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Structural Features of Condensed Tannins Influence Their Antimethanogenic Potential in Forage Plants

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Abstract

Despite years of research on the antimethanogenic potential of condensed tannins (CT), their large-scale application is inhibited by a substantial variability in previous studies with regards to their impact on ruminant nutrition. This variability mainly results from the complexity of CT structures, and their impact on methane emissions is often unaccounted for. Hence, this study (a) evaluated the variability in antimethanogenic potential across six forage species, (b) linked methane emissions to tannin activity, and (c) determined the impact of CT structural features on methane abatement. Six forage species were grown in a greenhouse under controlled environmental conditions, namely, sainfoin (*Onobrychis viciifolia*), birdsfoot trefoil (*Lotus corniculatus*), big trefoil (*Lotus pedunculatus*), plantain (*Plantaga lanceolata*), sulla (*Hedysarum coronarium*) and lucerne (*Medicago sativa*). The plants were harvested at the flowering stage and leaf samples were analysed for chemical composition, condensed tannin concentration and structural features, before being incubated in rumen fluid for 24 hours. Lucerne was used as negative control (without tannins) and an additional polyethylene glycol (PEG) treatment was included, to inactivate tannins and link any effect on fermentation characteristics to tannin activity only. A strong variability across the species ($P<0.0001$) was observed on methane emissions. Sulla had the highest antimethanogenic potential and decreased methane emissions by 47% compared to lucerne. All species rich in CTs decreased both methane and total gas production, yet the PEG treatment did not alter the methane proportion in the total gas produced. In addition to CT concentration (R= -0.78), methane emissions were found to be negatively correlated with the CT structural features, prodelphinidin percentage (R= -0.6) and mean degree of polymerisation (R= -0.57). This study demonstrated that antimethanogenic potential of forages depends on CT concentration as well as on structural features and incorporating them in the studies can efficiently assess their impact on ruminant nutrition.

Introduction

Incorporating tannin containing forages in ruminant feed has been found to benefit animal health by improving nutrient utilisation, reducing incidences of bloat and parasitic burden. Concomitantly, they have have the potential to reduce methane emissions (Mueller-Harvey 2006). The latter is very important from an environmental perspective, as methane is a potent greenhouse gas and it has been calculated that 20% of the radiative forcing since 1750, are resulted from methane emissions, which are largely generated from enteric fermentation in ruminants (Thorpe 2008). Although, a number of studies have analysed the antimethanogenic potential of these forages, studies incorporating structural features of tannins and structure-activity relationships are limited. Astringency of tannins is usually measured with their ability to form complexes with macromolecules such as proteins. It is crucial to understand CT-protein interactions and their impact on biological systems as when supplied in excessive quantities, they are found to exert antinutritional effects by reducing palatability and digestibility of the feed (Zeller 2019). As, CTs are vastly diverse even within individual plants, characterisation is usually done by analysing their structural features such as the mean degree of polymerisation (mDP) and prodelphinidin percentage (PDP) (Adamczyk et al. 2011; Mueller-Harvey et al. 2017). Affinity of CTs to bind proteins has been found to increase with polymer size (mDP), as well as with increasing PDP, but so far it has rarely been tested how these structural characteristics affect methane formation in ruminants (Naumann et al. 2017; Aboagye and Beauchemin 2019). In this study a range of CT containing temperate forages with promising agronomic potential were grown under constant climatic conditions. The forages were subsequently analysed for their antimethanogenic potential and CT composition. Hence, the aim of our study was to determine to which extent these structural characteristics influence the methane abatement potential.

Methods and Study Site

A greenhouse experiment was conducted with five CT containing species, namely *Hedysarum coronarium* (HC), *Lotus corniculatus* (LC), *Lotus pedunculatus* (LP), *Onobrychis viciifolia* (OV) and *Plantago lanceolata* (PL). *Medicato sativa* (MS) was used as a tannin-free negative control. Plants were grown in 24x24x40 cm (LxWxH) flower pots with five plants per pot. The total of 24 experimental pots were arranged in a complete randomised design (6 species x 4 replicates). Harvesting was done at the flowering stage and plants were separated into leaves, stems and flowers, before being freeze dried and stored at 80 °C to maintain structural integrity of CTs. Samples were ball milled and subsequently extracted with 80% aqueous acetone solution and analysed with UPLC-MS/MS system as described in the protocol by Malisch et al. (2015). In addition to quantifying polyphenol and tannin concentration, this method also analysed structural features of CT such as PDP and mDP. Antimethanogenic potential of the species was analysed using the Hohenheim gas test (Menke 1988), an *in vitro* fermentation technique using in 6 replicates (2 consecutive days of 3 replicates). To separate the effect of tannin from forage quality effects or other bioactive compounds present in the plants, a treatment including polyethylene glycol (PEG) was added, as PEG precipitates the CT and allows the quantification of methane emissions in absence of CT. Total gas and methane production were measured after 8 and 24 hours of incubation. An infrared methane analyzer (Pronova Analysentechnik GmbH & Co. KG, Berlin, Germany) was used to measure methane concentration in the total gas (Jayanegara, Makkar, and Becker 2015). Statistical analysis of the data was performed using R programming language. The effect of the factors was determined using linear mixed effect model with species, treatment and their interaction as fixed factors with pot and replicates used as random factors. Pearson correlation formula was used to determine the association between methane production and structural features of CT.

