Structural features of condensed tannins affect in vitro ruminal methane production and fermentation characteristics

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Short title: Structural of condensed tannins and fermentation

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SUMMARY

An *in vitro* study was conducted to investigate the effects of condensed tannin (CT) structural properties, i.e. average polymer size (or mean degree of polymerization), percentage of *cis* flavan-3-ols and percentage of prodelphinidins in CT extracts on methane (CH_4) production and fermentation characteristics. Condensed tannins were extracted from eight plants in order to obtain different CT types: blackcurrant leaves, goat willow leaves, goat willow twigs, pine bark, redcurrant leaves, sainfoin plants, weeping willow catkins and white clover flowers. They were analysed for CT content and CT composition by thiolytic degradation, followed by high performance liquid chromatography (HPLC) analysis. Grass silage was used as a control substrate. Condensed tannins were added to the substrate at a concentration of 40 g/kg, with

or without polyethylene glycol (+ or −PEG 6000 treatment) to inactivate tannins, then incubated for 72 h in mixed buffered rumen fluid from three different lactating dairy cows per run. Total cumulative gas production (GP) was measured by an automated gas production system. During the incubation, 12 gas samples (10 μl) were collected from each bottle headspace at 0, 2, 4, 6, 8, 12, 24, 30, 36, 48, 56 and 72 h of incubation and analysed for CH_4. A modified Michaelis-Menten model was fitted to the CH_4 concentration patterns and model estimates were used to calculate total cumulative CH_4 production (GP_{CH4}). Total cumulative gas production and GP_{CH4} curves were fitted using biphasic and monophasic modified Michaelis-Menten models, respectively. Addition of PEG increased GP, GP_{CH4}, and CH_4 concentration compared to the −PEG treatment. All CT types reduced GP_{CH4} and CH_4 concentration. All CT increased the half time of GP and GP_{CH4}. Moreover, all CT decreased the maximum rate of fermentation for GP_{CH4} and rate of substrate degradation. The correlation between CT structure and GP_{CH4} and fermentation characteristics showed that the proportion of prodelphinidins within CT had the largest effect on fermentation characteristics, followed by average polymer size and percentage of *cis*-flavan-3-ols.

INTRODUCTION

Methane (CH_4) is the second most important greenhouse gas from domestic ruminants with a major contribution to anthropogenic methane emissions (Boucher *et al*. 2009). Besides the contribution of methane to greenhouse gas emissions, methane synthesis in the rumen also represents a loss of dietary energy of *c*. 2-12%, depending on the composition and quality of the diet (Johnson & Johnson 1995). Minimizing methane synthesis during rumen fermentation without altering animal production is, therefore, desirable both as a strategy to reduce global warming and as a means to improve feed conversion efficiency. Methane emissions from the rumen can be reduced by feeding extra fermentable starch (Ellis *et al*. 2008) and adding oils

(Alexander *et al*. 2008; Castillejos *et al*. 2008) or secondary plant compounds, such as condensed tannins (CT), to the diet (Carulla *et al*. 2005; Waghorn 2008). Condensed tannins have been considered an anti-nutritional factor in animal diets, but now it is acknowledged more and more that certain CT may also have beneficial effects (Mueller-Harvey 2006). Negative or positive effects of CT on animal performance depend on the type and level of tannins in the plant (McNabb *et al*. 1993; Wang *et al*. 1996; Min *et al*. 2003), the amount ingested and animal species involved (Mueller-Harvey 2006). Singh & Bhat (2001) indicated that the negative effects associated with dietary CT are reduction of feed intake and lower digestibility when supplementing high concentrations of dietary CT (60-120 g/kg DM). Low concentrations of these tannins (20-40 g/kg DM), however, can have positive effects on protein degradation, animal performance, urinary nitrogen secretion; they can reduce the occurrence of rumen bloat in cattle and can increase milk production and milk protein content in dairy cows (Min *et al*. 2003; Ramírez-Restrepo & Barry 2005). Condensed tannins negatively affect ciliate protozoa, fibre-degrading bacteria and methanogenic archaea depending on the structure and concentration of dietary CT (Min *et al*. 2003; Kumar *et al*. 2014). As a result, CT can reduce CH_4 production (Ramírez-Restrepo & Barry 2005; Bhatta *et al*. 2009, 2013; Grainger *et al*. 2009). By increasing CT concentration in ruminant diets, it was found that methane production and size of archaeal and protozoal populations decreased (Bhatta *et al*. 2009; Hariadi & Santoso 2010). Few studies, however, are available about which structural CT features are most responsible for reducing ruminal CH_4 production. The three main CT features are; i) the mean degree of polymerization (mDP), ii) the ratio between prodelphinidins:procyanidins (PD:PC) (proportion of PD) and iii) the ratio between *cis:trans* flavan-3-ols (proportion of *cis*) within CT (Gea *et al*. 2011).

The objective of the current study was to examine the relationship between tannin structures and CH_4 production and fermentation characteristics during *in vitro* incubation by

using eight different tannin extracts with a wide range of mean degree of polymerization, proportion of PD and *cis* within CT.

MATERIALS AND METHODS

Plant samples for extracting condensed tannins

Blackcurrant (*Ribes nigrum*) and redcurrant (*Ribes rubrum*) leaves were collected at Hildred pick your own (PYO)-farm, Goring-on-Thames, UK; goat willow (*Salix caprea*) leaves and twigs were collected from Goring-on-Thames, UK; weeping willow (*Salix babylonica*) catkins were collected on Evesham Rd., Emmer Green, Reading, UK; white clover (*Trifolium repens*) flowers were collected from the National Institute of Agricultural Botany (NIAB, Cambridge, UK); whole sainfoin (*Onobrychis viciifolia*, var. Esparsette) plants were collected from Barham, Kent, UK; pine bark was provided by University of Turku, Finland. After collection, the plant materials were freeze-dried and then ground to pass a 1-mm sieve using an impeller mill (Retsch GmbH, SM1, Haan, Germany).

Extraction of condensed tannins

The CT extracts were prepared according to a slightly modified method of Gea *et al* (2011). Finely ground plant powder (50 g) was extracted with acetone/water (500 ml; 7:3; v/v) under constant stirring for 60 min. The mixture was transferred into a Buchner funnel, fitted with a Whatman filter paper and filtered under vacuum. The filtrate was extracted with dichloromethane (CH_2Cl_2) to remove lipids and chlorophyll and concentrated in a rotary evaporator at <37 °C. The remaining aqueous solution was centrifuged for 6 min at 4500 rpm (Thermo Electron Corporation, Jouan CR3i Multifunction Centrifuge, Basingstoke, UK) in order to remove residual chlorophyll and any other insoluble material. Extracts were frozen, freeze-dried and stored at -20 °C.

