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Addition of Purified Tannin Sources and Polyethylene Glycol Treatment on Methane Emission and Rumen Fermentation *in Vitro*

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ABSTRACT

The objectives of this experiment were (1) to observe the effects of purified tannins and polyethylene glycol (PEG) on *in vitro* rumen fermentation and methanogenesis, and (2) to assess the accuracy of volatile fatty acid (VFA) profiles in predicting methane emission. Hydrolysable and condensed tannins were extracted and purified from chestnut, sumach, mimosa and quebracho. Hay and concentrate mixture (70:30 w/w, 380 mg) was incubated in Hohenheim glass syringe containing 10 mL rumen liquor + 20 mL buffer. The purified tannins were injected into the syringes at a concentration of 1.0 mg/mL each, either without or with PEG 6,000 addition in three replicates. Results revealed that a decrease of methane emission (20%-27%) was observed when the purified tannins were added into basal diet as compared to control (P<0.05), and PEG addition increased methane emission (P<0.05). All purified tannins decreased total gas and total VFA production (P<0.05). The H_2 recovery of the treatments ranged from 86.7% to 95.3%. Estimation of methane emission by using VFA profiles revealed an accurate result with a very low root mean square prediction error (1.75%). It is concluded that tannins mitigate methane emission while PEG neutralize such effect, and VFA profiles are accurate predictors of the emission.

Key words: tannin, polyethylene glycol, methane, rumen, stoichiometry

ABSTRAK

Penelitian ini bertujuan (1) untuk mengevaluasi pengaruh tanin murni dan polietilen glikol (PEG) terhadap fermentasi rumen dan metanogenesis secara *in vitro*, dan (2) untuk menganalisis akurasi profil *volatile fatty acid* (VFA) dalam memprediksi emisi metana. Tanin terhidrolisis dan terkondensasi diekstrak dan dimurnikan dari *chestnut, sumach, mimosa* dan *quebracho*. Rumput hay dan konsentrat (70:30 w/w, 380 mg) diinkubasi di dalam *syringe* Hohenheim bersama dengan 10 ml cairan rumen + 20 ml larutan *buffer*. Tanin dimasukkan ke dalam *syringe* pada konsentrasi 1,0 mg/ml dengan atau tanpa penambahan PEG dalam tiga ulangan. Hasil menunjukkan bahwa terjadi penurunan emisi metana (20%-27%) ketika tanin murni ditambahkan ke dalam ransum basal dibandingkan dengan kontrol (P<0,05), dan penambahan PEG meningkatkan emisi gas metana (P<0,05). Semua tanin murni menurunkan produksi gas total dan VFA total (P<0,05). *Recovery* H_2 berkisar 86,7%-95,3%. Estimasi emisi metana secara stoikiometri dari profil VFA menunjukkan hasil yang akurat dengan nilai *root mean square prediction error* yang sangat rendah (1,75%). Disimpulkan bahwa tanin dapat menekan emisi metana sementara PEG menetralkan efek tersebut, dan profil VFA merupakan prediktor yang akurat terhadap emisi metana.

Kata kunci: tanin, polietilen glikol, metana, rumen, stoikiometri

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INTRODUCTION

Ruminants have the ability to convert roughages such as grasses and agricultural by-products into high quality foods for human such as milk and meat. This is possible due to the action of microbes residing in the rumen in degrading such fibrous materials into their monomers which are later on being utilized for various anabolic reactions. However, ruminants also produce methane (CH_4), another main greenhouse gas (GHG) that contributes to global warming after carbon dioxide (CO_2) (Monteny *et al.*, 2006). The presence of methanogenic archaea within rumen microflora enables formation of methane from CO_2 and H_2 or methanogenesis to occur (Morgavi *et al.*, 2010). Such methane emission from enteric fermentation, apart from its association with environmental problem, is also representing a certain amount of energy loss from the animals (Cottle *et al.*, 2011). It is therefore essential to develop various feeding strategies that simultaneously mitigate methane emission and increase the efficiency of energy utilization.

