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In vitro assessment of ruminal fermentation, digestibility and methane production of three species of *Desmanthus* for application in northern Australian grazing systems

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Abstract. Three species of *Desmanthus* adapted to the heavy clay soils of northern Australia were studied to determine their nutritive value and effects on *in vitro* fermentation with rumen fluid, compared with Rhodes grass (*Chloris gayana*) hay. Leaves and stems of *D. leptophyllus* cv. JCU 1, *D. virgatus* cv. JCU 2 and *D. bicornutus* cv. JCU 4 were collected in summer, winter and spring of 2014 and analysed for chemical composition. Apparent digestibility as *in vitro* organic matter digestibility (IVD-OM) and fermentation parameters including methane (CH_4) production were measured during 72-h fermentations using rumen fluid from steer donors grazing tropical grasses and legumes. *Desmanthus bicornutus* was on average more digestible than both *D. leptophyllus* and *D. virgatus* at 24, 48 and 72 h of incubation. This species also demonstrated an anti-methanogenic potential, in particular when harvested in summer with a reduction in CH_4 production of 26% compared with Rhodes grass hay after 72 h of incubation. At this time point, *D. leptophyllus* produced higher volatile fatty acids (VFA per g of organic matter fermented) compared with the other forages. This legume also reduced the CH_4 production up to 36% compared with the Rhodes grass hay reference. However, *D. leptophyllus* showed lower IVD-OM. Overall, *Desmanthus* species produced lower *in vitro* CH_4 and lower volatile fatty acids concentration compared with the reference grass hay. These effects may be due to presence of secondary compounds such as hydrolysable tannins, condensed tannins and/or their combination in *Desmanthus* species. The IVD-OM was influenced by the season after 72 h of incubation; the digestibility was higher in plants collected in spring. This study suggests that contrasting fermentative profiles in *Desmanthus* cultivars may offer the opportunity to reduce the greenhouse gas contribution of the beef industry. The next step in demonstration of these promising *in vitro* results is demonstration of *Desmanthus in vivo* as proof of concept confirming the productivity and CH_4 reduction ability of these legumes in the pastoral systems of northern Australia.

Additional keywords: digestion, greenhouse gas, legume, ruminant, tannins.

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Introduction

Grazing beef production systems in northern Australia are affected by annual dry periods extending usually from April to October. In this scenario, forage availability and its quality are limiting factors that reduce animal productivity and farm profitability (Cox and Gardiner 2013). To mitigate the effect

of low availability and low quality, the use of improved grass, shrub and legume species to increase nutritive quality of native pasture and beef productivity has been a common, but costly practice (Shelton *et al*. 1991; Dalzell *et al*. 2006; Hill *et al*. 2009). However, not all species adapt equally to variable soil and climatic conditions that prevail on rangelands (Vera and Seré 1985; Durmic *et al*. 2017; Vandermeulen *et al*. 2018). Therefore, finding adapted herbaceous and shrub legumes that thrive on the heavy clay soils (i.e. vertosols soils; grey, brown and or black cracking soils with a clay field texture in semiarid environments) is challenging (Isbell and NCST 2016; Vandermeulen 2016), but species from the genus *Desmanthus* are among the few to be successfully adapted to those conditions in northern Australia (Pengelly and Conway 2000; Gardiner and Swan 2008; Gardiner *et al*. 2013).

Recently, new *Desmanthus* cultivars (cv. JCU 1 to 5) described by Loch (2015) and Gardiner (2016), have become available. Among them, *D. leptophyllus* cv. JCU 1, *D. virgatus* cv. JCU 2 and *D. bicornutus* cv. JCU 4 have been marketed and established as a blend of the three species (Gardiner *et al*. 2013). These *Desmanthus* cultivars comprise a wide range of early to late maturity types, herbaceous to suffruticose plant habits, and edaphic and climatic tolerances. These legumes have also been associated with improved animal production. In Queensland, according to Gardiner and Parker (2012) steers grazing a *Desmanthus*-buffel grass (*Cenchrus ciliaris*) paddock during 90 days (i.e. winter) achieved 40 kg more weight gain than steers only grazing buffel grass. Furthermore, a mixture of *Desmanthus* with Mitchell grass (*Astrebla* spp.) increased wool production by up to 34% in grazing sheep (Rangel and Gardiner 2009).

In contrast, detrimental effects have been demonstrated in growing goats. Compared with *Leucaena leucocephala*, Kanani *et al*. (2006) showed reduced animal preference of *D. bicornutus* resulting in low liveweight gain. Although both species contain common secondary metabolites such as condensed tannins (CT; Gonzalez-V. *et al*. 2005; Tan *et al*. 2011) that mitigate pastoral CH_4 emissions in large (Grainger *et al*. 2009) and small ruminant systems (Ramírez-Restrepo *et al*. 2010), the toxic alkaloid mimosine is not present in *Desmanthus* spp. (Cook *et al*. 2005).