Analysis of condensed tannin extracts

Extracts were analysed for CT content and structural properties by thiolytic degradation, followed by high performance liquid chromatography (HPLC) analysis (Novobilský *et al*. 2011; Williams *et al*. 2014). This provided information on the proportion of flavan-3-ols (catechin, epicatechin, gallocatechin and epigallocatechin) in the CT terminal and extension units (Fig. 1). In addition, it allowed calculation of the mean degree of polymerization (mDP), prodelphinidins:procyanidins (PD:PC) and *cis*:*trans* flavan-3-ol ratio in the CT polymers based on the following formulae (1, 2, 3) (Gea *et al*. 2011) (Table 1):

$$\text{mDP} = \frac{\text{sum of extension and terminal flavan}-3-\text{ol units (mol)}}{\text{sum of terminal flavan}-3-\text{ol units (mol)}} \quad (1)$$

$$\text{PD} = \frac{\text{Proportion of GC+EGC units}}{\text{Proportion of C+EC+GC+EGC units}} \quad (2)$$

$$\textit{cis} = \frac{\text{Proportion of EC + EGC units}}{\text{Proportion of C + GC+EC+EGC units}} \quad (3)$$

where C = catechin, EC = epicatechin, GC = gallocatechin and EGC = epigallocatechin flavan-3-ols, proportion of PD + PC = 100; proportion of *cis* + *trans* = 100

Experimental design

The effects of CT structural features on CH_4 production and fermentation kinetics during *in vitro* incubation were evaluated using tannin-free grass silage as a control substrate. The chemical composition of grass silage was: dry matter (DM) = 937.0 g/kg; organic matter (OM) = 911.3 g/kg DM; crude protein (CP) = 151.4 g/kg DM, neutral detergent fibre (NDF) = 498.5 g/kg DM. Condensed tannin were added to the substrate at an effective concentration of 40 g CT/kg of substrate in the presence (+PEG) or absence (−PEG) of polyethylene glycol (PEG 6000) to inactivate the tannins (Makkar *et al*. 1995). Condensed tannins (10 mg) and

substrate (250 mg) with or without PEG (100 mg; CT: PEG = 1:10 w/w) (Pellikaan *et al.* 2011*b*) were weighed into duplicate 250 ml bottles (Schott bottle, GL45, Mainz, Germany) per tannin extract within each run, with two separate runs. Test substrate was incubated with a mixture of rumen liquid collected from three different rumen fistulated lactating Holstein-Friesian dairy cows per run (i.e., total of six rumen fistulated cows). These cows were fed a grass and maize silage mixture in the morning and afternoon and 9 kg of concentrate according to their requirements. The handling of the animals was approved by the Institutional Animal Care and Use Committee of Wageningen University, Wageningen, the Netherlands and in accordance with Dutch legislation on the use of experimental animals. Rumen fluid was collected before the morning feeding in pre-warmed thermos flasks, which were filled with carbon dioxide (CO_2) and transported directly to the nearby laboratory. All further manipulations were done under CO_2 to ensure anaerobic conditions. The rumen fluid was pooled and filtered through two layers of cheesecloth into a flask flushed with CO_2. Filtered rumen fluid was mixed with a buffer solution with constant stirring and continuous flushing with CO_2 and maintained in a water bath at 39 °C. Buffer solution was made as described by Williams *et al*. (2005). After adding 30 ml of the buffered rumen liquid mixture, bottles were directly placed in a shaking water bath at 39 °C and connected to an automated time-related gas production system (Pellikaan *et al*. 2011*a*) and gas production was measured over 72 h. Gas production (GP) in blanks (i.e. buffered rumen fluid mixture without substrate and CT extracts) was 2.73 ± 0.21 ml for run1 and 9.95 ± 0.35 ml for run 2. Condensed tannin extracts consisted of 46.2 to 87.4 (g/100 g extracts) of non-CT compounds, which may contribute to fermentation. Therefore, blanks were also included containing CT extracts only, with and without PEG. The average gas production in CT extracts with and without PEG in run 1 were 10.4 and 4.8 ml, and in run 2 were 16.7 and 4.8 ml, respectively. After 72 h incubation, the fermentation fluid pH was recorded (Mettler Toledo FE20/EL20 pH meter,

Schwerzenbach, Switzerland) and fermentation fluid from each bottle was collected for volatile fatty acid (VFA) and ammonia (NH_3) analysis.

In vitro gas and methane production

Total cumulative gas (GP) and methane (GP_{CH4}) production were measured using an automated gas production system at the laboratory of the Animal Nutrition Group of Wageningen University, the Netherlands (Pellikaan *et al.* 2011*a*). Methane concentration in the headspace of the fermentation bottle was measured by gas chromatography (GC8000Top, CE Instruments, Milan, Italy). Fermentation bottles were modified (Pellikaan *et al.* 2011*a*) to sample CH_4 from the headspace. In brief, bottles were fitted with a glass extension that was sealed with a screw cap and an air-tight septum (Grace, XLB-13 Septa 1/2). Ten µl aliquots of the bottle headspace gas were sampled through the septa at distinct time points of incubation (0, 2, 4, 6, 8, 10, 12, 24, 30, 36, 48, 56 and 72 h) using a gas-tight syringe (Gastight ® # 1701 Hamilton 1701N, 10 µl Syringe, Point style 5, Bonaduz, Switzerland). They were injected directly into the gas chromatography, which was equipped with a stainless steel column (6 m long, 0.53 mm internal diameter, 25 µm film thickness and packed with PoraPack Q50-80 mesh Grace, Breda, the Netherlands) and connected to a flame ionization detector. The temperature of the injector, column and detector were maintained at 150 °C, 60 °C and 150 °C, respectively. The carrier gas was nitrogen, and the pressure for nitrogen, hydrogen and air was set at 100, 50 and 100 kPa, respectively. The CH_4 concentration was calculated by external calibration using a certified gas mixture with a known CH_4 concentration in synthetic air (Linde Gas Benelux, Schiedam, and the Netherlands). Peak areas were determined by automatic integration system software (Chrom-Card data system Version 2.3.3, September, 2005, Rodano Milan, Italy) for gas chromatography.

