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Evaluating effects of tannins on extent and rate of *in vitro* gas and CH_4 production using an automated pressure evaluation system (APES)

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ABSTRACT

An *in vitro* study was conducted to investigate effects of tannins on extent and rate of gas and CH_4 production using an automated pressure evaluation system (APES). In this study three condensed tannins (CT; quebracho, grape seed, green tea tannins) and four hydrolysable tannins (HT; tara, valonea, myrabolan, chestnut tannins) were evaluated with lucerne as a control substrate. CT and HT were characterised by matrix assisted laser desorption ionisation–time of flight mass spectrometry (MALDI–TOF MS). Tannins were added to the substrate at an effective concentration of 100 g/kg, either with or without polyethylene glycol (PEG 6000), and incubated for 72 h in pooled buffered rumen liquid from four lactating dairy cows. After inoculation, fermentation bottles were immediately connected to the APES to measure total cumulative gas production (GP). During the incubation, 11 gas samples were collected from each bottle at 0, 1, 4, 7, 11, 15, 23, 30, 46, 52 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_{CH_4}). GP and GP_{CH_4} curves were fitted using a modified monophasic Michaelis–Menten model. Addition of quebracho reduced (P=0.002) GP, whilst the other tannins did not affect GP. Addition of PEG increased GP for quebracho (P=0.003), valonea (P=0.058) and grape seed tannins (P=0.071), suggesting that these tannins either inhibited, or tended to inhibit, fermentation. Addition of quebracho and grape seed tannins reduced ($P \leq 0.012$) the maximum rate of gas production, indicating that microbial activity was affected. Quebracho, valonea, myrabolan and grape seed decreased ($P \leq 0.003$) GP_{CH_4} and the maximum rate ($0.001 \leq P \leq 0.102$) of CH_4 production. Addition of chestnut, green tea and tara tannins did not affect total gas nor CH_4 production. Vvalonea and myrabolan tannins have the most promise at reducing CH_4 production as they had only a minor impact on gas production.

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Abbreviations: CP, crude protein; CT, condensed tannins; DM, dry matter; GP, gas production; GP-R_{max}, the maximum rate of gas production; GP_{CH_4}-R_{max}, the maximum rate of CH_4 production; GP-$T_{1/2}$, half-time of total gas production; GP_{CH_4}-$T_{1/2}$, half-time of CH_4 production; HT, hydrolysable tannins; MALDI–TOF MS, matrix assisted laser desorption ionisation–time of flight mass spectrometry; MW, molecular weight; OM, organic matter; VFA, volatile fatty acids.

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1. Introduction

Methane is produced by the archaea which reside in the rumen and utilize metabolic H_2 formed by rumen microbiota (Demeyer and Van Nevel, 1975; McAllister and Newbold, 2008). An increasing number of studies have shown that tannins reduce CH_4 emissions by ruminants (Kamra et al., 2006; Waghorn et al., 2002; Woodward et al., 2001). Tannins are a diverse group of secondary plant polyphenolic compounds which are generally categorized into two subgroups based on their chemical structures, being hydrolysable (HT) and condensed (CT) tannins. Hydrolysable tannins contain several gallic acid units (gallotannins) and a central polyol such as glucose. Galloyl groups may be oxidatively cross-linked to form more complex structures (ellagitannins; Mueller-Harvey, 2001). Condensed tannins (*syn.* proanthocyanidins) are a diverse group of polymeric flavanols with multiple phenolic groups that chelate metal ions and form complexes with macro-molecules such as proteins and polysaccharides (Schofield et al., 2001).