Among a number of nutritional attempts for mitigating enteric methane emission, plant secondary compounds such as tannins, saponins and essential oils are considered as the promising substances due to their abundant in nature (Benchaar & Greathead, 2011; Jayanegara *et al.*, 2010; Jayanegara *et al.*, 2012). With regard to tannins, previous studies have reported that tannin-containing plants or tannin extracts decreased methane emissions both in *in vitro* and *in vivo* experiments (Animut *et al.*, 2008; Jayanegara *et al.*, 2009a; Jayanegara *et al.*, 2012). Some purified tannins had also been tested regarding their efficacy in mitigating methane, but the magnitude of methane decrease was low due to the low concentration applied, i.e. 0.5 mg/mL (Jayanegara *et al.*, 2009b). In the present experiment, the concentrations of purified tannins from chestnut (*Castanea* sp.), sumach (*Rhus typhina*), mimosa (*Mimosa tenuiflora*) and quebracho (*Schinopsis balansae*) were doubled than that of Jayanegara *et al.* (2009b); the first two sources were hydrolysable tannins and the others were condensed tannins. Further, polyethylene glycol (PEG), a tannin binding agent, was added in conjunction with the purified tannins to observe whether such addition would influence methane emission or not.

On the other hand, measurement of methane emission from ruminants *in vivo* requires relatively sophisticated and expensive equipment. Therefore *in vitro* methods have been developed to measure the gas emission. Standard method for such measurement is by using infrared methane analyser (Jayanegara *et al.*, 2009a, 2009c) or gas chromatograph (Jayanegara *et al.*, 2011). Unfortunately, there are limited animal nutrition laboratories that have such devices in Indonesia. Indirect estimation was attempted in the country to quantify methane emission such as by using CO_2 entrapment technique (Yogianto *et al.*, 2014; Yuliana *et al.*, 2014) or by employing a stoichiometric equation from volatile fatty acid (VFA) profiles (Jayanegara *et al.*, 2013). Regarding the latter approach, root mean square prediction error (RMSPE) of estimated methane value from VFA against value obtained from direct measurement was still high, indicating that there was a substantial bias in the methane values between the two methods (Jayanegara *et al.*, 2013). Therefore, as a second objective, this study was aimed at confirming whether VFA profiles could accurately predict actual methane emission or not.

MATERIALS AND METHODS

Tannin Extraction and Purification from Plant Materials

Plant materials of chestnut and sumach (leaves of the plants) were obtained from University of Hohenheim botanical garden, Stuttgart, Germany, whereas plant materials of mimosa and quebracho (barks of the plants) were obtained from Mongolia (Jayanegara *et al.*, 2009a). Extraction of tannins was performed in an ultrasonic water bath at 135 W (Branson 3210, Connecticut, USA) by following the procedure of Makkar (2003). An amount of 1 g of each tannin source was mixed with 50 mL of 50% aqueous methanol and put in the water bath for 25 min at room temperature. The extraction was done twice of each sample, both supernatants were pooled and centrifuged at 10,000 g for 10 min at 4 °C.

After the tannins were extracted, they were purified by using a Sephadex LH-20 column as outlined by Makkar & Becker (1994). Briefly, supernatant obtained from the extraction procedure was added with 0.1% of ascorbic acid to avoid tannin oxidation. The supernatant was passed through swollen slurry of Sephadex LH-20 prepared in 50% aqueous methanol and washed five times with the respective solvent. Non-tannins were removed out through this procedure. The remaining tannins were then eluted using 70% aqueous acetone. Acetone was removed under vacuum at about 30 °C and subsequently the aqueous solution containing tannins was lyophilized. The tannins were further used in the following *in vitro* rumen fermentation experiment.