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

$$CH_4 = \sum_{i=1}^{i=1+n}\{V_{HS}(C_{i+1} - C_i) + G_{i+1}C_{i+1}\} \quad (4)$$

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

Curve fitting and calculations

Total cumulative gas (GP) and CH_4 production curves were fitted with biphasic and monophasic Michaelis-Menten equations, respectively (Groot *et al.* 1996) using the non-linear least squares regression procedure in SAS (2002):

$$OMCV = \sum_{i=1\ or\ 2}^{n} \frac{A_i}{1+\left(\frac{C_i}{t}\right)^{B_i}} \quad (5)$$

where *OMCV* = GP or CH_4 production (ml/g of incubated OM), *A* = the asymptotic gas production (ml/g of incubated OM), *B* = the switching characteristics of the curve, *C* = time at which half of the asymptotic gas production is reached (half-time, $T_{½}$, h) and t = the time (h). The value of *i* indicates the number of phases (*i* = 1 or 2). The maximum rate of gas production (R_{max}, ml/h) was calculated using the estimated *A*, *B* and *C* values as described by Bauer *et al.* (2001).

$$R_{max} = \frac{A \times C^B \times B \times \left[TR_{max}^{(-B-1)}\right]}{\left[1+C^B \times TR_{max}^{(-B)}\right]^2} \quad (6)$$

where TR_{max} is the time at which R_{max} occurs; $TR_{max} = C \times \left\{\left[\frac{B-1}{B+1}\right]^{\{1/B\}}\right\}$ (7)

The maximum rate of substrate degradation (R_M, %/h) was calculated from the *A*, *B* and *C*-values as estimated from the CH_4 production curves (Groot *et al*. 1996).

$$R_M = (B \times tR_M^{(B-1)}) / (C^B + tR_M^B) \quad (8)$$

where tR_M is the time at which R_{max} occurs; $tR_M = C \times (B\text{-}1)^{1/B}$ (9)

Chemical analysis

Grass silage was air dried, ground through a 1-mm sieve using a cross beater mill (Peppink 100 AN, Deventer, The Netherlands) and analysed for DM (ISO 6496, 1999), ash (ISO 5984, 2002) and nitrogen (N) (ISO 5983, 2005). Crude protein content was calculated as: CP = 6.25 × N. Neutral detergent fibre was analysed according to Van Soest *et al*. (1991) after a pre-treatment with a heat stable amylase and corrected for residual ash.

Fermentation fluid, sampled for VFA analysis (750 µl), was acidified with 750 µl of ortho-phosphoric acid solution. The ortho-phosphoric acid solution was 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 2-methylvaleric acid solution. Volatile fatty acid concentration was analysed by gas chromatography following procedures of Pellikaan *et al.* (2011*b*) with the carrier gas modified by using hydrogen instead of helium to enhance baseline separation. Isocaproic acid was included as the internal standard. The total VFA (tVFA) concentration in the fermentation fluid was expressed as mmol/g of incubated OM. Fermentation fluid samples for NH_3 analysis (750 µl) were mixed with 750 µl of 10% trichloroacetic acid solution. Ammonia was determined using a colorimetric method (Pellikaan *et al.* 2011*b*) after deproteinizing the supernatant with 100 g/l trichloroacetic acid and the resulting chromophore was measured at 623 nm using an ultra-violet (UV) spectrophotometer (Evolution 201-Themo Scientific).

Statistical analysis

Effects of the CT structural properties on the substrate (grass silage) in combination with (+PEG) or without (−PEG) on fermentation kinetics and fermentation end-products were tested by analysis of variance using the MIXED procedure of SAS (2002) as:

$$Y_{ijk} = \mu + T_i + P_j + R_k + (T \times P)_{ij} + \varepsilon_{ijk} \quad (8)$$

where Y_{ijk} = the dependent variable, μ = the overall mean, T_i = the tannin extract type (i= 1 to 9), P_j = the effect of PEG (j=1 to 2), R_k = run (k=1 to 2), $(T \times P)_{ij}$ = the effect of tannin extract type and PEG interaction and ε_{ijk} = the residual error term. The statistical unit was the average of replicate *in vitro* bottles within run. Differences among treatment means were analysed using Tukey-Kramer's multiple comparison procedure in the LSMEANS statement in SAS with effects considered significant at a probability value of $P < 0.05$, and trends at a probability value of $P < 0.10$.

Relationships between CT content, mDP, proportion of PD and proportion of *cis* and fermentation parameter estimates were analysed using the multiple stepwise regression procedure in SAS (2002) where CT content (g CT/100 g extract), mDP, proportion of PD and proportion of *cis* were included as independent variables. The criteria to include variables in the model were a combination of a low value for the Mallow's Cp-criterion, a high coefficient of determination (R^2), and setting the residual degrees of freedom (D.F.) in the regression model at > 65% of the total D.F.

RESULTS

Effect of CT source on total gas and methane production

Table 2 shows that tannin source and PEG addition affected GP, GP_{CH4} and CH_4 concentration. In general, CT addition gave an increase in GP and reduced GP_{CH4} except for SF, and reduced CH_4 concentration (all $P < 0.001$). Addition of PEG resulted in a general

increase ($P < 0.001$) in GP, GP_{CH4} and CH_4 concentration. There was an effect of interaction between tannin source and PEG ($P < 0.001$) on GP, GP_{CH4} and CH_4 concentration. Addition of CT without PEG did not affect total GP compared to grass silage (control), except for sainfoin CT, which increased GP ($P < 0.001$). However, CT without PEG reduced both GP_{CH4} and methane concentration (both $P < 0.001$). Redcurrant CT gave the lowest GP_{CH4} ($P < 0.001$) and CH_4 concentration ($P < 0.001$) compared to other CT extracts. In general, PEG addition increased GP ($P < 0.001$) by 18%, GP_{CH4} ($P < 0.001$) by 47% and CH_4 by 25% ($P < 0.001$) compared to the no PEG treatments.

Effects of CT source on fermentation parameters and kinetics

The time at which half of the asymptotic gas production (GP-T1½; half time) in the first phase was reached was not affected ($P = 0.170$) by CT source (Table 3). The half times of GP for the second phase (GP-T2½) and of methane production (GP_{CH4}-T½) were in general increased ($P < 0.001$) when CT was added to the control, whilst GP_{CH4}-R_{max} and R_M were decreased ($P = 0.004$; Table 3). The addition of PEG significantly decreased ($P = 0.006$) the values for GP-T1½, GP-T2½ and GP_{CH4}- T½, and increased ($P < 0.001$) the GP-$R2_{max}$, GP_{CH4}-R_{max} and R_M. An effect of interaction between tannin source and PEG was found ($P = 0.003$) for GP-T2½, GP-$R2_{max}$, GP_{CH4}-T½, and GP_{CH4}-$R_{max.}$ Individual CT types affected GP-T2½ and GP_{CH4}-T½ differently, with CT extracts from redcurrant leaves and sainfoin causing the largest increase compared to the control ($P \leq 0.001$). The maximum rate of GP in the second phase (GP-$R2_{max}$) and CH_4 production (GP_{CH4}-R_{max}) both decreased ($P < 0.001$) when CT was added to the control. Adding blackcurrant leaf CT to the control created the largest decrease ($P = 0.031$) in GP-$R2_{max}$, whereas redcurrant leaf CT generated the largest decrease ($P < 0.001$) in GP_{CH4}-R_{max}. Addition of CT decreased ($P = 0.004$) the rate of substrate degradation (R_M) as derived from the CH_4 production curves, with the largest reduction caused by sainfoin CT

(2.6 %/ h). Figures 2 and 3 describe the CH_4 production profiles that resulted from the CT additions without PEG. The profiles also show the large CT effects from redcurrant leaves, blackcurrant leaves and white clover flowers on the extent and kinetics of CH_4 production.