Results and Discussion

Condensed tannin concentration and structural characteristics across the forage species

The distribution of CT composition in the leaves varied widely among species ($P< 0.05$) and ranged from 0% in MS and PL to 2.6% in HC (Table 1). The concentration of CT and its structural characteristics for the plants overlapped with the values reported in previous studies for OV, HC, LC and LP (Piluzza et al. 2000; McNabb et al. 1993; Malisch et al. 2015). PL and MS leaf extracts were void of CTs and had only minor polyphenol concentrations. Condensed tannins in plantain are reportedly within the range from 0.4 -1% (Stewart 1996).

Table 1 Polyphenol and condensed tannin concentration across the species, as well as the mean degree of polymerization (mDP) and prodelphinidin percentage (PDP) of condensed tannins

Plant	Total polyphenols (mg/g DM)	CT (mg/g DM)	mDP (n)	PDP (%)
HC cv. Sudda	25.5 ± 2.8	25.2 ± 2.8	19.3 ± 1.3	91 ± 1
LC cv. Bull	3.1 ± 1.1	2.3 ± 1.1	9.7 ± 1.0	33 ± 8
LP cv. Lot 29	21.4 ± 2.2	19.9 ± 2.3	16.7 ± 0.2	91 ± 2
MS cv. Galaxy	0.2 ± 0.1	NA	NA	NA
OV cv. CPI 63750	20.2 ± 1.7	17.0 ± 1.2	14.3 ± 0.7	83 ± 4
PL cv. PLA60	0.6 ± 1.3	NA	NA	NA

Antimethanogenic potential of the forage species

A significant effect ($P< 0.0001$) of PEG treatment as well as the species was observed on total gas, methane production and proportion of methane in total gas (Table 2). With the addition of PEG, total gas production of all tannin containing species were similar to MS, indicating that forage quality at the flowering stage in absence of CT has a negligible effect on digestibility across species. In the absence of PEG, CTs from sulla and big trefoil were able to decrease methane emissions by 44 and 31% respectively. However, in both the species there was a concomitant decrease in total gas production, indicating their negative effect on ruminal fermentation. This is in accordance with previous findings, in which both HC and LP have been reported to possess anti nutritional effects when supplied as a sole diet, due to their high tannin concentration (Douglas and Foote 2000; Tavendale et al. 2005). Methane suppression by OV in absence of PEG was comparatively less than former species i.e. 17%. These findings confirm that the antimethanogenic potential of these species is a direct result of their CTs (Jayanegara, Makkar, and Becker 2015). However, despite its absence of CTs, PL leaves produced the lowest methane and total gas emissions, and in line with the absence of CTs, the PEG treatment had no effect. This is probably due to presence of bioactive compounds in PL such as aucubin acetoside and catapol. These compounds are found to possess antimicrobial property and can influence ruminant fermentation (Stewart 1996; Navarrete et al. 2016).

Table 2. In vitro total gas, methane emissions and methane proportion in total gas produced during in vitro fermentation of temperate forage species with or without the addition of polyethylene glycol (PEG).