In Vitro Incubation

An amount of 380 mg hay and concentrate mixture (70:30 w/w) was incubated in 100 mL Hohenheim glass syringe containing 10 mL rumen liquor + 20 mL buffer following the procedure of Makkar *et al.* (1995). The hay and concentrate used were similar to those used in Jayanegara *et al*. (2009b). Rumen fluid (including solid material) was collected before morning feeding from two non-lactating dairy cows fed on roughage and concentrate based diets. The rumen fluid was mixed, strained and filtered by passing through a 100 μm nylon net. The lyophilized tannins were solubilized in distilled water (30 mg tannins/mL distilled water) and subsequently an amount of 1 mL tannin solutions were injected into the syringes to make up the tannin concentration of 1.0 mg/mL fermentation fluid. Additionally, polyethylene glycol (PEG) with the molecular weight of 6,000 kDa, a tannin binding agent (Jayanegara & Sofyan, 2008), was added in the amount of 750 mg in each syringe that contained 30 mL of fermentation fluid. The following treatments were tested:

T1 : Control

T2 : T1 + chestnut tannins
T3 : T1 + chestnut tannins + PEG
T4 : T1 + sumach tannins
T5 : T1 + sumach tannins + PEG
T6 : T1 + mimosa tannins
T7 : T1 + mimosa tannins + PEG
T8 : T1 + quebracho tannins
T9 : T1 + quebracho tannins + PEG

Allocation of treatments into experimental units was based on a completely randomized design in which each syringe represented as the experimental unit. *In vitro* incubation was carried out at 39 °C for 24 h. Variables measured after 24 h incubation were total gas production, methane emission, protozoal number and volatile fatty acid (VFA) profile, i.e. acetate (C_2), propionate (C_3), butyrate (C_4), *iso*butyrate (*iso*C_4), valerate (C_5) and *iso*valerate (*iso*C_5). Total VFA was obtained by summing up the all individual VFA concentrations. This experiment was performed in three replicates, represented by one Hohenheim glass syringe per replicate.

Variable Measurements

Total gas production was recorded from the scale on the syringe after 24 h of incubation. Methane emission was measured by using an infrared methane analyzer (Pronova Analysentechnik GmbH & Co. KG, Berlin, Germany) as previously conducted by (Jayanegara *et al.*, 2009a). Apart from being directly measured, methane emission was also estimated stoichiometrically from VFA profile by following Moss *et al*. (2000) equation:

$CH_4 = 0.45\ C_2 - 0.275\ C_3 + 0.40\ C_4$ (mmol)

Hydrogen recovery was calculated from the ratio between hydrogen utilized and hydrogen produced as follow:

H_2 produced = $2\ C_2 + C_3 + 4\ C_4$ (mmol)
H_2 utilized = $4\ CH_4 + 2\ C_3 + 2\ C_4$ (mmol)
H_2 recovery = H_2 utilized/H_2 produced × 100%

Since the amount of methane emission is influenced by hydrogen recovery (Jayanegara *et al.*, 2013), adjustment was made by considering a hydrogen recovery of 90% (Moss *et al.*, 2000).

For analysis of VFA profile, 1 mL of fermentation fluid was aliquoted into 1.5 mL Eppendorf cup and immediately kept on ice to stop the fermentation process. The sample was subsequently centrifuged at 30,000 g, 10 min and 4 °C. After centrifugation, supernatant and pellet were carefully separated. An amount of 630 µL of the supernatant was transferred into a fresh vial and 70 µL of internal standard, i.e. methylvaleric acid was added. All samples were kept at 4 °C over night to precipitate soluble proteins and then were centrifuged (30,000 g, 10 min, 4 °C) to remove the precipitate. Samples were finally injected into a gas chromatograph device (GC 14A, Shimadzu Corp., Kyoto, Japan) with a stainless steel column packed with 10% SP™-1000, 1% H_3PO_4, Chromosorb WAW (Suppelco Inc., Bellafonte, PA, USA). Individual VFA peaks were detected by the chromatograph and the peak area was converted into concentration (in mmol/L).

