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Divergence between purified hydrolysable and condensed tannin effects on methane emission, rumen fermentation and microbial population *in vitro*

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ABSTRACT

This study aimed to investigate the effects of purified hydrolysable (chestnut and sumach) and condensed tannins (mimosa and quebracho) on methane production, rumen fermentation and microbial population structure. The tannins were extracted and purified from their original plant sources, and were characterized for their protein precipitation capacity. The purified tannins were added into 380 mg hay:concentrate substrate (70:30 w/w) at three different concentrations (0.5, 0.75 and 1.0 mg/ml). *In vitro* incubation was carried out for 24 h at 39 °C in 100 ml calibrated glass syringe containing 30 ml of medium (10 ml rumen liquor and 20 ml double strength buffer). Parameters measured after the incubation were gas production, methane concentration, short chain fatty acids (SCFA: C_2, C_3, C_4, *iso*C_4, C_5, *iso*C_5, total SCFA and ratio of C_2/C_3), *in vitro* organic matter digestibility (IVOMD) and microbial population structure. The experiment was performed in three runs, represented by two incubation units per run. Results revealed that the protein precipitation capacity of chestnut and sumach tannins was greater than that of mimosa and quebracho tannins. An interaction between different tannins and doses existed with regard to methane concentration ($P \leq 0.05$). All the tannins decreased methane concentration either linearly or quadratically, but their magnitudes were different; the magnitude of decrease was greater for the hydrolysable tannins than the condensed ones, and correlated with their protein precipitation capacity. Increasing levels of all tannins decreased IVOMD by following a quadratic pattern ($P \leq 0.05$) and there was a tendency that the condensed tannins decreased IVOMD more than the hydrolysable tannins ($P \leq 0.1$). All the purified hydrolysable and condensed tannins decreased total methanogen population ($P \leq 0.05$) than that of control when added at 1.0 mg/ml; the decrease ranged from 22.3 to 36.7% from control. Additions of purified tannins at all levels generally decreased *Fibrobacter succinogenes* population ($P \leq 0.05$). Sumach tannins at all addition levels decreased the population of *Ruminococcus flavefaciens* ($P \leq 0.05$), and the magnitude of decrease was much greater than those of other tannins. It is concluded that hydrolysable tannins had a greater effect in reducing methane emission with less adverse effect on digestibility than those of condensed tannins.

Abbreviations: ANOVA, analysis of variance; BSA, bovine serum albumin; GHG, greenhouse gas; NDF, neutral detergent fibre; IVOMD, *in vitro* organic matter digestibility; PF, partitioning factor; qPCR, quantitative real-time polymerase chain reaction; SCFA, short-chain fatty acid.

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1. Introduction

Carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) are important greenhouse gases (GHG) in the atmosphere, and their global atmospheric concentrations have considerably increased especially during the last century (Monteny et al., 2006). Accumulation of these gases raises the earth's temperature and contributes to global warming (Rosenzweig et al., 2008). Agricultural sector is among the major sources of GHG in which about 0.20–0.35 of the global GHG emission is originated from the sector. Approximately 0.70 of methane emission arises from anthropogenic sources in which agriculture accounts for about two-third from the figure (EPA, 2010). Enteric fermentation from ruminants, globally, is estimated to account for between one-quarter and one fifth of anthropogenic methane emissions (Thorpe, 2009).

On the other hand, apart from their contribution to global warming, ruminants are capable of converting fibrous materials such as grasses and agricultural by-products into high quality foods such as milk and meat. Other non-food products from the animals could also be utilized for human benefit such as skin, wool, faeces, *etc.* With regard to the context of ruminant production, methane emission from enteric fermentation is not only associated with environmental problems, but it also represents considerable amount of energy losses from the animals. It has been estimated that around 0.06–0.10 of the gross energy of the ruminant diet is lost through methane (Immig, 1996). Therefore, developing feeding strategies to minimize methane emissions is desirable both for conserving the environment as well as for increasing the efficiency of energy utilization.