Therefore, it is obvious that improvements in productivity of low input beef pastoral systems require at least the association of palatable and productive grasslands to complement native forage communities (Department of Primary Industries Queensland 1988, 1999; Vera and Ramírez-Restrepo 2017; Ramírez-Restrepo and Vera 2018). However, any attempt to achieve sustainable tropical pastoral production requires an integrated mitigation framework that considers profiles of plant-rumen fermentation characteristics as one of the key elements (Ramírez-Restrepo and Charmley 2015). This approach will help improve nutrition balance, body growth, reproduction and cattle welfare (Provenza *et al*. 2007; Manteca *et al*. 2008; Provenza and Villalba 2010; Vera and Ramírez-Restrepo 2017). In this respect, *Desmanthus* cultivars may contribute to improve nutrition for pastures and as an additional effect may mitigate CH_4 emission from grazing cattle.

Agronomic attributes of members of the *Desmanthus* genus have been previously demonstrated (Jones and Brandon 1998; Pengelly and Conway 2000; Cook *et al*. 2005). However, given that forage quality varies markedly with seasons, there are no data that consider the effects of *Desmanthus* spp. on nutritive values and the fermentability. This includes concentration of tannins and the anti-methanogenic characteristics using grazing animals as donors of rumen content for *in vitro* incubations, an important omission. The objectives of the present study were to assess nutritive value of three *Desmanthus* species using herbage collected during three separate seasons and determine their effects on *in vitro* fermentation. The primary variables of interest were the potential to reduce *in vitro* CH_4 production in fermentations inoculated with rumen fluid from *Bos indicus* Brahman grazing steers.

Materials and methods

Study site

The *in vitro* experimentation constantly used the same *Bos indicus* Brahman breed rumen-cannulated steers ($n = 4$, 407 ± 9.45 kg liveweight) as ruminal fluid donors. The experiment was conducted at CSIRO Agriculture at the Australian Tropical Sciences and Innovation Precinct (ATSIP) in Townsville and at the Lansdown Research Station (19°39′S, 146°50′E). The study followed CSIRO Animal Ethics Committee approved guidelines (A12/2014) and the Australian code of practice for the care and use of animals for scientific purposes (NHMRC 2013).

Plant material harvesting and preparation

Desmanthus leptophyllus (cv. JCU 1), *D. virgatus* (cv. JCU 2) and *D. bicornutus* (cv. JCU 4) were grown in pots under identical agronomic practices in a semi-enclosed greenhouse at the faculty of Agriculture at the University of Queensland. Each species was composed of four groups of five pots and all plants were cut back to ~10 cm on the 29 January 2014 (Day 0). In parallel, one sample for each *Desmanthus* species was harvested by Agrimix Pty Ltd (Eagle Farm, Qld, Australia) from the same number of pots at three different growth stages, in summer (March – Day 51), winter (August – Day 189) and spring (October – Day 273) of 2014. The harvesting approach represented the patterns of seasonal dynamics of forage quality and dry matter (DM) production relative to net forage accumulation (Ramírez-Restrepo *et al*. 2006*a*). Thus, the initial day of cut (Day 0) was staggered so further cuts (i.e. Days 51, 189 and 273) provided successive overlapping studied seasons (Ramírez-Restrepo *et al*. 2006*a*). After collection, samples were immediately stored at −20°C and freeze-dried. Rhodes grass (*Chloris gayana*) was representatively sampled from commercial local hay and oven-dried at 55°C to be used as the reference. All substrates were ground to pass a 1-mm mesh screen using a Cyclotec 1093 Sample Mill (Foss Tecator, Hillerød, Denmark).

Chemical composition

Proximate analysis

Forage analysis was performed following standard methodologies (Association of Official Analytic Chemists, AOAC 1995). Procedures 967.03, 942.05, 984.13 and 930.9 were used for dry matter (DM), ash, nitrogen (N) and ether extract (EE) content, respectively. Neutral detergent fibre

(NDF) without heat-stable α-amylase, acid detergent fibre (ADF), cellulose and acid detergent lignin (ADL (sa)) were determined following the procedures of Van Soest *et al*. (1991). The NDF and ADF were expressed including residual ash.

Phenols and tannins analyses

Prior to the phenolic fraction assays, pigments were removed from ground samples using diethyl-ether with acetic acid (99 : 1 v/v) according to the modified method of Ammar *et al*. (2004). Depigmented samples were oven-dried at 40°C to avoid deterioration of phenolic compounds. The phenol contents i.e. total phenols (TP), total tannins (TT) and CT were extracted using aqueous acetone (70 : 30 v/v; Makkar 2003).

The TP and non-tannins phenols (NTP) contents in the plant extract were measured by means of the Folin-Ciocalteu reagent method. The TT fraction was determined by the difference between TP and NTP (Makkar 2003). Total phenols, NTP and TT were expressed as tannic acid equivalent. The total CT fraction was expressed as leucocyanidin equivalent and estimated by the n-butanol-HCl method described by Porter *et al*. (1985). Hydrolysable tannins (HT) content in plant substrate extract were calculated as the difference between the TT and CT (Singh *et al*. 2005).