A uniform aliquot of the syringe contents after 24 h of incubation was used for microscopic counts of protozoa. An equal volume (500 µL each) of the aliquot was mixed with the fixative containing bromocresol green (0.06 g/100 mL), sodium chloride (0.8 g/100 mL) and 1:10 diluted formalin in water. The counting was performed on Neubauer counting chamber (Paul Marienfield GmbH & Co. KG, Lauda-Königshofen, Germany) with counting depth 0.1 mm and counting area 1 mm^2.

Data Analysis

Data were analyzed by using one-way analysis of variance (ANOVA) with the following statistical model:

$$Y_{ij} = \mu + \tau_i + \varepsilon_{ij}$$

where Y_{ij} is the observed value for the j^{th} replicate of the i^{th} treatment, μ is the overall mean, τ_i is the treatment effect for the i^{th} treatment (fixed effect), and ε_{ij} is the random residual error (Kaps & Lamberson, 2004). The differences among treatments were compared by using Duncan's multiple range test when the ANOVA result of each variable showed significant different at $P<0.05$.

Total gas production and methane emission data with PEG addition were divided by their corresponding data without PEG addition and multiplied by 100% as previously conducted by Jayanegara & Sofyan (2008) and Jayanegara *et al*. (2009a), respectively. Such data indicated the potency of each purified tannins in decreasing rumen fermentation and mitigating methane emission, respectively.

Methane emission estimated from VFA profile was plotted against its observed value. For an ideal model, the estimated methane emission is equal to the observed methane. Assessment of prediction error was made through mean square prediction error (MSPE) according to Kebreab *et al*. (2008):

$$\text{MSPE} = \sum_{i=1}^{n} \frac{(O_i - P_i)^2}{n}$$

where n= number of run, O_i= the observed methane emission and P_i= the predicted or estimated methane emission. Root MSPE (RMSPE) was used as a measure of prediction accuracy. The RMSPE value was expressed as a proportion (percentage) from the observed methane emission. Further, a linear regression analysis was performed between the estimated and the observed methane.

RESULTS AND DISCUSSION

Addition of all purified tannins at 1 mg/mL decreased total gas production as compared to control ($P<0.05$; Table 1). The magnitude of gas decrease was higher for condensed tannins than those of hydrolysable tannins ($P<0.05$). When PEG was added, total gas production increased to almost similar to control; this was true for all purified tannins and the range of increase was between 10%-20% (Figure 1). The decrease of gas production due to tannins is possible through interaction between tannins and feed components such as protein

Table 1. Gas production, methane emission and protozoa population of purified tannin additions without or with polyethylene glycol (PEG)

Treatment	Gas (mL)	CH4 (mL)	CH4 (mmol)	Protozoa (log count/mL)
Control	95.8^{e}	19.4^{d}	0.76^{d}	5.13
Chestnut				
No PEG	79.3^{b}	14.9^{b}	0.58^{b}	4.99
With PEG	91.3^{de}	18.5^{c}	0.72^{c}	5.04
Sumach				
No PEG	83.8^{c}	15.4^{b}	0.60^{b}	5.07
With PEG	92.5^{de}	18.5^{c}	0.72^{c}	5.19
Mimosa				
No PEG	77.0^{ab}	14.9^{b}	0.58^{b}	5.13
With PEG	91.7^{de}	18.4^{c}	0.72^{c}	5.08
Quebracho				
No PEG	74.0^{a}	14.2^{a}	0.56^{a}	5.13
With PEG	89.0^{d}	18.2^{c}	0.71^{c}	5.04
SEM	1.52	0.38	0.015	0.015
P-value	<0.001	<0.001	<0.001	0.061

Note: Means in the same column with different superscripts differ at P<0.05.