A number of nutritional attempts have been made to lower enteric methane emission from ruminants as reviewed by some authors (Takahashi et al., 2005; Beauchemin et al., 2008; Hristov et al., 2013). With a rapidly growing concern on food safety, natural compounds such as those originated from plants for mitigating the methane emissions are preferable over the synthetic ones (Makkar et al., 2007). Accordingly, essential oils, saponins and polyphenols or tannins are the prospective plant natural compounds for mitigating methane emissions (Benchaar and Greathead, 2011; Bodas et al., 2012; Jayanegara et al., 2014). With regard to tannins, previous studies have reported that feeding of tannin-containing forages to ruminants reduced methane emissions (*e.g.*, Puchala et al., 2005; Animut et al., 2008). However, in most of those studies, the reduction in methane was obscured by changes in forage composition and quality that may affect the emission as well. Thus, there is a considerable uncertainty about the effectiveness of tannin-containing forages to reduce enteric methane emissions from ruminants.

In the present study, other confounding components were omitted by extracting and purifying tannins from some plant sources, *i.e.* chestnut (*Castanea* sp.), sumach (*Rhus typhina*), mimosa (*Mimosa tenuiflora*) and quebracho (*Schinopsis balansae*). The first two plants were rich in hydrolysable tannins and the others were rich in condensed tannins; both tannin types may elicit different responses on rumen methanogenesis due to their distinct chemical structures in which such kind of study is limitedly investigated to date. Although tannins particularly hydrolysable tannins may cause toxicity responses to ruminants when consumed at excessive amounts, they provide beneficial effects when used at low to moderate concentrations (Reed, 1995). Condensed tannins are not usually toxic to ruminants since they are not absorbed (Reed, 1995), but they may bind parts of the nutrients irreversibly, making them unavailable. Also they can bind to gastrointestinal tract, causing adverse effects (Makkar et al., 2007); this is a drawback of condensed tannins as opposed to that of hydrolysable tannins. The working hypotheses were that the purified tannins would reduce methane production and that different forms of the tannins would elicit different response on methane reduction *in vitro*.

2. Materials and methods

2.1. Extraction and purification of tannins

Chestnut and sumach plant materials were collected from the botanical garden of University of Hohenheim, Stuttgart, Germany. Mimosa and quebracho materials were collected from Mongolia in conjunction with other plants used in our previous study (Jayanegara et al., 2009). Extraction of tannins from the plant materials (1 g each in 50 ml of aqueous methanol, 1:1 v/v) was done in an ultrasonic water bath at 135 W (Branson 3210, Connecticut, USA) for two successive periods of 25 min each, pooling both the supernatants, and centrifugation at 10,000 × *g* for 10 min at 4 °C (Makkar, 2003). The purification procedure of tannins was carried out by using a Sephadex LH-20 column, according to the modified method from Makkar and Becker (1994). Supernatant obtained from the extraction procedure was added with 1 mg/g of ascorbic acid to prevent oxidation of tannins. This supernatant was passed through a swollen slurry of Sephadex LH-20 prepared in aqueous methanol (1:1, v/v). The Sephadex LH-20 was washed slowly on a sintered glass funnel with approximately 1.5 l of aqueous methanol (1:1, v/v) under gravity to remove non-tannins from the Sephadex. The tannins remained on the Sephadex LH-20 and gave it a brown colour. Tannins were eluted using aqueous acetone (7:3, v/v). Acetone was removed under vacuum at about 30 °C and then the aqueous solution containing tannins was lyophilized. The tannins were characterized and used in the subsequent *in vitro* rumen fermentation experiment.

2.2. Characterization of purified tannins

Characterization of purified tannins from chestnut, sumach, mimosa and quebracho were done by butanol–HCl-method (Makkar, 2003) and by protein precipitation method using blue dye bound bovine serum albumin (BSA) (Asquith and Butler,

1985). Butanol–HCl-method was used to confirm that mimosa and quebracho tannins were condensed tannins and chestnut and sumach were not. Briefly, a volume of 0.5 ml purified tannins was diluted with aqueous acetone (7:3, v/v) in a 100 mm × 12 mm glass tube. Then to it was added 3 ml butanol–HCl reagent (butanol–HCl 95:5, v/v) and 0.1 ml ferric reagent (20 mg/g ferric ammonium sulphate in 2 N HCl). The tubes were covered with a glass marble each and transferred into a heating block adjusted at 97–100 °C for 60 min. After cooling, the absorbance of each tube was recorded at 550 nm and subtracted with a suitable blank (the absorbance of the unheated mixture).