In vitro *ruminal fermentation*

Rumen inoculum preparation

Rumen-cannulated Brahman steers grazed together a mix of native and naturalised pasture characterised by farm management at the beginning of the study following a visual assessment of predominant species on the paddock. In decreasing proportion they were *Cenchrus ciliaris*, *Chloris* spp., *Macroptilium* spp., *Panicum* spp., *Urochloa* spp., *Stylosanthes* spp. The approach ensured the absence of *Desmanthus* species on the paddock to avoid the impact of any confounding factor on the fermentative studied traits. On collection days (6 a.m.), liquid and particulate fractions of the rumen content were manually collected from the four quadrants of the rumen of the steers through fistula and conditioned as described by Ramírez-Restrepo *et al*. (2014) until further processing.

In vitro *rumen inoculation and incubation*

The inoculation and *in vitro* fermentation were performed following the methods described by Kinley *et al*. (2016). Briefly, the rumen fluid incubation medium was prepared by combining the strained rumen fluid with the Goering and Van Soest (1970) buffer at a ratio of 1 : 4 (v/v). Rumen media (125 mL) was added anaerobically to the 39°C incubation bottle containing 1 g of organic matter (OM) of forage sample. Further the inoculated bottles were purged with N_2 and capped gas tight with an Ankom RF1 gas production module (Macedon, NY, USA). Bottles were incubated at 39°C in an orbital incubator (Ratek, OM11, Boronia, Vic., Australia) at 85 rpm.

The experimental scheme yielded a total of 44 bottles and was replicated over three independent fermentation runs as follows:

[3 *Desmanthus* cultivars (i.e. species) × three seasons + one Rhodes grass hay reference and one blank (rumen medium only)] × four bottles × three fermentation runs.

During each fermentation run, one bottle for each substrate was stopped after 24 and 48 h and the remaining two bottles were stopped after 72 h, yielding three replicates for measures taken at 24 and 48 h and six replicates for measurements done at 72 h, i.e. measurements of total gas, CH_4 and volatile fatty acid (VFA) productions and the *in vitro* apparent digestibility of substrate organic matter (IVD-OM). The run was used as the experimental unit.

Total gas and methane production

The total gas production (TGP) was measured continuously over 72 h of incubation according to Kinley *et al*. (2016), with the cumulative pressure recorded every 20 min. The cumulative TGP was obtained by converting the pressure readings to mL/g OM and mL/g OM fermented.

In vitro CH_4 concentration was measured in headspace samples collected into pre-vacuumed 10-mL vials at the predetermined time series points of incubation (24, 48 and 72 h). Gas concentration was determined as described by Kinley *et al*. (2016) using gas chromatography (GC-2014, Shimadzu Corporation, Kyoto, Japan) equipped with a Restek (Bellefonte, PA, USA) ShinCarbon ST 100/120 micropacked column (2 m × 1 mm) and both flame ionisation detector and thermal conductivity detector. Methane concentration in headspace was converted to mL/g OM and mL/g OM fermented using the TGP by application of the natural gas law.

In vitro *apparent digestibility of substrate organic matter and volatile fatty acids analysis*

The IVD-OM and VFA analysis were performed as described by Kinley *et al*. (2016). Briefly, after gas sample collection, the *in vitro* fluid was filtered with a 0.5-cm layer of filtration sand. The solid residues were dried (105°C) and burned (550°C; 8 h) to determine the IVD-OM. The concentration in VFA in the *in vitro* fluid samples was measured using a Shimadzu GC-17A equipped with a flame ionisation detector and a Restek Stabilwax-DA fused silica column (30 m × 0.25 mm × 0.25 µm), and with ultra purity N as the carrier gas. Peak detection and integration of VFA were performed with the Shimadzu GC Solution Software.

Statistical analysis

All statistical analyses were performed using the Statistical Analysis System version 9.4 (SAS Institute, Cary, NC, USA). The chemical composition (i.e. CP, EE, NDF, ADF, cellulose and ADL), the phenols and tannins constituents (i.e. TP, NTP, TT, CT, HT), the *in vitro* fermentation parameters (i.e. IVD-OM; TGP and CH_4 (mL/g OM fermented and mL/g OM) and VFA (mmol/L and mmol/g OM fermented) were assessed using the MIXED procedure in a model that considered the fixed effects of *Desmanthus* species (i.e. *D. leptophyllus* cv. JCU 1, *D. virgatus* cv. JCU 2 and *D. bicornutus* cv. JCU 4), seasons (i.e. summer, winter and spring) and the resulting interaction between species and seasons. The least-squares means (LSM) have been classified according to this interaction. Rhodes grass was used as a reference and was not included in the statistical analysis (Grosse Brinkhaus *et al*. 2017). The MEANS procedure was performed to calculate the mean of each parameter of the grass hay reference. Pearson's correlations were used to determine the interaction between CH_4 production at different incubation

time points and the phenolic compounds concentration in forage samples. Least-squares means ± s.e.m. were considered significantly different at $P < 0.05$ and tending to differ when $P \leq 0.10$.

