and the evaluation of the corresponding effect on the fermentation. *Acacia etbaica* contain PF with a 5-deoxy group in close proximity to the interflavanol bond (López-Andrés et al., 2013), and this could lower PEG binding to the PA structure which may result in the presence of active tannins leading to a decline in fermentation. The relatively lower effect of PA, despite the high PA concentrations in *E. racemosa*, *R. natalensis* and *M. senegalensis* could be due to the presence of highly polymerized PA which lower the solubility and protein binding properties (Kumar, 1983). Moreover, high amount of fibre-bound PA lowers the fibre digestion and PEG binding and, therefore, reduces the PA counteracting activity of PEG.

Graphical representation (Fig. 1) of the GP pattern of *M. senegalensis*, *D. cinerea* and *S. singueana* shows the effect of PA on the rate of GP. The ability of microorganisms to ferment depends on substrate type and composition resulting in differences in GP profiles (Groot et al., 1996). A rapid increase in GP during the first 3 h for these browse is attributable to the fermentation of water soluble fractions (Groot et al., 1996). Although the PA seems to affect GP at the earlier time points of incubation (before 6 h) significantly as indicated by the addition of PEG (Fig. 1 and Table 2), the PA source seems to be of minor influence here. The effect of PA to reduce the rate of fermentation was low in *S. singueana*, moderate in *D. cinerea* and markedly higher in *M. senegalensis*. *Maytenus senegalensis* and *S. singueana* had comparable GP but the fermentation pattern was different indicating that the higher PA concentrations in *M. senegalensis* influenced its rate of GP. Huyen et al. (2016) reported that PA extracts added to grass silage (4%) decreased the rate of *in vitro* substrate degradation. Gas production profiles of the three browse species in Fig. 1 were not consistent with the respective PA concentrations. Although fermentation was delayed in *M. senegalensis*, the cumulative GP was higher than *D. cinerea*. *Senna singueana* with a lower PA concentration had a higher cumulative GP. The higher CH_4 as percent total GP in *M. senegalensis* regardless of its high

PA content suggests that PA affected fermentation more than methanogenesis. It could also be that the differences in CH_4 as percent total GP in the three species were due to differences in the number of PD monomeric units although these were not quantified. Thus, the effect of PA on GP and CH_4 is determined by its type and composition.

The increase in the production of CH_4 after the PEG addition ranged from 53% in *E. racemosa* to 601% in *A. etbaica*. Bhatta et al. (2012) also reported increased in CH_4 (10–798%) *in vitro* with PEG addition in tropical tannin-containing forages. The reduction in the production of CH_4 is accompanied by a concomitant reduction in GP, or vice versa. The latter indicates impaired fermentation and hence affects rumen microbes. However, it is important to place the results in perspective considering the CH_4 reduction and the decline in fermentation. Bhatta et al. (2012) suggested that the ratio between the reduction in CH_4 and GP (determined as differences using PEG) can be used to compare tannin-containing forages for CH_4 reducing potential with a higher ratio indicating a better CH_4 mitigating potential. Accordingly, the ratios for the browse, where a significant reduction in CH_4 was observed, were: *D. cinerea* (0.32), *S. singueana* (0.32), *R. natalensis* (0.27), *E. racemosa* (0.26), *M. senegalensis* (0.26), *A. etbaica* (0.24) and *D. angustifolia* (0.22). Bezabih et al. (2014) evaluated 24 tropical grass species *in vitro* for 72 h and reported a higher average GP to CH_4 ratio of 0.23 *vs* 0.14 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. The biological activities of tannins are dependent on their concentrations but also mainly on their structures (Salminen and Karonen, 2011; Bodas et al., 2012). The high PD:PC ratio in sainfoin was associated with reduced CH_4 production *in vitro* (Hatew et al., 2014; Huyen et al., 2016), and could justify the CH_4 reduction activity of PA-containing browse. Prodelphinidins have more hydroxyl groups compared to PC and thus they may have higher inhibitory activity (Patra and Saxena, 2009), resulting in reduced CH_4 production. The presence of flavonol derivatives in browse species also appeared to lower the CH_4 production. The CH_4 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 derivatives in high concentrations in *S. singueana* is likely to significantly contribute to its CH_4 lowering property as Oskoueian et al. (2013) indicated that kaempferol significantly reduced the CH_4 production without reducing the GP. In *D. cinerea*, the CH_4 depressing activity could be mainly due to the presence of higher levels of quercetin and myricetin derivatives. Myricetin reduced both the GP and CH_4 production but the quercetin derivatives increased the GP while decreasing the CH_4 production. Oskoueian et al. (2013) showed that myricetin reduced bacterial and methanogen copy numbers and the activity of a number of bacterial enzymes, which is in line with the decreased GP and CH_4 production and concomitant VFA produced. In case of quercetin methanogen copy numbers decreased whereas bacterial copy numbers and bacterial enzyme activity were not affected, suggesting no effect on overall fermentation, which agrees with the amount of VFA produced compared to their control. However, the shifts in molar proportions directed towards acetate and butyrate in combination with lower CH_4 could imply that less H_2 and CO_2 is used as substrate by methanogens, resulting in more moles of gas, and hence, a higher GP. The contribution of galloyl derivatives to CH_4 reduction was less evident in *D. angustifolia*. This browse contained negligible quantities of galloyl derivatives but could markedly reduce the CH_4 production, in a proportion comparable to *D. cinerea* which contain the highest content of galloyl derivatives. Since *D. angustifolia* has moderate amount of PA consisting of PC monomeric units and lower concentrations of other polyphenols, its CH_4 inhibitory activity could be due to the presence of phenolic compounds not quantified in this study.