Blue dye bound BSA method was used to characterize the ability of each tannin sources to precipitate protein. A volume of 2 ml blue BSA solution (containing 2 mg blue BSA/ml) was pipetted into a screw cap tube. Purified tannins in aqueous methanol (1:1, v/v) were added to make 1 ml of the sample, which was added to the blue dye. The tubes were kept in cold over night and then centrifuged for 15 min at 3000 × *g*. The supernatant was carefully poured off without disturbing the blue precipitate. Then 3.0 ml of isopropanol/SDS/TEA (200 ml/10 g/50 ml in 1 l of water) was added to the precipitate and vortexed vigorously to completely redissolve the precipitate. The absorbance was read at 590 nm and the amount of protein precipitated was calculated from the calibration curve prepared using BSA.

2.3. In vitro rumen fermentation experiment

A mixture of hay and concentrate (70:30, w/w) in the amount of 380 mg was incubated in 100 ml calibrated glass syringe containing 30 ml of medium (10 ml rumen liquor and 20 ml double strength buffer) by following the procedure of Makkar et al. (1995a), which is a modified protocol from the original method of Menke et al. (1979). The concentrate mixture (in g/kg) constituted of wheat bran (350), barley (470), soya (150) and rapeseed oil (10). The rumen fluid and particulate matter were collected before the morning feeding from two cattle fed on roughage and concentrate based diets, mixed, homogenized, strained and filtered through 100 μm nylon net. The glassware used was kept at approximately 39 °C and flushed with CO_2 before use. The lyophilized tannins were solubilized in distilled water at a concentration so that injection of up to 1 ml of the tannin solutions gave final concentrations of 0.5, 0.75 and 1.0 mg tannins/ml in the syringes. The tannin solutions were injected into the syringes from syringe nozzle shortly before dispensing the incubation medium. The 30 ml buffered medium containing rumen microbes was dispensed into the syringes and incubation done at 39 °C for 24 h. Parameters measured after 24 h incubation were gas production, methane production, short chain fatty acids (SCFA: C_2, C_3, C_4, *iso*C_4, C_5, *iso*C_5, total SCFA and ratio of C_2/C_3), *in vitro* organic matter digestibility (IVOMD) and microbial population structure. This experiment was performed in three runs, represented by two incubation units per run.

2.4. Post-fermentation analyses

After 24 h of incubation, total gas production was recorded from the calibrated scale on the syringe. Methane concentration was measured using an infrared (0–300 ml/l range) methane analyzer (Pronova Analysentechnik GmbH & Co., KG, Berlin, Germany) calibrated against 106 ml/l methane (Jayanegara et al., 2009). After measuring the total gas volume, the tubing of the syringe outlet was inserted into the inlet of the methane analyzer. Data on methane were presented as a proportion to the total gas produced.

The analysis of SCFA was performed according to Hoeltershinken et al. (1997). Aliquots of 1 ml volume were pipetted into prepared sampling tubes (1.5 ml Eppendorf cups) kept on ice to immediately stop the fermentation processes. To ensure the withdrawal of homogenous samples, contents were vigorously stirred before pipetting; wide bored tips were used to avoid plugging by feed particles. The samples then were centrifuged (30,000 × *g*, 10 min, 4 °C) and supernatant and pellet were carefully separated. Aliquot of 630 μl of the supernatant was transferred into a fresh vial and 70 μl of internal standard (methylvaleric acid) was added. These samples were kept at 4 °C over night to precipitate soluble proteins. They were centrifuged again (30,000 × *g*, 10 min, 4 °C) to remove the precipitate. Then 500 μl of the acidified, deproteinized supernatants were transferred into 1.5 ml glass vials (VWR/Merck 548-0003), sealed with serum caps (VWR/Merck 548-0413) for SCFA analysis. Samples were injected into a gas chromatograph (GC 14A, Shimadzu Corp., Kyoto, Japan) with a stainless steel column packed with 100 mg/g SP™-1000, 10 mg/g H_3PO_4, Chromosorb WAW (Suppelco Inc., Bellafonte, PA, USA). The gas chromatography programme detects the individual SCFA-peaks and converts the peak area to concentration (μmol/ml or mM). SCFA in test syringes were corrected for SCFA in the corresponding blanks to obtain net SCFA production. The corresponding blank consisted of buffered medium without the substrate but containing treatments at the similar levels.

For IVOMD determination, the contents of the syringes after 24 h of incubation were digested with neutral detergent solution and the undigested feed was recovered on crucibles, washed, dried, and ashed. The IVOMD of substrate after 24 h was calculated by subtracting this value from the organic matter incubated in the syringe (Blümmel et al., 1997).