Plant	Total gas		Methane		Methane in total gas (%)	
	No PEG	With PEG	No PEG	With PEG	No PEG	With PEG
HC cv. Sudda	121.82 Bb	225.28 Bca	28.12 Eb	50.43 Ca	23.23 Aba	22.42 Aa
LC cv. Bull	226.23 Aa	238.36 AB	54.52 Aa	55.84 Aa	24.16 Aa	23.48 Aa
LP cv. Lot 29	132.86 Bb	215.69 Ca	33.61 Bb	49.32 Ba	25.77 Aa	23.07 ABa
MS cv. Galaxy	225.28 Ab	241.2 Aa	54.04 Aa	54.53 Aa	24.02 Ab	22.6 Aa
OV cv. CPI 63750	212.97 Ab	247.92 Aa	47.14 Ca	51.36 ABCa	22.19 Bb	20.7 BC
PL cv. PLA60	220.11 Aa	226.8 BCa	41.94 Da	44.6 Ba	19.09 Ca	19.61 Ca

Different upper-case letters denote the differences between cultivars for each treatment, while different lower-case letters denote differences between treatments for each species. Column means without a common superscript differ (P< 0.05).

As hypothesized, among the CT containing species, those with high CT concentration, as well as high mDP PDP (LP and HC) had the highest antimethanogenic potential, which was clearly linked to CT by the difference between methane emissions with and without PEG.

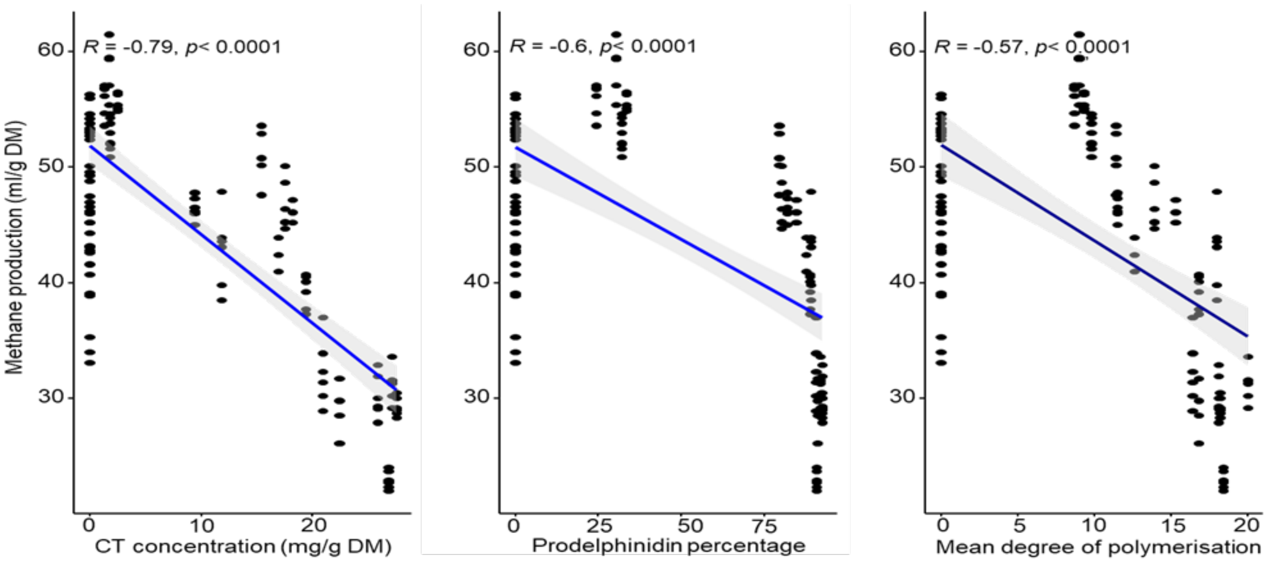

Figure 1 Correlation between CT concentration and composition with methane production.

Consequently, correlations were conducted between the produced methane and the CT concentration of the species resulted in a negative correlation with *R* of 0.79 (P< 0.0001). Prodelphinidin percentage (*R* = -0.6, *P*< 0.0001) and mDP (*R*= -0.57, *P*< 0.0001) were negatively correlated to methane production (Figure 1). The correlation coefficients strongly suggest that CT structural characteristics in conjunction with its concentration, play an important part in determining the extent of methane suppression. This is in accordance to the study by Hatew et al. (2015), where the differences in PD percentage were found to be an important source of variation in methane production for sainfoin cultivars.

Conclusion

In vitro techniques are useful screening tools in understanding the potential impact of CTs on ruminant nutrition. The extent of methane reduction varied greatly across the species and could be attributed to both CT concentration and their structural features. The assessment of their structural features and bioactivity is important in order to strategically decrease methane emissions in ruminants while preventing their negative impact on animal productivity. Consequently, a large degree of the variability in previous findings can likely be traced back to the missing characterisation of the CTs structural features. However, the simultaneous reductions in total gas will make the in vivo trials necessary, to identify the impact on the production from potentially reduced forage digestibilities.

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