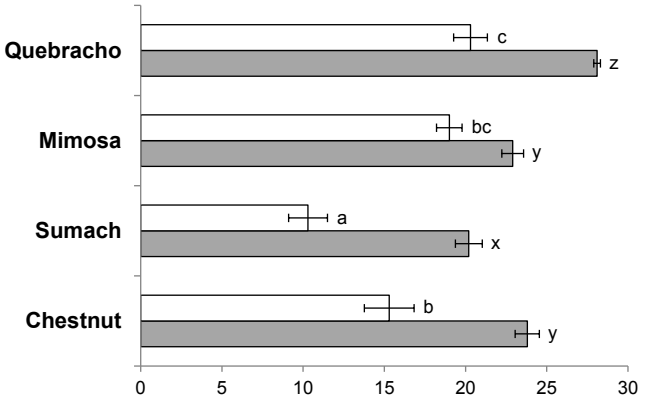

Figure 1. Percent gas (□) and methane (■) increase of various purified tannin sources due to polyethylene glycol addition. ^{a,b,c} Significantly different between purified tannin sources for percent gas increase; ^{x,y,z} Significantly different between purified tannin sources for percent methane increase.

and carbohydrate (both structural and non-structural carbohydrate) (Jayanegara *et al*., 2012). The main interaction between tannins and those macromolecules is via hydrogen bond (Mueller-Harvey, 2006). Such bind prevents degradation and fermentation of the molecules partially to form gas and thus lowers its production. Since the bind is stronger for condensed tannins and there is a partial degradation of hydrolysable tannins by certain rumen microbial species particularly at the sugar attachment sites (Mueller-Harvey, 2006), it is unsurprising that the gas decrease was higher on addition of the condensed tannins than the other ones. Addition of PEG neutralizes the effect of tannins since the PEG has higher affinity to tannins compared to the macromolecules (Makkar *et al*., 1995). The increase of gas production due to neutralization effect of PEG on tannins (present in green tea and black tea by-products) was also observed by Kondo *et al.* (2014).

A decrease of methane emission was observed when the purified tannins were added into basal diet as compared to control (P<0.05); the range of decrease was between 20.6% and 26.8% and between 21.1% and 26.3% when expressed in mL and mmol, respectively. The magnitude of decrease due to tannins in the present study was higher than the previous study of Jayanegara *et al.* (2009b) that ranged between 13.3% and 13.9%. Apparently this was due to the lower concentration of tannins used in that study, i.e. only half (0.5 mg/mL) of the concentration used in the present experiment. No clear distinction between condensed and hydrolysable tannins was observed with regard to methane emission. With regard to the mechanisms in which tannins are able to mitigate ruminal methane emissions, two mechanisms have been proposed by Tavendale *et al.* (2005), i.e. (1) indirectly, through reduction in fibre digestion which in turn decreases H_2 production as a precursor of methane, and (2) directly, through inhibition of the growth or activity of methanogens. It seems that both mechanisms are reflected in the present study through simultaneous depression in total gas production and methane emission due to tannin additions. Furthermore, tannins are known to decrease protozoa population (Bhatta *et al*., 2009) in which part of the methanogens are living together (Morgavi *et al*., 2010) and contribute to the lower methane emission. However, the reduced population of protozoa was not proven in the present experiment. This was supported by a meta-analysis study conducted by Jayanegara *et al.* (2012); across all studies, increasing levels of tannins did not significantly reduce log protozoa population in both *in vitro* and *in vivo* experiments.

Similar to the pattern of total gas production, methane emission increased after PEG addition (Figure 1). The increase of methane emission due to PEG ranged from approximately 20% to 27%. Such increase of methane was also observed in our previous study (Jayanegara *et al*., 2009a) in which the PEG was added into a number of tannin-containing plants obtained from Mongolia and Germany. This response confirms the effect of tannins in mitigating ruminal methane emission since PEG has a specific affinity to tannins over other compounds (Makkar *et al*., 1995; Kondo *et al*., 2014). The PEG binds tannins, making the plant secondary compounds lost their biological activities in interacting with other macromolecules such as protein and carbohydrate as well as lost their anti-microbial properties such as fibre-degrading bacteria and methanogens. It has to be noted that PEG has various molecular weight (e.g., 1,000, 4,000, 6,000 and 20,000) and its interaction with tannins may vary according to the respective weight. Other polymers that have similar properties like PEG in deactivating tannin biological activity are polyvinyl pyrrolidone (PVP) and polyvinyl polypyrrolidone (PVPP) (Kondo *et al*., 2014).