2.5. Analysis of microbial population structure

The analysis of microbial population structure was performed according to a modified method of Denman and McSweeney (2006) as described by Goel et al. (2009). In brief, after 24 h of incubation, a uniform aliquot (1 ml) of the syringe content was taken in an Eppendorf tube for DNA isolation and preserved at −70 °C until analysis. Different microbial groups, *i.e.* methanogens, *Fibrobacter succinogenes*, *Ruminococcus flavefaciens* and total fungi were determined in the samples using SYBR green quantitative real-time polymerase chain reaction (qPCR) assay. The real-time PCR machine used was from

Table 1
Absorbance at 550 nm of purified tannins using the butanol–HCl-method and their protein precipitation capacities (PPC) on bovine serum albumin (BSA).

Tannin	Absorbance	PPC (mg BSA/mg sample)
Chestnut[a]	0.043	5.8
Sumach[a]	0.067	7.1
Mimosa[b]	0.421	4.6
Quebracho[b]	0.167	4.1

The assay was comprised of 250 ml of extract in aqueous acetone (7:3, v/v), 1.5 ml of butanol–HCl reagent and 50 ml of the iron reagent.
[a] 1 mg/ml in aqueous acetone (7:3, v/v).
[b] 0.2 mg/ml in aqueous acetone (7:3, v/v).

Bio-Rad (iQ5 Real-Time PCR detection system, Veenendaal, The Netherlands). The population of different microbial groups was determined as relative to the total bacterial populations. Relative population sizes of methanogens, *F. succinogenes*, *R. flavefaciens* and total fungi were expressed as a proportion of total rumen bacterial 16S rDNA at 24 h. The threshold cycle (ΔCt) values were calculated by subtracting the *Ct* value of target gene from the *Ct* value of reference gene (16S rDNA of total bacteria at 24 h). The relative expression of different groups has been calculated from the delta *Ct* values as $2^{-\Delta Ct}$. The shifts in microbial communities owing to purified tannins addition were determined by taking the microbial population in the control substrate as 100.

2.6. Observations and calculations

Net gas (ml) was calculated from differences of the gas in the test syringe and the corresponding blanks, and similarly net methane produced (ml) was determined by subtracting the methane in the blank syringe from that in the test syringe. Methane concentration was expressed as net methane produced in net gas (ml/l). The Partitioning Factor (PF), a measure of efficiency of microbial protein synthesis (Blümmel et al., 1997) was calculated as:

$$\frac{\text{IVOMD (mg)}}{\text{Net gas produced (ml)}}.$$

2.7. Statistical analysis

Data obtained from the experiment were analyzed by using analysis of variance (ANOVA) with the following statistical model:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \gamma_k + \varepsilon_{ijk}$$

where Y_{ijk} is the observed value, μ is the overall mean, α_i is the effect of different purified tannins (fixed effect), β_j is the effect of tannin doses (fixed effect), $(\alpha\beta)_{ij}$ is the interaction effect between different tannins and doses (fixed effect), γ_k is the block effect or replicate (fixed effect), and ε_{ijk} is the random residual error. In the case of insignificant interaction, the main factors, *i.e.* tannin sources or doses were further tested. Tannin sources were compared with orthogonal contrast tests and tannin doses were tested with polynomial terms, *i.e.* linear and quadratic. When the interaction was significant, each tannin source was tested for its polynomial term. For microbial population data, Dunnett's test was conducted to compare addition of purified tannins from the control group. Significance of an effect was stated at the probability level of $P \leq 0.05$ and a tendency was stated at $P \leq 0.1$. All the statistical analyses were performed by employing SAS software version 9.1 (SAS Institute Inc., Cary, NC, USA).

3. Results

Absorbance at 550 nm of condensed tannins using butanol–HCl–iron method is presented in Table 1. Mimosa and quebracho tannins are known to be condensed tannins, which is evident from the greater absorbance. The absorbance for chestnut and sumach tannins was negligible, confirming that tannins present in these plants are the hydrolysable ones. Protein precipitation capacity of the condensed tannins (mimosa and quebracho) was lower than that of the hydrolysable tannins (chestnut and sumach) as also presented in Table 1.