Results

Chemical composition

As displayed in Table 1, the chemical composition was different between species and seasons. The CP concentration was higher in *D. leptophyllus* in winter and in *D. bicornutus* in spring. Compared with the other legumes, *Desmanthus virgatus* had a high concentration of fibre. Among the legumes, *D. bicornutus* in winter contained more CT whereas *D. leptophyllus* in spring more HT (Table 2). Rhodes grass contained less TP, TT, CT and HT than the legumes, and furthermore CT was not detected in the Rhodes grass.

In vitro *fermentation*

The *in vitro* fermentation parameters of the three *Desmanthus* species and the Rhodes grass hay reference are presented in Table 3. The IVD-OM was significantly different between the three legume species at 24, 48 and 72 h ($P < 0.001$; Table 3). *Desmanthus bicornutus* had on average higher IVD-OM after 24, 48 and 72 h of incubation in rumen fluid ($P < 0.01$) than *D. virgatus*, *D. leptophyllus* (Table 3). The IVD-OM values of this species were in the range of those of the reference at 48 h and 72 h. After 72 h of incubation, the IVD-OM was significantly different between season ($P < 0.001$; Table 3); the collection of *Desmanthus* cultivars in spring led to a higher digestibility *in vitro.*

Total gas produced (TGP, mL/g OM fermented) and CH_4 (CH_4, mL/g OM fermented; Table 3) were different between *Desmanthus* species and seasons after 72 h (Table 3). The TGP (mL/g OM) by *D. leptophyllus* was lower than the other forage species after 72 h of incubation ($P < 0.05$). However, when this parameter was expressed relative to the digestibility (mL/g OM fermented), differences became smaller as, TGP of this legume species was similar to that of *D. virgatus* in summer and spring, and to that of *D. bicornutus* in winter. In terms of CH_4 production (mL/g OM; Table 3), irrespective of the season, *D. leptophyllus* produced less *in vitro* CH_4 compared with the other substrates after 72 h of incubation ($P < 0.001$). Fermentations including the *Desmanthus* species emitted less CH_4 (mL/g OM incubated) than the reference grass hay at 72 h of incubation. The higher TGP by Rhodes grass hay was associated to greater CH_4 production (Table 3). This was also observed with *D. virgatus* in winter. When expressed to the OM fermented (mL CH_4/g OM fermented), *D. leptophyllus* in summer and winter and *D. bicornutus* in summer displayed lower CH_4 production after 72 h ($P < 0.001$; Table 3). Both species produced less CH_4 per g of OM fermented than the reference after 48 and 72 h.

Methane production (mL/g OM) was negatively correlated with TP, with *r* values in the range –0.55 to –0.61 ($P < 0.01$). This was also observed with TT ($r = –0.58$ to –0.61; $P < 0.001$) but not with NTP. Correlation between CH_4 production (mL/g OM) and HT was higher ($r = –0.71$ at 24 and 48 h; $r = –0.64$ at 72 h; $P < 0.001$) than with CT ($P > 0.05$ at 24 and 48 h; $r = –0.33$ and $P < 0.05$ at 72 h). The same trend was also observed with CH_4 per g of OM fermented at 24 ($r = –0.33$, $P < 0.1$ for CT vs $r = –0.60$, $P < 0.001$ for HT) and 48 h ($r = –0.42$, $P < 0.05$ for CT vs $r = –0.49$, $P < 0.05$ for HT) but with lower *r*-values. However, after 72 h of incubation the correlation was greater between CH_4 production per gram of OM fermented and CT ($r = –0.62$; $P < 0.001$ vs $r = –0.44$; $P < 0.01$ for HT).

Table 1. Chemical composition (g/kg DM) of the *Desmanthus* species and Rhodes grass (*Chloris gayana*) hay samples processed as techno-analytical triplicates in each season for each forage legume

Least-squares means values among the legumes within the same column followed by the same letters are not significantly different ($P < 0.05$). Comparisons between species, seasons and their interaction in each row for each measurement time are declared at $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and tending to differ when $P \leq 0.10$. ADF, acid detergent fibre; ADF, acid detergent lignin; CP, crude protein (CP; N × 6.25); DM, dry matter; ether extract (E); NA, not applicable; NDF, neutral detergent fibre; s.e.m., standard error of the mean

Species	Cultivar	Season	CP	EE	NDF	ADF	Cellulose	ADL
D. leptophyllus	JCU1	Summer	170b	49.2ab	470d	271b	212c	59.4bc
		Winter	189a	32.4d	495c	240d	184e	64.9b
		Spring	135cd	29.7d	470d	238d	184e	51.5de
D. virgatus	JCU2	Summer	126d	29.6d	525b	340a	277a	72.7a
		Winter	112e	18.8f	584a	340a	271b	76.1b
		Spring	132cd	25.2e	458d	257c	199d	56.7cd
D. bicornutus	JCU4	Summer	139c	41.7c	478d	273b	197d	47.9ef
		Winter	174b	51.0a	398e	172e	130f	41.3 g
		Spring	182ab	46.6b	403e	171e	128f	43.7fg
C. gayana[A]	NA	NA	135	22.4	658	342	297	27.6
Pooled s.e.m.	NA	NA	4.09	1.43	6.76	4.17	1.88	1.92
P								
Species	–	–	***	***	***	***	***	***
Season	–	–	**	***	***	***	***	***
Species × season	–	–	***	***	***	***	***	***

[A]Reference (not included in the statistical analysis).