Effects of CT source on fermentation end-products

Tannin source affected tVFA, the molar proportion of individual VFA (acetate [HAc], propionate [HPr], butyrate [HBu] and valerate [HVa]), the molar proportion of branched chain VFA (HBc) and non-glucogenic to glucogenic VFA (NGR) ratio, as well as pH and NH_3 ($P = 0.014$; Tables 4 and 5). Addition of PEG showed a similar significant ($P < 0.001$) effect except for pH and HVa, which were not affected. In general, CT addition gave an increase ($P < 0.001$) in tVFA and the molar proportion of HPr, whereas the molar proportions of HAc and HBc decreased ($P < 0.001$), as did NGR ratio and the NH_3 concentration. Addition of PEG resulted in a general increase ($P < 0.001$) in tVFA, the molar proportions of HAc and HBc, the NGR ratio and NH_3 concentration, whilst molar proportions of HPr were decreased ($P < 0.001$). There was an effect of interaction between tannin source and PEG ($P = 0.035$) on HAc, HPr, HBu, HBc, NGR ratio and NH_3 concentration. Addition of CT without PEG, tVFA and pH were similar among treatments (Table 4). There were significant differences in tVFA and pH (both $P \leq 0.001$) between sainfoin and goat willow twig treatments: sainfoin gave the highest tVFA (10.08 mmol/g OM) and the lowest pH (6.24), whereas goat willow twigs gave the highest pH (6.48) and the lowest tVFA (7.13 mmol/g OM) compared to the other CT extracts. All CT additions without PEG decreased ($P = 0.014$) the NH_3 concentrations compared to control. Redcurrant leaf extract gave the lowest ($P = 0.041$) NH_3 concentration (1.56 mmol/g OM). Addition of CT decreased ($P < 0.001$) the molar proportion of HAc, except for CT from goat willow twigs, and increased ($P < 0.001$) the molar proportion of HPr with the highest proportion found with the redcurrant leaf CT.

Addition of CT decreased ($P < 0.001$) the molar proportion of HBc and the NGR ratio, with the largest reduction caused by sainfoin and redcurrant leaf CT. In general, PEG addition increased ($P < 0.001$) NH_3 and tVFA concentration, increased the molar proportions of HAc, HBc as well as the NGR, and decreased the molar proportion of HPr ($P < 0.001$).

Relationships between CT structure and fermentation kinetics and end-products

Table 6 shows the relations between CT content (g CT/100 g extract), mean degree of polymerization (mDP), proportion of PD, and proportion of *cis*-flavan-3-ols within CT and fermentation kinetics or fermentation end-products. Condensed tannin content affected ($P = 0.011$) GP, GP_{CH4}-$T_{½}$ and fermentation end-products and had a relatively strong effect on most fermentation end-products as R^2 values ranged from 0.64 to 0.91, except for NH_3 (0.46) and CH_4 concentration (0.27). Prodelphinidins affected ($P = 0.035$) GP, GP-$R2_{max}$, HPr, HBc, and NGR, however, the relation between PD and fermentation end-products had higher R^2 values (0.79 to 0.91) compared to the fermentation kinetic parameters ($R^2 \leq 0.60$). The proportion of *cis* flavan-3-ols affected fermentation kinetics of methane production, but the R^2 values ranged only from 0.27 to 0.65.

DISCUSSION

Waghorn *et al*. (1994) suggested that > 50 g CT/kg *Lotus pedunculatus* may negatively affect feed intake whereas lower dietary CT concentrations have no influence on feed intake by ruminants. Therefore, in the current study, tannin contents were accounted for and the final concentration adjusted to 40 g CT/kg initial substrate. Addition of 40 g CT/kg substrate did not affect GP and tVFA compared to control, except for sainfoin CT, which gave higher GP and tVFA values. This result could be due to the fact that tannin extracts differed considerably in CT content (12.6 g/100 g in sainfoin to 53.8 g/100 g in goat willow twigs extracts). Non-

tannin compounds present in the tannin extracts may be fermented; therefore, in addition, blanks containing CT extracts were incubated with and without PEG in the current study (average GP data presented in materials and methods section). The GP of blanks with sainfoin extracts, with and without PEG, were 42.7 ml and 26.1 ml, respectively. Thus, non-CT compounds in the extracts may have influenced the current findings, despite the corrections made for blanks. In spite of having no effect on GP, addition of CT decreased the maximum rate of GP in the second phase and increased the half time of asymptotic GP in the second phase. This suggests that CT inhibited the initial rate of fermentation but not the extent of dry matter degradation. The current study demonstrated clearly that CT delayed the initial rate of fermentation, when added CT decreased the rate of substrate degradation from 7.2%/h in grass silage to 4.2%/h on average. In addition, a reduction of ruminal NH_3 and HBc means that CT can act as a protein protectant against ruminal microbial degradation and will increase rumen escape protein (Waghorn 1990; Bunglavan & Dutta 2013).

A reduction of ruminal NH_3 and HBc in the present study agrees with previous reports by Bhatta *et al*. (2009) and Pellikaan *et al.* (2011*b*). Ammonia is a product of amino acid deamination, whereas *iso*-butyrate and *iso*-valerate are breakdown products of the carbon skeleton of the essential amino acids valine and leucine, respectively, during rumen fermentation (Van Soest 1994). Tannins can bind proteins to form insoluble complexes and thus reduce protein degradation in rumen fluid (Mueller-Harvey 2006; Patra & Saxena 2011); hence, this explains the decrease of NH_3 and branched chain VFA concentrations. On the other hand, the reduction in NH_3 and HBc could be due to enhanced utilization of HBc and NH_3 for microbial protein synthesis (Waghorn & Shelton 1997). Pellikaan *et al*. (2011*b*) also reported lower *in vitro* NH_3 and HBc concentrations when lucerne substrate was supplemented with quebracho CT. Condensed tannins have higher affinities for proteins than

polysaccharides (Patra & Saxena 2011). This may explain the more profound effect of tannins on NH_3 concentration compared to their impact on tVFA production.

In the current study, all CT sources reduced CH_4 production and CH_4 concentration compared to the control. This reduction could be due to lower fibre degradation caused by the formation of CT and lignocellulose complexes, which in turn prevent microbial fermentation, or by direct inhibition of cellulolytic micro-organisms or a combination of both (McSweeney *et al*. 2001). Moreover, lower CH_4 production could also stem from a lower acetate proportion and a higher propionate proportion due to CT. The mechanism of CT effect on acetate and propionate production could possibly be the result of a change in microbial profile or composition and microbial activity. Jones *et al*. (1994) found that CT inhibited the growth of *Butyrivibrio fibrisolvens* bacteria, which are involved in fibre fermentation. Fermentation of organic matter to acetate and butyrate liberates $2[H]^-$ ions, which are used in the rumen to produce CH_4, whereas propionate production is considered to be a $[H]^-$ ion sink (Tavendale *et al*. 2005; Beauchemin *et al*. 2009). Addition of CT did not change the ruminal pH and all pH values (6.24 to 6.46) were in the normal range for optimal microbial digestion of fibre and protein (Van Kessel & Russell 1996).