An interaction between different tannins and doses existed with regard to methane concentration ($P \leq 0.05$; Table 2). All the tannins decreased methane concentration either linearly (mimosa and quebracho) or quadratically (chestnut and sumach), but their magnitudes were different; the magnitude of decrease was greater for the hydrolysable tannins than the condensed ones, and correlated with their protein precipitation capacity. Among all the tannin sources, sumach tannins apparently were the best in mitigating the methane emissions. Different patterns were obtained when methane was expressed as decrease of methane per unit of digestible OM in which the interaction between tannin sources and doses turned to be insignificant. All purified tannins linearly decreased methane per unit of digestible OM ($P \leq 0.05$) but no difference between the hydrolysable and the condensed tannins. All the tannins decreased total gas production and IVOMD by

Table 2
Effect of addition of purified tannins on gas production, methane production and *in vitro* organic matter digestibility.

Treatment	Dose (mg/ml)	Gas (ml)	CH_4 (ml/l)	IVOMD (g/kg)	PF (mg/ml)	CH_4/digestible OM (ml/g)
Control	0	83.8	198	818	3.15	62.9
Chestnut	0.5	82.2	195	815	3.20	61.1
	0.75	80.7	192	802	3.21	59.8
	1.0	78.7	185	777	3.19	58.1
Sumach	0.5	79.3	194	815	3.31	58.6
	0.75	78.4	191	803	3.30	57.9
	1.0	74.7	183	789	3.41	53.8
Mimosa	0.5	80.1	194	790	3.18	61.2
	0.75	77.3	193	773	3.22	59.9
	1.0	74.2	192	750	3.26	58.9
Quebracho	0.5	78.6	195	794	3.25	59.6
	0.75	76.2	193	760	3.22	60.2
	1.0	72.6	191	747	3.32	57.6
SEM		0.454	1.19	3.09	0.011	0.500
P-value						
Treatment		0.624	0.014	<0.001	0.744	0.925
Treatment		0.624	0.014	<0.001	0.744	0.925
Control *vs* Tannin		0.598	na	0.010	0.314	0.644
HT *vs* CT		0.388	na	0.660	0.425	0.967
Chestnut *vs* Sumach		0.701	na	0.700	0.566	0.548
Mimosa *vs* Quebracho		0.590	na	0.681	0.687	0.894
Dose		<0.001	<0.001	<0.001	0.006	<0.001
Linear		<0.001	na	<0.001	<0.001	<0.001
Quadratic		0.201	na	0.026	0.942	0.942
Treatment*Dose		0.320	0.001	0.079	0.233	0.351
Chestnut*Linear		na	<0.001	na	na	na
Chestnut*Quadratic		na	0.008	na	na	na
Sumach*Linear		na	<0.001	na	na	na
Sumach*Quadratic		na	0.029	na	na	na
Mimosa*Linear		na	<0.001	na	na	na
Mimosa*Quadratic		na	0.568	na	na	na
Quebracho*Linear		na	<0.001	na	na	na
Quebracho*Quadratic		na	0.770	na	na	na

CT, condensed tannin; HT, hydrolysable tannin; IVOMD, *in vitro* organic matter digestibility; na, not applicable; PF, partitioning factor.

following a linear and a quadratic patterns, respectively ($P \leq 0.05$). No interactions between tannin sources and doses were observed for these parameters. Higher levels of all purified tannins linearly improved PF ($P \leq 0.05$).

Total SCFA production showed an interaction effect between tannin sources and doses ($P \leq 0.05$; Table 3). Increasing levels of tannins generally decreased total SCFA ($P \leq 0.05$) except for sumach tannins. The proportion of acetate decreased with increasing levels of tannin additions while the proportion of propionate increased, and as a consequence, the ratio of acetate to propionate also decreased. Such patterns were at different magnitudes among the tannins as shown by the significant interaction effects for these parameters ($P \leq 0.05$).

All the purified hydrolysable and condensed tannins decreased total methanogen population than that of control when added at 1.0 mg/ml (Table 4); the decrease ranged from 22.3 to 36.7% from control. However, there was no difference for the lower levels of purified tannin additions except for mimosa tannins at 0.75 mg/ml. Additions of purified tannins at all levels in general decreased *Fibrobacter succinogenes* population, except those of sumach tannins at 0.75 and 1.0 mg/ml which were indifferent from control. Sumach tannins at all addition levels decreased the population of *Ruminococcus flavefaciens*, and the magnitude of decrease was much higher than those of other tannins. Decrease of anaerobic fungi population was observed by adding all purified tannins especially at higher levels.