The pattern of total VFA production was similar to that of total gas; addition of all purified tannins significantly reduced total VFA (P<0.05) and PEG addition increased back the variable although could not completely recover the concentration similar to control (Table 2). This was also true for all individual VFA, i.e. acetate (C_2), propionate (C_3), butyrate (C_4), *iso*butyrate (*iso*C_4), valerate (C_5) and *iso*valerate (*iso*C_5). Such VFA is a major energy source for ruminants (Jayanegara *et al*., 2006). The mechanism whereby tannins decreased total and individual VFA is similar as explained above since VFA is also an end product of rumen microbial fermentation (Jayanegara *et al*., 2006). Purified tannins decreased acetate to propionate ratio significantly (P<0.05). An inhibition on the growth of cellulolytic bacteria in the presence of tannins is apparently a causal factor of such VFA shift (Jayanegara *et al*., 2012) thus lowers the acetate production as a major VFA resulted from cellulolytic bacteria fermentation. Although propionate in general was decreased due to purified tannin additions, the magnitude of decrease was relatively small as compared to acetate decrease. The decrease of acetate to propionate ratio due to purified tannin additions is favourable towards mitigation of methanogenesis since fermentation of glucose to acetate yields H_2, a main substrate of the methane formation, and on the contrary, the fermentation of glucose to propionate consumes H_2 (Morgavi *et al*., 2010; Hristov *et al*., 2013). It has to be noted that butyrate concentrations in all treatments were rather high as compared to that of the typical one in the rumen. Since this was consistently observed across all treatments, therefore it was unlikely due to the treatment effects. Apparently the high population of butyrate-producing bacteria in the inoculum is a plausible explanation of such pattern, which may be as a result of high-fibre intake of the donor cows or the presence of resistance starch in the concentrate diet (Li *et al*., 2012).

Less H_2 was produced on the addition of purified hydrolysable and condensed tannins as compared to control (P<0.05; Table 3). Depression of substrate degradation due to tannins is apparently a plausible explanation. Addition of PEG improved the H_2 production but still hardly similar to control. Metabolic hydrogen is produced during degradation and fermentation of feed polymers (mainly carbohydrates, both structural and non-structural carbohydrates) through Emden-Meyerhof-Parnas pathway, which is an oxidative process under anaerobic condition in the rumen (Moss *et al*.,

Table 3. Hydrogen (H_2) balance of purified tannin additions without or with polyethylene glycol (PEG)

Treatment	H_2 produced (mmol)	H_2 utilized (mmol)	H_2 recovery (%)
Control	4.79^{d}	4.47^{d}	93.2^{c}
Chestnut			
No PEG	4.01^{a}	3.60^{a}	90.3^{b}
With PEG	4.51^{c}	4.21^{c}	93.3^{c}
Sumach			
No PEG	4.30^{b}	3.75^{b}	87.2^{a}
With PEG	4.70^{d}	4.25^{c}	90.6^{b}
Mimosa			
No PEG	4.06^{a}	3.62^{a}	89.0^{b}
With PEG	4.38^{bc}	4.18^{c}	95.3^{d}
Quebracho			
No PEG	4.05^{a}	3.51^{a}	86.7^{a}
With PEG	4.51^{c}	4.19^{c}	93.0^{c}
SEM	0.054	0.067	0.59
P-value	<0.001	<0.001	<0.001

Note: Means in the same column with different superscripts differ at P<0.05.