Table 2. Phenols and tannins constituents (g/kg DM) of the leguminous and grass species samples processed as techno-analytical triplicates in each season for each forage legume

Least-squares means values among the legumes within the same column followed by the same letters are not significantly different ($P < 0.05$). Comparisons between species, seasons and their interaction in each row for each measurement time are declared at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and tending to differ when $P \leq 0.10$. CT, condensed tannins, DM, dry matter; HT, hydrolysable tannins; NA, not applicable; ND, not detected; NS, not significant; NTP, non-tannin phenolics; s.e.m, standard error of the mean; TP, total phenolics; TT, total tannins

Species	Cultivar	Season	TP	NTP	TT	CT	HT
D. leptophyllus	JCU1	Summer	79.9b	8.65ab	71.3b	34.1b	37.1b
		Winter	78.2bc	7.41bc	70.8bc	36.7b	34.1b
		Spring	96.2a	8.64ab	87.6a	37.2b	50.4a
D. virgatus	JCU2	Summer	36.9f	6.57bc	30.3f	14.5d	15.9d
		Winter	40.8f	4.93c	35.9ef	23.0c	12.9d
		Spring	49.9e	10.8a	39.0e	25.1c	14.0d
D. bicornutus	JCU4	Summer	60.7d	5.18c	55.5d	34.7b	20.8cd
		Winter	73.0c	7.54bc	65.4bc	45.3a	20.1cd
		Spring	73.2c	9.34ab	63.9c	34.2b	29.6bc
C. gayana[A]	NA	NA	7.77	6.08	1.68	ND	1.68
Pooled s.e.m.	NA	NA	1.96	1.09	2.43	1.92	3.43
P							
Species	–	–	***	NS	***	***	***
Season	–	–	***	**	***	***	*
Species × season	–	–	**	= 0.09	*	**	NS

[A]Reference (not included in the statistical analysis).

The total VFA concentration was not different between *Desmanthus* species in summer, winter and spring seasons (Table 4 for 72 h of incubation; data not shown for 24 h and 48 h). However, it was on average different between the legume species as *D. bicornutus* and *D. virgatus* produced more VFAs (mmol/L) than *D. leptophyllus* ($P < 0.05$). The total VFA concentration (mmol/L) was overall higher for the Rhodes grass hay reference after 72 h of incubation than the *Desmanthus* species (Table 4). Similar effect was obtained for acetate and propionate concentrations. However, when total VFA were expressed relative to the digestibility (mmol/g OM fermented; Table 4), the legume cultivars produced similar amounts of VFA compared with Rhodes grass hay; these values were higher for *D. leptophyllus*.

Discussion

The objective of the present study was to investigate the effects of the three *Desmanthus* species collected in three seasons on *in vitro* fermentation profile including CH_4 production using rumen fluid from grazing Brahman steers. The main finding was that, according to the season, *D. leptophyllus* and/or *D. bicornutus* exhibited the highest anti-methanogenic potential after 72 h of *in vitro* rumen fermentation compared with *D. virgatus*. Compared with the Rhodes grass reference, *in vitro* CH_4 production per gram of OM fermented was reduced by 15–36% with *D. leptophyllus* (values at 72 h and 24 h respectively), and by 10–26% with *D. bicornutus* (values at 24 h and 48 h respectively). This may be due to the presence of secondary compounds such as CT because both species contain the highest concentrations of these molecules. Indeed significant correlations were found between CT and CH_4 production.

Previous studies on sheep and cattle demonstrated the potential of CT to lower CH_4 emissions, either with purified CT extracts (Grainger *et al*. 2009; Tan *et al*. 2011) or CT-containing forages (Tavendale *et al*. 2005; Hess *et al*. 2006; Ramírez-Restrepo *et al*. 2010). However, although the direct effect of HT on the fermentation was not assessed, the anti-methanogenic effect of HT or the combination of tannins present in *Desmanthus* herbage cannot be ruled out because the systemic interrelation among polyphenolic compounds, protein and carbohydrate fractions is complex (Tedeschi *et al*. 2014; Tedeschi and Fox 2016).

Jayanegara *et al*. (2015) investigated the impacts of purified HT and CT on CH_4 emission. They highlighted the anti-methanogenic effect of HT associated to less detrimental impact on digestibility, as CT have been demonstrated to present digestibility issues (Kamalak *et al*. 2004; Animut *et al*. 2008). Our study supports the hypothesis that HT could have significant anti-methanogenic properties as we found a significant negative correlation between HT concentration in *Desmanthus* forages and CH_4 emission per g of OM fermented. *Desmanthus leptophyllus* and *D. bicornutus* produced lower CH_4 at 72 h of incubation, and contained higher HT than both *D. virgatus* and the reference hay (Tables 3 and 2).