The current study confirmed that PEG, which was used as a CT neutralizing agent (Silanikove *et al*. 2001; Tavendale *et al*. 2005; Beauchemin *et al*. 2009), clearly influenced the fermentation characteristics. It has been used in many studies (Makkar *et al*. 1995; Getachew *et al.* 2000*a*, 2002; Calabrò *et al*. 2012) in order to measure the effect of tannin-containing substrates on rumen fermentation *in vitro*. Polyethylene glycol is an inert compound able to inactivate CT (Makkar *et al.* 1995). It is supposed to make tannins inert by forming tannin–PEG complexes and even to be able to displace protein from a tannin–protein complex. This would explain the interaction effect observed between tannin sources and PEG in the current study. The results of the current study showed an increase in GP, GP_{CH4} and

CH_4 concentration, a decrease in half time and an increase in the maximum rate of fermentation for GP and GP_{CH4} in the presence of PEG. This is probably caused by an increase in available nutrients to rumen microbes, especially nitrogen (Getachew *et al.* 2000*b*) due to protein being released from a tannin–protein complex. When more nitrogen is available, in the presence of sufficient carbohydrate, more amino acids are absorbed into the bacterial cells, resulting in greater microbial growth, and consequently, an increased fermentation activity with higher gas production and total volatile VFA production (Calabrò *et al.* 2012).

The individual CT source had different effects on fermentation kinetics and fermentation end-products, although, in the current study, the same amount of CT was added to the control (40 g CT/kg of grass silage substrate). This ensured that the effects of tannins on fermentation kinetics and fermentation end-products could be assessed. The effects varied depending on the CT source, agreeing with observations in the literature (Beauchemin *et al.* 2007; Pellikaan *et al.* 2011*b*; Hassanat & Benchaar 2013). In the current study, the purity of CT (12.6 g to 53.8 g CT/100 g extract) affected both fermentation kinetic and end-product parameters. These results could be due to non-tannin compounds in the extract, such as sucrose and flavonoids (Marais *et al.* 2000; Regos *et al.* 2009). Effects of the CT purity on gas production were also reported by Pellikaan *et al.* (2011*b*), where CT content ranged from 14.5 g to 95.1 g CT/100 g extract.

In the current study, the degree of polymerization affected NH_3 concentration negatively and delayed the half time of asymptotic GP in the second phase. The mDP of CT ranged from 2.3 (weeping willow catkins) to 9.8 (redcurrant leaves). Addition of CT from redcurrant leaves had the largest mDP and gave the lowest NH_3 concentration. This agrees with Makkar *et al.* (1988), who reported that the protein precipitation capacity of CT depends

on the degree of polymerization. Interestingly, Hatew *et al.* (2015) also found a negative correlation between mDP and protein degradation.

The proportion of PD negatively affected GP, HBc and NGR and positively affected GP-$R2_{max}$ and HPr. Prodelphinidins have more hydrogen bonding sites than procyanidins and this may enhance their affinity to fibres and proteins. This agrees with Hatew *et al.* (2015), who also found that prodelphinidins had a greater effect on fibre than protein digestion. Condensed tannins with a higher PD proportion have more hydrogen bonding sites, which may enhance the affinity of this type of CT to bind to fibre and create CT-fibre complexes, thus reducing fibre degradation in the rumen fluid (Hatew *et al.* (2015). The formation of insoluble CT-fibre and CT-protein complexes is likely to have reduced the fermentation. This could be seen as a reduction in GP and an increase in GP-$R2_{max}$ in the current study. The PD:PC ratio (expressed as proportion of PD within the CT) was the most important factor among all of the CT features and showed a negative correlation with the total GP production and acetate proportion (Hatew *et al.* 2015). Molan *et al.* (2001) also found that PD, rather than PC, inhibited particularly the growth of proteolytic bacteria.

The proportion of *cis* flavan-3-ols negatively affected the GP_{CH4}-R_{max} and CH_4 concentration and increased GP-$T2_{½}$ and GP_{CH4}-$T_{½}$, but the coefficients of determination (R^2) with GP_{CH4}-R_{max} and CH_4 concentration were only 0.34 and 0.27, respectively. Hatew *et al*. (2015) also found no correlation (neither positive nor negative) between proportion of *cis* and fermentation kinetics or fermentation-end products; however, they only evaluated CT from different sainfoin (*Onobrychis viciifolia*) accessions, where mDP, proportion of PD and proportion of *cis* values were correlated and thus these CT offered limited opportunities for assessing the effects of the different CT structural features. The present study, in contrast, evaluated a greater diversity of CT that originated from eight different plant sources. Based on the coefficient of determination (R^2), the CT content and prodelphinidin were the most

important factors among the CT features that affected fermentation kinetics and fermentation end-products with R^2 values ranging from 0.6 to 0.9.

CONCLUSIONS

Condensed tannins extracted from different plants had diverse effects on the extent and rate of gas and CH_4 production and also reduced CH_4 production and concentration. The CT content and proportion of prodelphinidin were the most important factors among the CT properties that affected fermentation kinetics and fermentation end-products. Thus, higher CT and prodelphinidin contents contributed most to lower methane production without negatively affecting the overall fermentation.

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Table 1. *Condensed tannin (CT) content, mean degree of polymerization (mDP), proportion of prodelphinidins and cis in extracts obtained from eight plant sources*

Plant source	CT content (g CT/100 g extract)	mDP number of flavan-3-ols	PD Proportion of total flavan-3-ols	*Cis* Proportion of total flavan-3-ols
Blackcurrant leaves	29.2 ± 1.21	5.44 ± 0.33	94.2 ± 0.14	12.9 ± 0.32
Goat willow leaves	29.9 ± 4.12	3.87 ± 0.025	3.3 ± 0.35	2.8 ± 0.07
Goat willow twigs	53.8 ± 2.08	4.31 ± 0.025	25.2 ± 0.17	53.0 ± 0.07
Pine bark	49.2 ± 0. 85	2.54 ± 0.011	35.9 ± 0.67	79.6 ± 0.28
Redcurrant leaves	24.5 ± 1.40	9.8 ± 0.15	94.0 ± 0.21	77.3 ± 0.07
Sainfoin plants	12.6 ± 0.60	5.53 ± 0.039	74.7 ± 0.78	80.2 ± 0.14
White clover flowers	33.7 ± 1.08	4.4 ± 0.07	99.20 ± 0.001	65.7 ± 0.07
Weeping willow catkins	25.2 ± 0.39	2.34 ± 0.035	55.9 ± 0.11	77.7± 0.21

Values are means ± standard deviation.