4. Discussion

In this study, all the purified tannins were able to decrease ruminal methane emissions *in vitro*, and confirmed such an effect of tannins (Jayanegara et al., 2012). Concerning the mechanisms in which tannins reduce methane emissions from ruminants, Tavendale et al. (2005) have proposed two mechanisms, *i.e.* (1) through reduction in fibre digestion, which decreases H_2 production, and (2) through inhibition of the growth of methanogens. Both the mechanisms are reflected in the present study; tannins reduce the population of fibre-degrading bacteria (*i.e. F. succinogenes* and *R. flavefaciens*) and anaerobic fungi which primarily degrade the fibre components as compared to the control group. This is complemented with the reduction in total SCFA concentration, the main by-product of plant carbohydrate (including fibre) digestion in the rumen (van Houtert, 1993). Tannins also decrease the population of methanogens, the principal actor of methanogenesis in the rumen. The magnitudes of these microbial population reductions are generally higher at increasing levels of tannin additions. Furthermore, although this was not measured in the present study, tannins are known to decrease protozoal number (Bhatta

Table 3
Effect of addition of purified tannins on total (in mmol/l) and individual short-chain fatty acid (SCFA) profiles (as molar percentage to total SCFA).

Treatment	Dose (mg/ml)	Total SCFA	C_2	C_3	C_4	*iso*C_4	C_5	*iso*C_5	C_2/C_3
Control	0	52.2	63.4	19.2	14.8	0.67	1.36	0.52	3.30
Chestnut	0.5	49.7	62.9	19.2	15.2	0.66	1.39	0.58	3.28
	0.75	49.2	63.0	19.3	15.2	0.64	1.37	0.57	3.27
	1.0	47.9	61.3	21.1	14.6	0.74	1.46	0.83	2.91
Sumach	0.5	51.6	63.5	18.8	15.2	0.61	1.28	0.54	3.38
	0.75	51.7	62.7	19.5	15.2	0.66	1.32	0.65	3.23
	1.0	50.4	62.2	20.5	14.5	0.67	1.42	0.76	3.04
Mimosa	0.5	46.6	63.6	19.3	15.2	0.57	1.18	0.38	3.30
	0.75	46.6	63.5	19.6	14.8	0.55	1.20	0.34	3.24
	1.0	45.8	62.7	20.0	15.0	0.62	1.19	0.50	3.13
Quebracho	0.5	51.2	63.8	18.9	14.9	0.62	1.27	0.53	3.38
	0.75	47.3	62.5	20.0	15.1	0.61	1.27	0.49	3.13
	1.0	43.8	61.1	21.2	15.3	0.67	1.19	0.58	2.89
SEM		0.531	0.119	0.132	0.103	0.018	0.019	0.041	0.024
P-value									
Treatment		0.006	<0.001	<0.001	0.010	0.312	0.027	0.184	<0.001
Control *vs* Tannin		na	na	na	na	0.810	na	0.524	na
HT *vs* CT		na	na	na	na	0.300	na	0.572	na
Chestnut *vs* Sumach		na	na	na	na	0.313	na	0.192	na
Mimosa *vs* Quebracho		na	na	na	na	0.611	na	0.233	na
Dose		<0.001	<0.001	<0.001	0.076	0.060	0.185	<0.001	<0.001
Linear		na	na	na	na	0.685	na	0.018	na
Quadratic		na	na	na	na	0.024	na	0.029	na
Treatment*Dose		0.001	0.002	0.011	0.013	0.958	0.015	0.352	0.003
Chestnut*Linear		0.004	<0.001	0.003	0.977	na	0.116	na	0.001
Chestnut*Quadratic		0.861	0.012	<0.001	0.001	na	0.342	na	<0.001
Sumach*Linear		0.238	0.004	0.020	0.731	na	0.534	na	0.009
Sumach*Quadratic		0.637	0.043	0.003	0.017	na	0.002	na	0.002
Mimosa*Linear		<0.001	0.132	0.003	0.335	na	0.001	na	0.008
Mimosa*Quadratic		0.073	0.032	0.060	0.658	na	0.086	na	0.042
Quebracho*Linear		<0.001	<0.001	<0.001	0.022	na	0.001	na	<0.001
Quebracho*Quadratic		0.053	<0.001	<0.001	0.422	na	0.767	na	<0.001

C_2, acetate; C_3, propionate; C_4, butyrate; C_5, valerate; CT, condensed tannin; HT, hydrolysable tannin; na, not applicable.

Table 4
Shifts in rumen microbial population on the addition of purified tannins at different levels.