However, HT concentration seemed positively related to the lower IVD-OMD observed with *D. leptophyllus*, which was not the case with CT. Thus, it would seem that the results of our study are not in agreement with other studies (Jayanegara *et al*. 2015) that have shown that HT had a greater effect in reducing CH_4 emission with less adverse effect on digestibility than those of CT. Hydrolysable tannins have been reported to decrease methanogenesis via direct effect as the inhibition of the growth and/or activity of methanogens and/or hydrogen-producing microbes (Bhatta *et al*. 2009; Jayanegara *et al*.

Table 3. Effects of *Desmanthus* spp. on *in vitro* organic matter digestibility (IVD-OM), total gas and methane (CH_4) production at 24, 48 and/or 72 h of incubation

Three (24 and 48 h) and 6 (72 h) samples were initially incubated for each forage. Least-squares means values among the legumes within the same column followed by the same letters are not significantly different ($P < 0.05$). Comparisons between species, seasons and their interaction in each row for each measurement time are declared at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and tending to differ when $P \leq 0.10$. NA, not applicable; NS, not significant; s.e.m., standard error of the mean

Species	Cultivar	Season	IVD-OM			Total gas (mL/g OM fermented)			CH_4 (mL/g OM fermented)			Total gas (mL/g OM)	CH_4 (mL/g OM)
			24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	72 h	
D. leptophyllus	JCU1	Summer	0.369d	0.423d	0.454f	203.8bc	229.2ab	247.3bc	17.50d	27.56cd	30.71de	111.1e	13.64c
		Winter	0.403d	0.502d	0.502e	203.9bc	219.8b	232.5cd	19.45cd	26.01d	29.56e	114.6e	14.62c
		Spring	0.378d	0.492d	0.514e	235.8ab	248.6ab	259.5b	22.53bc	29.81bcd	33.13cd	132.9d	18.06b
D. virgatus	JCU2	Summer	0.562bc	0.622bc	0.625cd	218.5a	240.3ab	257.8b	23.77bc	29.55bcd	36.86b	159.8b	22.76a
		Winter	0.507c	0.577c	0.597d	250.7a	279.0a	287.5a	28.74a	37.95a	39.90a	169.8a	23.54a
		Spring	0.574bc	0.660ab	0.662bc	220.6abc	235.6ab	238.4c	26.16ab	33.52ab	34.76bc	157.6b	23.00a
D. bicornutus	JCU4	Summer	0.593ab	0.655abc	0.657c	197.5c	208.9b	217.3d	21.95bcd	27.80cd	28.71e	142.7c	18.87b
		Winter	0.601ab	0.684ab	0.701ab	215.9abc	227.3ab	228.4cd	24.35ab	30.52bcd	32.94cd	159.3b	22.99a
		Spring	0.650a	0.713a	0.732a	201.5bc	218.2b	219.6d	24.35ab	31.17bc	32.58cd	160.5b	23.82a
C. gayana[A]	NA	NA	0.547	0.656	0.698	245.3	276.2	260.7	27.13	38.09	38.75	183.9	27.35
Pooled s.e.m.		Summer	0.025	0.027	0.015	11.75	17.69	6.64	1.54	1.82	0.93	2.95	0.65
		Winter	0.025	0.024	0.015	11.75	16.53	6.83	1.54	1.59	0.95	3.07	0.66
		Spring	0.025	0.024	0.015	11.75	15.38	6.45	1.54	1.48	0.92	2.98	0.66
P													
Species		–	***	***	***	= 0.06	= 0.08	***	***	**	***	***	***
Season		–	NS	= 0.06	***	NS	NS	NS	*	= 0.06	*	***	***
Species × season		–	NS	NS	= 0.09	NS	NS	***	NS	= 0.06	NS	NS	**

[A]Reference (not included in the statistical analysis).

Table 4. Comparative volatile fatty acids concentration (mmol/L) between *Desmanthus* spp. over 72 h of *in vitro* incubation with rumen fluid from grazing Brahman steers

Six samples were initially incubated for each forage. VFA, volatile fatty acids. A : P, acetic to propionic acid ratio; A : B, acetic to n-butyric acid ratio. Least-squares means values among the legumes within the same column followed by the same letters are not significantly different ($P < 0.05$). Differences between species, seasons and their interaction in each row for specific measurement times are declared at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and tending to differ when $P \leq 0.10$. NA, not applicable; NS, not significant; s.e.m., standard error of the mean