The huge diversity in tannin structures may explain their variable effects on methanogenesis and rumen function with observed responses depending on source, type and level of tannin (Mueller-Harvey, 2006; Patra et al., 2006; Waghorn and McNabb, 2003). Several studies indicate both HT and CT have anti-methanogenic activity, either by direct inhibition of methanogens or indirectly though inhibition of protozoa (Animut et al., 2008; Bhatta et al., 2009; Jayanegara et al., 2009). Observations of Field et al. (1989) and Tavendale et al. (2005) suggest that tannins of lower molecular weights (MW; oligomeric tannins) could be more effective against methanogens than their monomeric precursors or tannins of higher MW, possibly because oligomeric tannins have a higher H-bonding strength and the ability to penetrate bacteria and bind microbial enzymes. Bhatta et al. (2009) reported differences in anti-methanogic activity between HT and CT from different tannin sources at inclusion levels of 50 and 100 g/kg. Jayanegara et al. (2009) and Mueller-Harvey (2001) argued that colorimetric assays, which are widely used to quantify tannins, cannot provide accurate quantitative data (or useful structural information) nor are they indicative of the biological activity of tannins. In contrast, the matrix assisted laser desorption ionisation–time of flight mass spectrometry (MALDI–TOF MS) technique can provide detailed information on the presence of individual tannin components (Reed et al., 2005). Although detailed information on the chemical structure of tannins is becoming available (*e.g.*, Deaville et al., 2007; Frazier et al., 2010), their anti-methanogenic acitivity is largely uninvestigated.

Gas and CH_4 production can be measured by using batch culture systems (Bodas et al., 2008; Patra et al., 2006; Soliva et al., 2008). However, limited information is available on kinetics of CH_4 production in batch culture systems, and most studies have measured CH_4 after 24 h of incubation. Depending on type of substate, and type and concentration of tannins, changes in gas and CH_4 production may occur over the entire incubation period (Pellikaan et al., 2010). Consistent with this, Hariadi and Santoso (2010) reported shifts in the relative ranking of the impact of tanniniferous plants on CH_4 production after incubations of 6, 24 and 48 h.

Our objective was to investigate effects of a range of hydrolysable and condensed tannins on extent, rate and kinetics of *in vitro* gas and CH_4 production using an automated pressure evaluation system (APES). MALDI–TOF MS was used to characterise the tannin mixtures and to probe whether differences in chemical compositions could explain *in vitro* responses.

2. Materials and methods

2.1. MALDI–TOF MS analyses of tannin isolates

Three sources of condensed tannin extracts from *Camellia sinensis* leaves (green tea tannins), *Schinopsis lorentzii* wood (quebracho) and *Vitis vinifera* seeds (grape seed extract), and four sources of hydrolysable tannins from *Caesalpinia spinosa* fruits (tara tannins), *Castanea sativa* wood (chestnut), *Quercus aegilops* (valonea) and *Terminalia chebula* (myrabolan) were used. Quebracho tannins (Unitan Ordinary Solid, tannin content 33.6 g/100 g dry matter (DM) extract), myrabolan tannins (14.5 g/100 g DM) and valonea tannins (27.0 g/100 g DM) were donated by Forestal Ltd. (Reading, UK), grape seed tannins (Grapemax Extra Pure, 33.3 g/100 g DM) were donated by Burgundy Botanical Extracts (Reyssouze, France), chestnut tannins (Tannino CE, 91.6 g/100 g DM), tara tannins (Tannino T-80, 95.1 g/100 g DM) and green tea tannins (Tannino TVR, 84.9 g/100 g DM) were donated by Silva Group (San Michele Mondovi, CN, Italy). Tannin analyses were completed at the Chemistry & Biochemistry Laboratory of Reading University (Reading, UK). Tannins were isolated as previously described (Frazier et al., 2010) and characterised by MALDI–TOF MS. Quebracho, chestnut and tara tannins were analysed by MALDI–TOF MS following the procedure described for mimosa tannins (Frazier et al., 2010). The MALDI–TOF MS analysis of grape seed tannins was reported by Frazier et al. (2010). Green tea, valonea and myrabolan tannins (18 mg/ml) were dissolved in acetonitrile/methanol (2:1, v/v); the matrix, super-DHB (20 mg/ml) was prepared by mixing 2,5-dihydroxybenzoic acid (2,5-DHB) with 2-dihydroxy-5-methoxybenzoic acid (1:1, w/w) and dissolved in acetonitrile/demineralised water (7:3, v/v) containing 0.1% (v/v) trifluoroacetic acid (TFA), and the salt solution consisted of 0.5 M NaCl in demineralised water. The sample platten was loaded with the tannin solution (4 μl), then the matrix solution (4 μl) and finally the NaCl solution (2 μl) using the dried droplet method, and subjected to MALDI–TOF MS as described for grape seed tannins (Frazier et al., 2010).