Table 2. *Total gas (GP) and CH_4 (GP_{CH4}) production, and the calculated CH_4 concentration from fermentations that contained different tannin types in the presence or absence of polyethylene glycol (+PEG or −PEG)*

	GP (ml/g OM)		GP_{CH4} (ml/g OM)		CH_4 (ml/100 ml GP)	
	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG
Grass silage	196	194	36	35	18	18
Blackcurrant leaves	169	234	21	37	13	16
Goat willow leaves	218	226	29	37	14	16
Goat willow twigs	184	206	31	35	17	17
Pine bark	188	225	22.7	41	12	18
Redcurrant leaves	169	233	12	39	7	17
Sainfoin plants	241	292	31	47	13	16
White clover flowers	207	222	24	38	13	16
Weeping willow catkins	177	240	26	34	13	15
S.E.M.	6.6		2.0		1.0	
P-values						
Tannin source (T)	<0.001		<0.001		<0.001	
PEG (P)	<0.001		<0.001		<0.001	
RUN	0.009		0.240		0.863	
T×P	<0.001		<0.001		<0.001	

GP, GP_{CH4} = volume of total gas and methane produced per gram OM initial substrate weight at time t = 72 h; CH_4 = methane concentration in total gas produced; +PEG, −PEG = with/without PEG addition; SEM = standard error of the mean.

Table 3. *Total gas (GP) and CH_4 (GP_{CH4}) production kinetic parameters ($T_{½}$, R_{max}) from fermentations containing different condensed tannin types with (+) and without (−) PEG were added*

	GP-T1½ (h)		GP-T2½ (h)		GP-R2$_{max}$ (ml/g OM/h)		GP_{CH4}-$T_{½}$ (h)		GP_{CH4}-R_{max} (ml/g OM/h)		R_M (%/h)	
	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG
Grass silage	2	6	12	10	11	10	12.7	12.1	1.9	2.0	7.15	8.36
Blackcurrant leaves	8	4	22	11	6	12	23.8	12.7	0.7	1.9	3.37	7.14
Goat willow leaves	2	3	15	12	9	13	18.0	12.8	1.2	1.9	5.63	7.18
Goat willow twigs	8	2	14	12	9	12	17.5	13.3	1.2	1.8	4.47	6.80
Pine bark	11	9	23	11	7	12	18.1	12.9	0.9	2.1	5.14	6.51
Redcurrant leaves	11	5	25	11	6	13	30.3	13.7	0.5	1.9	5.49	6.49
Sainfoin plants	9	2	23	12	7	14	32.0	17.7	0.8	1.8	2.64	5.04
White clover flowers	8	2	22	12	6	12	25.8	13.0	0.8	1.9	3.49	7.14
Weeping willow catkins	10	3	20	14	7	10	27.0	13.4	0.8	1.7	3.51	7.30
S.E.M.	2.7		1.3		1.0		1.7		0.10		0.007	
P-values												
Tannin source (T)	0.170		<0.001		0.406		<0.001		<0.001		0.004	
PEG (P)	0.006		<0.001		<0.001		<0.001		<0.001		<0.001	

RUN	0.272	0.895	0.048	0.008	0.016	0.632
T×P	0.238	<0.001	0.001	0.003	<0.001	0.289

$GP\text{-}T1_{½}$ = half time of asymptotic gas in first phase; $GP\text{-}T2_{½}$ = half time of asymptotic gas in second phase; $GP_{CH4}\text{-}T_{½}$ = half time of asymptotic methane production; $GP\text{-}R2_{max}$ = maximum rate of gas production in second phase; $GP_{CH4}\text{-}R_{max}$= maximum rate of methane production; +PEG, −PEG = with/without PEG addition; R_M = rate of substrate degradation; SEM = standard error of the mean.

Table 4. *Fermentation end-products produced in in vitro incubations containing different condensed tannin types with (+) and without (−) PEG*

	tVFA (mmol/g OM)		HAc (mol/100 mol tVFA)		HPr (mol/100 mol tVFA)		HBu (mol/100 mol tVFA)		HVa (mol/100 mol tVFA)		HBc (mol/100 mol tVFA)		NGR Ratio	
	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG
Grass silage	7.8	8.2	61.7	60.9	22.7	23.5	10.0	9.9	2.3	2.5	3.4	3.3	3.30	3.14
Blackcurrant leaves	8.2	9.4	56.2	60.9	34.4	24.1	5.9	9.8	1.7	2.3	1.8	3.0	1.93	3.08
Goat willow leaves	8.8	9.3	57.5	61.2	27.9	24.1	9.7	10.2	2.1	1.4	2.7	3.0	2.63	3.20
Goat willow twigs	7.1	7.9	61.2	61.4	26.1	23.9	8.3	9.7	1.3	1.7	3.1	3.2	2.87	3.17
Pine bark	8.2	8.7	58.8	61.5	29.5	22.9	7.2	10.1	1.9	2.2	2.5	3.2	2.37	3.28
Redcurrant leaves	8.2	9.5	55.7	59.9	35.0	24.7	5.2	9.8	2.3	2.5	1.7	3.0	1.85	2.97
Sainfoin plants	10.1	10.9	55.4	57.4	34.5	29.0	6.6	8.6	1.7	2.5	1.6	2.4	1.95	2.45
White clover flowers	8.9	8.8	56.1	61.4	32.4	24.2	7.0	9.5	2.1	1.9	2.3	3.0	2.08	3.10
Weeping willow catkins	8.7	9.4	56.1	61.2	31.5	23.3	7.4	10.1	2.5	2.2	2.4	3.1	2.20	3.20
S.E.M.	0.36		0.65		0.54		0.37		0.28		0.11		0.072	
P-values														
Tannin source (T)	<0.001		<0.001		<0.001		<0.001		0.027		<0.001		<0.001	

PEG (P)	<0.001	<0.001	<0.001	<0.001	0.292	<0.001	<0.001
RUN	0.285	0.535	<0.001	<0.001	0.469	0.390	<0.001
T×P	0.680	<0.001	<0.001	<0.001	0.294	<0.001	<0.001

+PEG, −PEG = with/without PEG addition; tVFA = total volatile fatty acid (tVFA = HAc + HPr + HBu+ HVa+ HBc); HAc = acetic acid; HPr = propionic acid; HBu = butyric acid; HVa = valeric acid; HBc = branched –chain volatile fatty acids (HBc = *iso*-butyric + *iso*-valeric acid); NGR= non–glucogenic to glucogenic VFA ratio [NGR = (HAc + 2 × HBu + 2 × *iso*-butyric + HVa + *iso*-valeric)/(HPr + HVa + *iso*-valeric)]; SEM = standard error of the mean.