Treatment	Population (% of control)			
	Methanogens	*Fibrobacter succinogenes*	*Ruminococcus flavefaciens*	Anaerobic fungi
Chestnut				
0.5 mg/ml	104 ± 35.1	44.4 ± 16.9*	91.8 ± 6.22	27.4 ± 2.39*
0.75 mg/ml	89.3 ± 11.3	16.6 ± 1.60*	67.9 ± 6.11*	9.6 ± 0.94*
1.0 mg/ml	63.3 ± 6.89*	14.2 ± 1.30*	67.4 ± 21.0*	7.3 ± 2.28*
Sumach				
0.5 mg/ml	106 ± 17.4	75.5 ± 8.41*	49.6 ± 5.53*	40.4 ± 6.11*
0.75 mg/ml	85.5 ± 12.0	87.7 ± 16.8	43.8 ± 3.34*	26.8 ± 1.51*
1.0 mg/ml	77.7 ± 7.35*	108 ± 11.5	44.4 ± 4.43*	23.3 ± 4.88*
Mimosa				
0.5 mg/ml	125 ± 26.7	57.8 ± 13.1*	131 ± 17.4	43.5 ± 8.82*
0.75 mg/ml	69.8 ± 6.61*	29.6 ± 7.17*	92.2 ± 20.2	49.2 ± 3.14*
1.0 mg/ml	71.4 ± 9.47*	25.9 ± 6.35*	90.0 ± 11.5	20.0 ± 2.80*
Quebracho				
0.5 mg/ml	134 ± 73.1	55.5 ± 8.89*	88.9 ± 14.9	94.5 ± 15.1
0.75 mg/ml	86.1 ± 17.6	40.2 ± 7.24*	91.1 ± 16.8	74.9 ± 15.4*
1.0 mg/ml	75.9 ± 4.17*	37.0 ± 2.49*	75.5 ± 15.4*	19.7 ± 3.81*

* Significantly different in the treatment from the control group at $P < 0.05$.

et al., 2009) and the decrease in methane emission could be mediated through decrease in protozoal number; both microbial species have symbiotic relationship in the rumen (Morgavi et al., 2010). Lower proportion of acetate and simultaneous higher proportion of propionate (thus lower acetate to propionate ratio) also contributes to the decrease of methane at increasing levels of tannin additions. Acetate formation from pyruvate in the rumen produces metabolic hydrogen, which is a main substrate of methanogenesis; while on the contrary, propionate formation from pyruvate consumes hydrogen (Moss et al., 2000).

Comparing between different types of tannins, chestnut and sumach tannins, which are hydrolysable tannins, had greater ability to decrease methane concentration than the purified mimosa and quebracho tannins, which are condensed tannins. This response was correlated to their BSA protein precipitation capacity, *i.e.* the greater ability of a tannin to precipitate BSA, the greater its ability to reduce ruminal methane emission. Protein precipitation capacity is a reflection of their affinity to bind proteins on microbial cell membranes and/or enzymes (McSweeney et al., 2001) thus represents the biological activity of tannins. Apparently the measure of biological activity of tannins is more accurate to be used for screening of methane mitigating properties of tannins from various plant sources rather than their concentrations. This was similar with our previous finding (Jayanegara et al., 2009) that observed a higher correlation coefficient between tannin biological activity (measured as percent increase of gas production on addition of polyethylene glycol, a tannin binding agent) and methane production reduction potential rather than that of the total tannin concentration.

Such differences between hydrolysable and condensed tannin effects on methane concentration could not be clearly seen from the different in methanogen populations. Although the methanogen populations are similar, hydrolysable and condensed tannins may affect the activity of methanogen differently which then may explain the effects. Another reason could be that the tannins could have affected the distribution of various methanogenic species (keeping the total methanogens unaffected), having different activities. The discrepancy between methane emission, methanogen population and methanogen activity may exist. In the study of Guo et al. (2008), for instance, it was revealed that addition of 0.4 mg/ml tea saponin into basal substrate decreased methane production by 8% than that of control but no change in the methanogen population was observed. In fact, the decrease of methane then was due to the reduction in methanogen activity as shown by the decrease of *mcrA* gene expression by 76% as compared to control.