Species	Cultivar	Season	Volatile fatty acids						A : P	A : B	Total VFA	
			Acetic	Propionic	*Iso*-butyric	Butyric	*Iso*-valeric	Valeric			mmol/L	mmol/g OM fermented
D. leptophyllus	JCU1	Summer	74.6	15.4cde	1.3	10.0	2.4ab	1.3	4.9ab	7.6a	105.1	29.40a
		Winter	75.1	15.3de	1.4	10.5	2.6ab	1.3	4.9ab	7.4ab	106.2	27.13ab
		Spring	75.3	14.9e	1.2	10.8	2.2b	1.2	5.1a	7.2ab	105.6	26.35bc
D. virgatus	JCU2	Summer	81.2	17.4ab	1.6	12.3	2.8ab	1.5	4.7ab	6.8ab	116.6	23.32d
		Winter	78.5	17.7a	1.4	12.6	2.5ab	1.3	4.4b	6.3b	114.0	23.91cd
		Spring	79.3	17.0abcd	1.5	12.4	2.8ab	1.5	4.7ab	6.5ab	114.4	21.61de
D. bicornutus	JCU4	Summer	75.7	15.7bcde	1.5	11.8	2.7ab	1.4	4.8ab	6.5ab	108.9	20.69e
		Winter	81.6	17.1abc	1.5	12.2	2.8ab	1.5	4.8abc	6.8ab	116.7	20.79e
		Spring	78.8	16.5abcde	1.6	12.2	3.0a	1.6	4.8abc	6.6ab	113.8	19.45e
C. gayana[A]	NA	NA	88.5	20.1	1.7	11.0	2.9	1.7	4.4	8.3	125.9	22.53
Pooled s.e.m.		Summer	2.9	0.63	0.14	0.95	0.27	0.12	0.12	0.42	4.7	0.88
		Winter	2.9	0.63	0.14	0.95	0.27	0.12	0.12	0.42	4.7	0.88
		Spring	2.9	0.63	0.14	0.95	0.27	0.12	0.12	0.42	4.7	0.88
P												
Species		–	NS	***	NS	*	NS	= 0.07	**	*	*	***
Season		–	NS	NS	NS	NS	NS	NS	NS	NS	NS	*
Species × season		–	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

[A]Reference (not included in the statistical analysis).

2010). Mechanisms involving HT in *Desmanthus* plants on methanogenesis need further investigation as this form of tannins is much less documented than CT (Bhatta *et al*. 2009).

Furthermore, when examining a range of plant secondary compounds for their CH_4 reduction attributes, Jayanegara *et al*. (2015) suggested that measuring the biological activity of metabolites, such as via the assessment of bovine serum albumin protein precipitation capacity (Asquith and Butler 1985), is more accurate than measuring the concentration of compounds in the plant. However, the test of astringency (ability to bind protein) of CT using bovine serum albumin is done in a medium with pH of 4.9 (McAllister *et al*. 2005), which is not the pH found in the rumen. Thus, verification of the biological activity of tannins in *Desmanthus* species are important using Rubisco as the model protein in a medium with pH 7 comparative to the pH in the rumen of animals on forage-based diets, and also because this enzyme represents the main protein in fresh fodder (McAllister *et al*. 2005).

Our results demonstrate that, among the two species presenting an anti-methanogenic potential, *Desmanthus bicornutus* was more digestible. *Desmanthus leptophyllus*, which had reduced *in vitro* gas and CH_4 production (mL/g OM), had lower IVD-OM irrespective of the season compared with the other two *Desmanthus* forages (Table 3). This observation revealed a contrasting trend when gas and CH_4 are expressed per g of OM fermented; *Desmanthus leptophyllus* and *D. bicornutus* produced then less CH_4 at 72 h of incubation. Although HT appear to decrease CH_4 more through a direct effect on reducing methanogenesis, CT would also act indirectly through reducing digestibility of the forage leading to reduced DMI and fibre digestion (Tiemann *et al*. 2008; Jayanegara *et al*. 2010). However, in a study designed to separate the effects of CT and fibre on digestibility of tropical legumes containing CT, Tiemann *et al*. (2008) concluded that low quality and reduced CH_4 production of CT-rich legumes are also partly explained by the degradation of fibre in the rumen. Properties of fibre in highly tanniferous plants seem determinant because lignin prevented mainly hemicellulose from degradation through the formation of indigestible complexes, whereas it is suggested that the extent of degradation of hemicellulose have influenced CH_4 production.

Moreover, in the Van Soest fibre method, the plant cell has two constituents that are cell wall (hemicellulose, cellulose and lignin) and mostly digestible cell contents (starch and sugars). Then it follows that the difference between plant cell and NDF content represents the cell contents, which is a good estimate of soluble carbohydrates (CHO) content. In our study, these highly fermentable CHO are higher in *D. bicornutus* and hence this would induce higher IVD-OM values of this species. This increased fermentability resulted in higher CH_4 production, but expressed per g of fermented OM, CH_4 production in *D. bicornutus* was on average as low as that of *D. leptophyllus*, showing the high potential of this species in supplying high value forage with lower methanogenic potential.

Reduced CH_4 production accompanied with lower digestibility has been observed in previous research (Animut *et al*. 2008; Tan *et al*. 2011). Supplementing goats fed sorghum-

sudangrass (*S. bicolor*) with different levels of the CT-containing legume *Lespedeza striata* (i.e. 1.00, 0.67, 0.33 and 0) reduced CH_4 emission, but at relatively low dietary CT levels this was not accompanied by considerable adverse effects on digestion such as total tract N digestibility (Animut *et al*. 2008). It is clear that CT bind protein in the rumen and the CT-protein complex remain stable at pH 5.5–7 (Jones and Mangan 1977), partially resistant to microbial degradation (McLeod 1974; Min *et al*. 2005). Rumen bypass protein then becomes available for lower digestive tract utilisation (Jones and Mangan 1977), which results in improved feed protein availability to the animal. Therefore, CT-containing *Desmanthus* spp. may have a beneficial impact on dietary N efficiency compared with Rhodes grass, but this should be considered within a framework of *in vivo* studies.