Table 5. *Fermentation end-products produced in in vitro incubations containing different condensed tannin types with (+) and without (−) PEG*

	pH		NH_3 (mmol/g OM)	
	−PEG	+PEG	−PEG	+PEG
Grass silage	6.41	6.41	2.4	2.3
Blackcurrant leaves	6.37	6.35	1.9	2.5
Goat willow leaves	6.35	6.32	2.2	2.4
Goat willow twigs	6.46	6.44	1.8	2.0
Pine bark	6.36	6.36	2.0	2.5
Redcurrant leaves	6.39	6.30	1.6	2.6
Sainfoin plants	6.24	6.22	1.9	2.5
White clover flowers	6.31	6.41	2.1	2.7
Weeping willow catkins	6.35	6.38	2.2	2.8
S.E.M.	0.029		0.14	
P-values				
Tannin source (T)	<0.001		0.014	
PEG (P)	0.619		<0.001	
RUN	0.006		0.048	
T×P	0.220		0.035	

+PEG, −PEG = with/without PEG addition; NH_3 = ammonia; SEM = standard error of the mean.

Table 6. *The relations between structural features of condensed tannins and fermentation kinetics and fermentation end-products as estimated by multiple stepwise regression*

Fermentation kinetics and end-products	n	α	CT content (g CT/100 g extract)	mDP	PD Proportion of total flavan-3-ols	*Cis* Proportion of total flavan-3-ols	R^2
GP, ml/g OM	12	286.44	-1.72		-0.477		0.600
GP-$T2_{1/2}$, h	12	11.79		1.22		0.072	0.600
GP-$R2_{max}$, ml/g OM/h	12	8.34			-0.025		0.402
GP_{CH4}, ml/g OM	12	–	–	–	–	–	–
GP_{CH4}-$T_{1/2}$, h	12	27.61	-0.33			0.118	0.660
GP_{CH4}-R_{max}, ml/g OM/h	12	1.37				-0.008	0.348
CH_4, ml/100 ml GP	12	15.67				-0.047	0.273
tVFA, mmol/g OM	16	11.16	-0.06	-0.13			0.642
HAc, mol/100 mol tVFA	16	54.83	0.11		-0.020		0.711
HPr, mol/100 mol tVFA	16	31.19	-0.11		0.062		0.912
HBc, mol/100 mol tVFA	16	2.11	0.02		-0.008		0.794

NGR Ratio	16	2.29	0.01		-0.007	0.877
NH_3, mmol/ g OM	16	2.37		-0.08		0.462

n = number of observations; α = intercept; CT= condensed tannin content (g/100 g extract); mDP = mean degree of polymerization (i.e. the average number of flavan-3-ol monomers per polymer); PD = Proportion of prodelphinidin subunits gallocatechin (GC) and epigallocatehin (EGC) units); *Cis* = Proportion of flavan-3-ols with *cis* configuration. GP, GP_{CH4} =volume of total gas and methane produced per gram OM initial substrate weight ; CH=methane concentration in total gas produced; GP-$T2_{1/2}$ = half time of asymptotic gas in second phase; GP_{CH4}-$T_{½}$ = half time of asymptotic methane production; GP-$R2_{max}$ = maximum rate of gas in second phase; GP_{CH4}-R_{max} = maximum rate of methane production; tVFA = total volatile fatty acid (tVFA = HAc + HPr + HBu+ HVa+ HBc); HAc = acetic acid; HPr = propionic acid; HBc = branched–chain volatile fatty acids (HBc = *iso*-butyric + *iso*-valeric acid); NGR= non–glucogenic to glucogenic VFA ratio [NGR = (HAc + 2×HBu + 2×*iso*-butyric + HVa + *iso*-valeric)/(HPr + HVa + *iso*-valeric)]; NH_3 = ammonia; R^2 = coefficient of determination.

Fig. 1. Thiolytic degradation of condensed tannin polymers. Extension subunits are released as flavan-3-ol benzyl mercaptan (BM) adducts, terminal subunits are released as the free flavan-3-ols (Gea *et al.* 2011).

Fig. 2. Methane production profiles of grass silage with four condensed tannins extracts without polyethylene glycol (−PEG) (proportion of prodelphinidins, PD ≤ 60)

Fig. 3. Methane production profiles of grass silage with four condensed tannins extracts without polyethylene glycol (−PEG) (proportion of prodelphinidins, PD > 60)

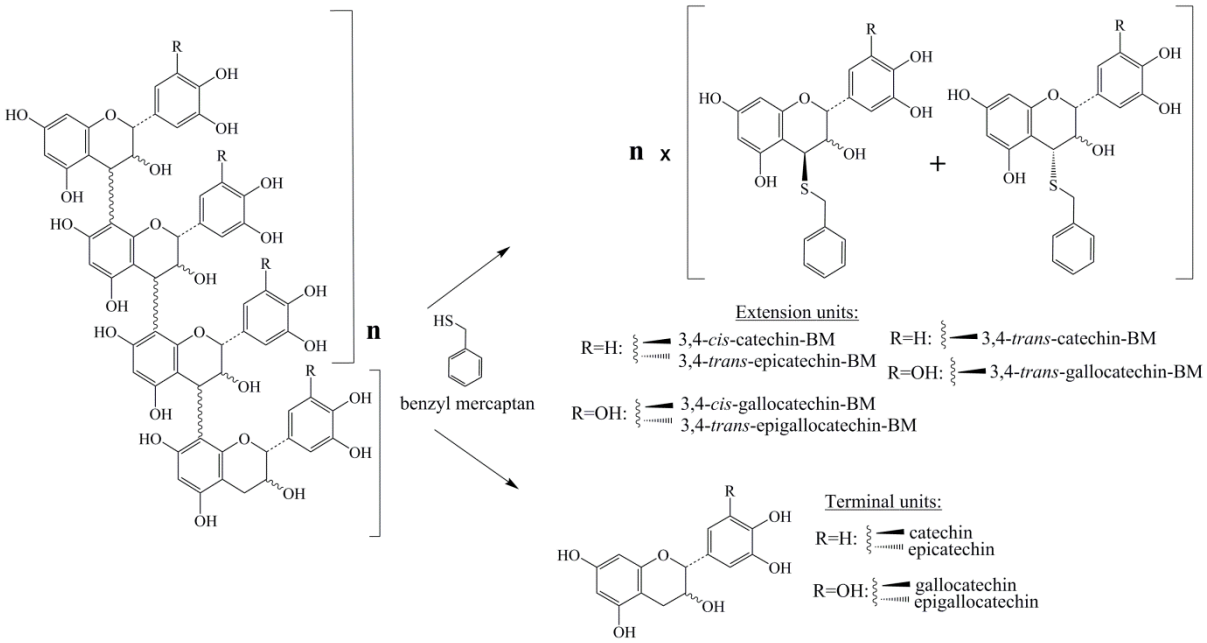
n
benzyl mercaptan
HS
n x
+
Extension units:
R=H: 3,4-cis-catechin-BM
3,4-trans-epicatechin-BM
R=H: 3,4-trans-catechin-BM
R=OH: 3,4-trans-gallocatechin-BM
R=OH: 3,4-cis-gallocatechin-BM
3,4-trans-epigallocatechin-BM
Terminal units:
R=H: catechin
epicatechin
R=OH: gallocatechin
epigallocatechin

Fig. 1.