4.2. Browse polyphenol composition and in vitro fermentation end products

Most phenolic compounds in forages exert antimicrobial activity by lowering the 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 with tropical browse species (Getachew et al., 2012; Gemeda and Hassen, 2015). However, the observed decrease in the molar proportion of acetic acid in *A. etbaica*, *D. cinerea*, *M. senegalenis* and *R. natalensis* by the addition of PEG contradicts results of the latter authors. Degradation of quercetin may increase the acetic acid pool (Lowry et al., 1996; Oskoueian et al., 2013), also suggesting the contribution of some polyphenols in the energy metabolism in the rumen. Similar to the reports of Getachew et al. (2002) in browse species, propionate and acetate decreased while butyrate increased in the presence of PEG. The increase in butyrate was therefore the main contributor for methanogenesis with the inclusion of PEG in most PA-containing browse species. The increase in branched chain fatty acids upon PEG addition agrees with results from PA-containing tropical browse (Gemeda and Hassen, 2015), associated with increased fermentation of the protein released from the tannin-protein complex.

The increase in ammonia concentrations in PA-containing browse after the PEG addition indicates that PA could have activity in reducing ammonia similar to the previous reports with hydrolysable and condensed tannin extracts (Pellikaan et al., 2011b; Huyen et al., 2016) and tannin-containing forages (Bhatta et al., 2012) *in vitro*. Ammonia production in general showed to be positively related to the CP content of browses (Table 3), which was predominantly explained by the high CP contents in the three non-PA-containing browses. Within PA-containing browses however, such a relationship was not evident regardless of PEG treatment. Therefore, within the PA-containing browses used in the current trial the range in CP (69–145 g/kg DM) was too narrow to show convincing relationship between protein availability in the substrates and NH_3 release. Data on the BCVFA (Table 3) support this, suggesting no relation between CP content and protein use as a substrate for microbial fermentation for the browses under investigation. The increase in GP and VFA production upon the addition of PEG to tannin-containing forages indicates increased organic matter digestibility. However, *E. racemosa*, *M. senegalensis* and *R. natalensis* had lower IVOMD after the PEG addition. The higher concentrations of PA in these browse and the possible tannin-protein complex formation increased the proportion of the undigested fraction in the incubation residue and hence lowers *in vitro* organic matter digestibility values (Makkar et al., 1995; Reed, 2001).

5. Conclusions

Seven of the ten browse species, except *C. farinosa*, *C. tomentosa* and *M. angolensis*, had a CH_4 inhibitory but also fermentation depressing activity. The use of PEG confirmed that the inhibitory activity was 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 the fermentation and vice versa. The ratio of the difference due to PEG addition in CH_4 production, to the differences in GP shows 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 production, considering CH_4 reduction as a proportion of GP reduction, total VFA and IVOMD, *S. singueana* is superior followed by *D. cinerea* with low to moderate contents of PA, respectively. Overall, the effect of tannin-containing browse was also due to additional inhibitory effects of other phenolic and non-phenolic compounds. Therefore, evaluation of the fermentative characteristics of browse needs to take into account co-occurring phenolic and non-phenolic compounds, in addition to tannins.

Conflict of interest

The authors declare that there is no conflict of interest.

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