The evolution of plant maturity (Tables 1 and 2) over seasons influenced the rumen fermentation as the IVD-OM and CH_4 were different between seasons after 72 h of incubation (Table 3). The lower fibre content in spring (Table 1) might be one reason for the higher plant digestibility during this season. Taking advantage of these differences over grazing season by associating in space and time various plants, including *Desmanthus* spp., might offer opportunities for ruminants to improve nutrition balance, health and well being (Provenza *et al*. 2007; Manteca *et al*. 2008).

Compared with the reference diet, the total VFA, acetate and propionate concentration after 72 h of incubation (Table 4) were overall reduced for the *Desmanthus* spp. Volatile fatty acids are the main source of metabolisable energy for ruminants, resulting from the rumen microbial fermentation (Bergman 1990). As a result, a reduction in VFA production is undesired. As VFA result from the fermentation of the diet in the rumen, a lower fermentability may explain lower VFA production, which was observed in this study. However, when VFA production was expressed relative to the IVD-OM, *Desmanthus leptophyllus* that had the lowest IVD-OM presented the highest VFA production (mmol/g OM fermented; Table 4). It is also known that plant secondary compounds such as tannins may modulate the microbial consortium of the rumen environment including notably modified microbial diversity and activity (McSweeney *et al*. 2001, 2002), and lead to a reduction in VFA production (Bhatta *et al*. 2009; Jayanegara *et al*. 2015). Polyphenolic secondary compounds in the legume species may explain the lower VFA concentration compared with Rhodes grass in which polyphenolics were mostly lower or not detected.

In an *in vitro* study on tropical legumes with various CT concentration (Barahona *et al*. 2003), CT-containing *Flemingia macrophylla* and *Desmodium ovalifolium* had produced lower total VFA than the non-tanniferous legumes *Leucaena macrophyllya* as it is observed in our study with *Desmanthus* forage containing CT compared with the non-tanniferous reference grass. However, the relation with CT in not straightforward as in the study of Barahona *et al*. (2003) on *Calliandra calothyrsus*, which had similar or higher CT content produced greater VFA concentration than both *F. macrophylla* and *D. ovalifolium*, and *Leucaena leucocephala* produced higher VFA concentration with CT content in the same range than *D. ovalifolium.*

Although lower VFA production, either due to a low DM digestibility or depressed feed intake, would imply less energy available to the animal; it has been reported that high dietary CT concentration (i.e. >50 g CT/kg DM) can lead to low feed intake (Barry and Duncan 1984; Waghorn *et al*. 1994; Bhatta *et al*. 2002), whereas low amounts in CT-containing legumes (i.e. <50 g CT/kg DM) did not (Wang *et al*. 1996; Carulla *et al*. 2005) even as the plant matured (Ramírez-Restrepo *et al*. 2006*b*). Other studies, however, have demonstrated that supplementation of *Desmanthus* spp. to steers fed buffel grass increased weight gain (Gardiner and Parker 2012) or sheep fed Mitchell grass boosted wool production (Rangel and Gardiner 2009).

Other factors than the depression of intake or VFA production might also play a role in animal performance. Because in our study *Desmanthus* cultivars contained less than 50 g CT/kg DM, it is reasonable to assume that the CT concentration in *Desmanthus* spp. could enhance rumen metabolism and systemic dynamic physiology (Tedeschi *et al*. 2014; Tedeschi and Fox 2016) in different ways. Certain CT chemistry (i.e. molecular size and monomer composition) is likely to influence the strength of interaction of tannins with dietary proteins and fibre, and thus forage quality (Barahona *et al*. 2003). For example, the astringency of *C. calothyrsus* seemed to be related to the tannin structure (Stewart *et al*. 2000; Lascano *et al*. 2003). However, it remains important to elucidate the magnitude of significant molecular interactions and the systemic exposure to analogous physiologic mechanisms.

Differences in fermentative traits between the *Desmanthus* cultivars along the seasons may provide opportunities to minimise the environmental footprint of pastoral systems in northern Australia. However, the degree of further collaboration between the academia, primary producers and agricultural industries will make a major difference to confront the challenges imposed by mitigation imperatives of climate change and the need to cope with a growing food security vulnerability.

Conclusion

In conclusion, *Desmanthus leptophyllus* and *D. bicornutus* demonstrated a greater potential to reduce enteric CH_4 after 72 h of *in vitro* fermentation compared with the third *Desmanthus* species. Among these legumes, *D. bicornutus* was on average more digestible. Collecting plants in different seasons influenced the fermentation as the IVD-OM was overall higher for spring plants after 72 h of incubation. Furthermore, *Desmanthus* spp. reduced *in vitro* CH_4 production and VFA concentrations compared with the Rhodes grass reference. The potential impact of *Desmanthus* spp. on the sustainability of tropical pastoral systems is important, requires in-depth characterisation, and seasonal pastoral experimentation with specific intended outcomes will be of great benefit to the Northern beef industry.

Conflicts of interest

The authors declare no conflicts of interest.

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