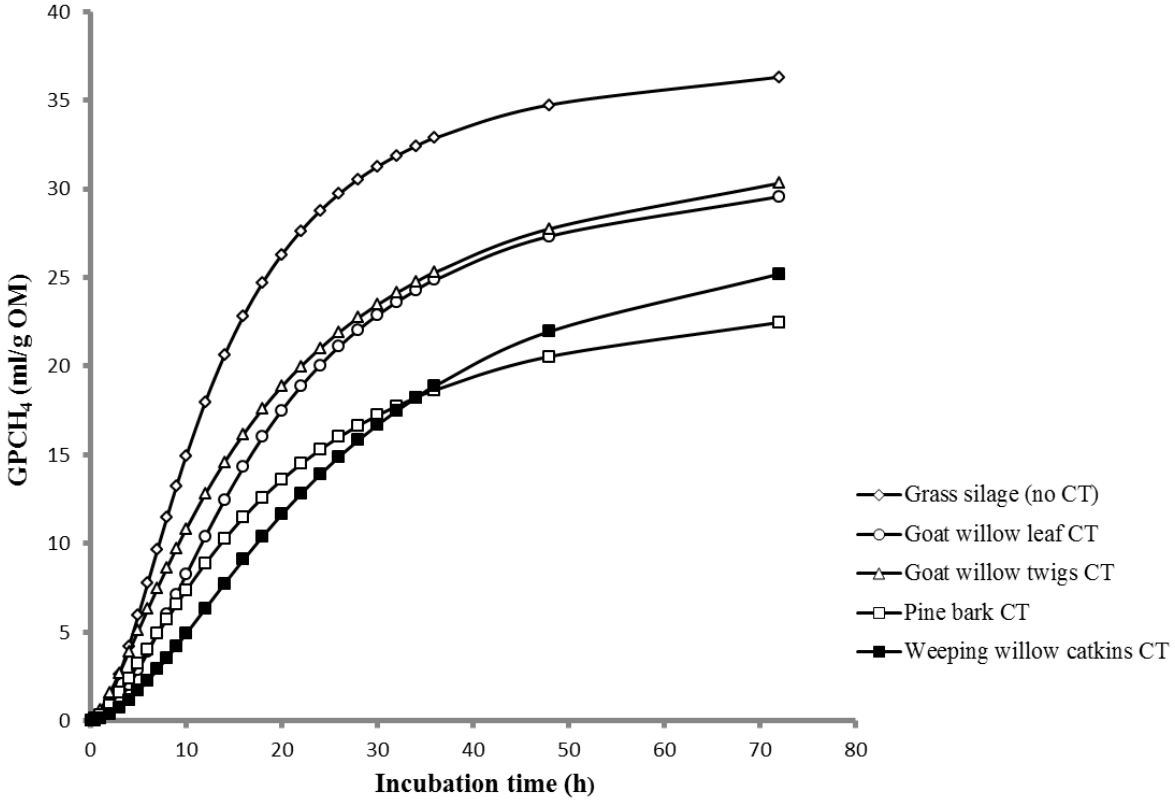
GPCH4 (ml/g OM)
Incubation time (h)
0
5
10
15
20
25
30
35
40
0
10
20
30
40
50
60
70
80
Grass silage (no CT)
Goat willow leaf CT
Goat willow twigs CT
Pine bark CT
Weeping willow catkins CT

Fig. 2.

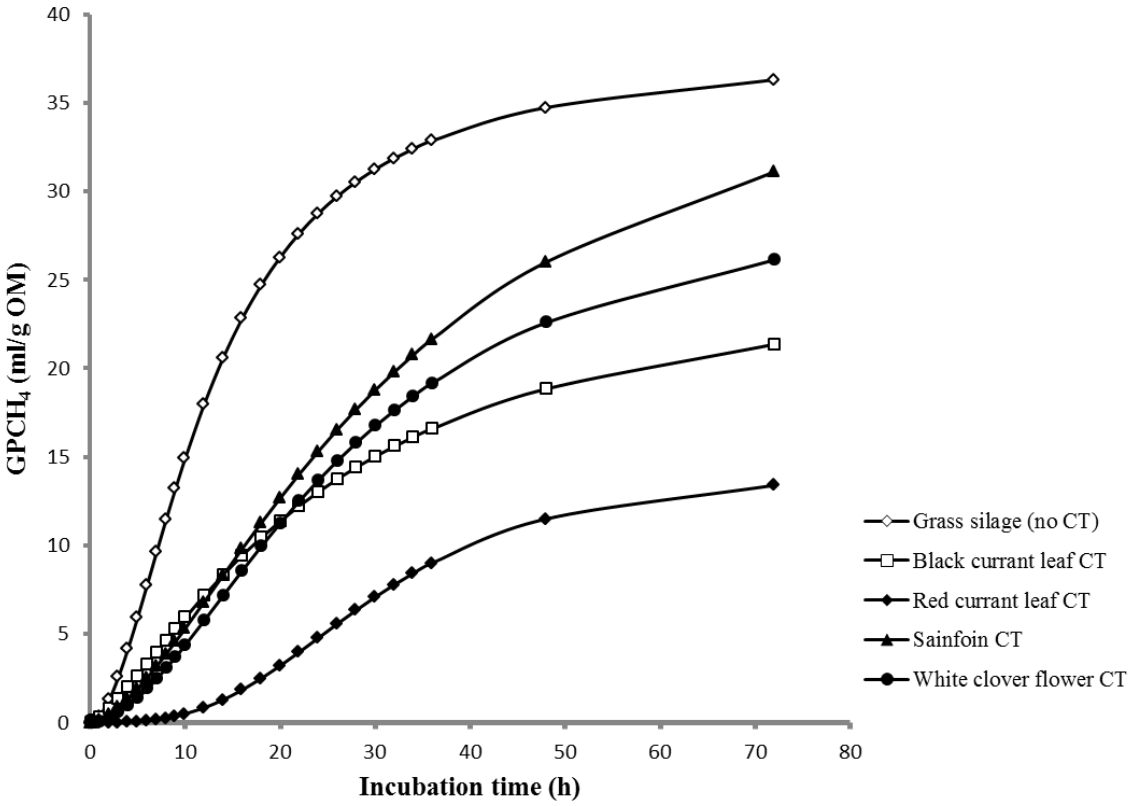
40
35
30
25
20
15
10
5
0
GPCH4 (ml/g OM)
0
10
20
30
40
50
60
70
80
Incubation time (h)
Grass silage (no CT)
Black currant leaf CT
Red currant leaf CT
Sainfoin CT
White clover flower CT

Fig. 3.