The condensed tannins tended to decrease IVOMD and decreased total SCFA production much greater than the hydrolysable tannins especially at higher application levels. Similar results were reported by Makkar et al. (1995b) who demonstrated that quebracho tannin (a condensed tannin) had a greater effect in decreasing rate of digestion and *in vitro* true dry matter digestibility compared to tannic acid (a hydrolysable tannin). Condensed tannins do not get degraded in the rumen environment (Makkar et al., 1995b; McSweeney et al., 2001) and therefore bind the nutrients stronger than that of hydrolysable tannins. On the other hand, hydrolysable tannins are easily degraded (Field and Lettinga, 1987) and may lose their ability to interact with other compounds. Despite the fact, the derivates of hydrolysable tannins degradation such as gallic acid, gallotannic acid and pyrogallol may still be highly toxic to methanogenic activity (Field and Lettinga, 1987; Scalbert, 1991) as confirmed by the lower total methanogen population than that of the control group. However, the mechanisms involved are poorly understood. A plausible mechanism for the toxicity may involve the "tanning" of proteins (such as enzymes) located at accessible sites in/on the methanogens (Field and Lettinga, 1987; McSweeney et al., 2001). Apparently the degradation products of hydrolysable tannins are also toxic to fibrolytic microorganisms as shown by *R. flavefaciens* and anaerobic fungi populations; even both these types of microorganisms seem to be more sensitive to hydrolysable tannins in comparison to condensed tannins. Thus from the data it is evident that hydrolysable tannins were more effective in decreasing methane emissions than condensed tannins, while at the same time hydrolysable tannins had lesser adverse effect on degradation of nutrients by the rumen microbes.

Regarding the role of condensed tannins in decreasing methane production through a depression in nutrient digestion especially that of fibre through formation of hydrogen bonds between free phenolic functional groups and the fibre (Silanikove et al., 2001), the mechanism proposed above was supported by SCFA profiles. The addition of mimosa and quebracho tannins decreased acetate, a major product of fibre fermentation to a greater extent than by hydrolysable tannins. However, such argument could not be clearly seen from the microbial population parameters, *i.e.* those of the fibre-degrading bacteria and the anaerobic fungi since the data were varied among the hydrolysable and condensed tannins. This result is in agreement with a study conducted by Carulla et al. (2005) who reported that addition of condensed tannin extract from *Acacia mearnsii* lowered methane production, and part of the reduction was due to a reduction in NDF (neutral detergent fibre) digestibility. Therefore the authors suggested that to minimize negative effects of condensed tannins on fibre digestibility, lower level of supplementations could be an alternative strategy. But on the other hand, the methane suppression effect might be less as well.

Despite the fact that hydrolysable tannins had a greater effect in reducing methane and less adverse effect in the term of digestibility, the methane reduction by tannins so far has been mainly attributed to condensed tannins. The reason might be the lower risk of toxicity of condensed tannins than hydrolysable tannins (Beauchemin et al., 2008). Ruminants consuming forage plants containing high levels of hydrolysable tannins such as *Clidemia hirta*, *Terminalia oblongata* (Murdiati et al., 1990, 1991) or oak species (Reed, 1995) showed toxicity symptoms and reduced their production levels. Although hydrolysable tannins are degraded by certain microbial species in the rumen (McSweeney et al., 2001), the toxicity appears to be due to absorption of degraded products of hydrolysable tannins and greater loads of phenols in the blood stream, which is beyond the capability of liver to detoxify them (Makkar et al., 2007). Thus a strategy to avoid the toxicity of hydrolysable tannins while obtaining their potential beneficial effects on ruminants is to administer them at low to moderate levels (Reed, 1995). A number of experiments have shown that feeding of hydrolysable tannins at these levels (typically $\leq$20 g/kg DM) did not cause any detrimental effects on production parameters (Sliwinski et al., 2002; Krueger et al., 2010; Toral et al., 2011). Nevertheless, chestnut and sumach tannins should be investigated *in vivo* for their potential to decrease methane production and to elicit toxicity, if any. Present research should be shifted from evaluating only the effect of condensed tannins in reducing methane emissions towards some potent hydrolysable tannins since the nature and the toxicity of hydrolysable tannins are quite diverse.

5. Conclusion

Hydrolysable tannins had greater ability to decrease methane concentration than condensed tannins. This response was correlated to their BSA protein precipitation capacity, *i.e.* the greater ability of a tannin to precipitate BSA, the greater its ability to reduce ruminal methane emissions. The condensed tannins appear to decrease methane more through a reduction in fibre digestion, while hydrolysable tannins act more through inhibition of the growth and/or activity of methanogens.

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