Table 2. Volatile fatty acid (VFA) profile (in mmol/L) of purified tannin additions without or with polyethylene glycol (PEG)

Treatment	Total VFA	C_2	C_3	C_4	isoC$_4$	C_5	isoC$_5$	C_2/C_3
Control	77.8^{d}	50.6^{e}	12.5^{b}	11.5^{d}	0.86^{e}	0.99^{f}	1.28^{d}	4.04^{b}
Chestnut								
No PEG	65.6^{a}	42.1^{a}	11.8^{a}	9.4^{a}	0.65^{a}	0.72^{a}	0.91^{a}	3.57^{a}
With PEG	73.3^{c}	48.5^{d}	11.7^{a}	10.4^{bc}	0.72^{bc}	0.84^{c}	1.06^{b}	4.13^{b}
Sumach								
No PEG	70.2^{b}	45.2^{b}	12.4^{b}	10.2^{b}	0.68^{ab}	0.81^{b}	1.00^{b}	3.65^{a}
With PEG	75.9^{d}	50.2^{e}	11.7^{a}	11.1^{d}	0.77^{d}	0.95^{e}	1.16^{c}	4.28^{c}
Mimosa								
No PEG	66.3^{a}	42.6^{a}	11.8^{a}	9.6^{a}	0.65^{a}	0.72^{a}	0.90^{a}	3.61^{a}
With PEG	71.2^{bc}	46.7^{c}	11.5^{a}	10.3^{bc}	0.74^{cd}	0.89^{d}	1.12^{c}	4.05^{b}
Quebracho								
No PEG	66.0^{a}	42.2^{a}	11.8^{a}	9.7^{a}	0.65^{a}	0.71^{a}	0.94^{a}	3.59^{a}
With PEG	73.2^{c}	47.9^{cd}	11.7^{a}	10.7^{c}	0.76^{cd}	0.93^{e}	1.17^{c}	4.08^{b}
SEM	0.84	0.63	0.07	0.13	0.014	0.02	0.025	0.052
P-value	<0.001	<0.001	0.003	<0.001	<0.001	<0.001	<0.001	<0.001

Note: Means in the same column with different superscripts differ at P<0.05.

2000). The pattern of H_2 utilization among the treatments was identical to that of H_2 production and ranged from 3.51 to 4.47 mmol. Utilization of H_2 is possible since a number of rumen microbes such as H_2-utilizing bacteria and methanogens are capable of consuming H_2 (Morgavi *et al.*, 2010). Further, the H_2 is also used during VFA synthesis or incorporated into microbial organic matter (Moss *et al.*, 2000). The H_2 recovery of the treatments ranged from 86.7% to 95.3%. Such range is within the normal range of H_2 recovery, i.e. between 78% and 96% (Moss *et al.*, 2000) or an average of 90% (Castro Montoya *et al.*, 2011).

Estimation of methane emission by using VFA profiles revealed an accurate result with a high coefficient of determination and a very low RMSPE, i.e. 1.75% (Figure 2). This indicates that stoichiometric estimation of methane from VFA is very useful when there is a lack of equipment to directly measure the gas. It has to be noted that all individual VFA values are requested to be converted into mmol since stoichiometric relationship is based on mol, not other units of measurements. The result was different with that of reported by Jayanegara *et al.* (2013) in which the stoichiometric equation overestimated the actual methane emission. An explanation was that the H_2 recovery obtained in that study was much lower compared to the present study, i.e. between 28.9% to 56.2%. Although adjustment by considering H_2 recovery improved the accuracy and lowered the RMSPE to 8.01% (Jayanegara *et al.*, 2013), the current study was much more accurate by providing lower RMSPE. Nevertheless, in any case, the consideration of H_2 recovery is essential to obtain a more accurate methane emission. In a circumstance that the RMSPE is still high, such stoichiometric prediction is useful in making an order or rank which feed produces lower or higher ruminal methane emission *in vitro*.

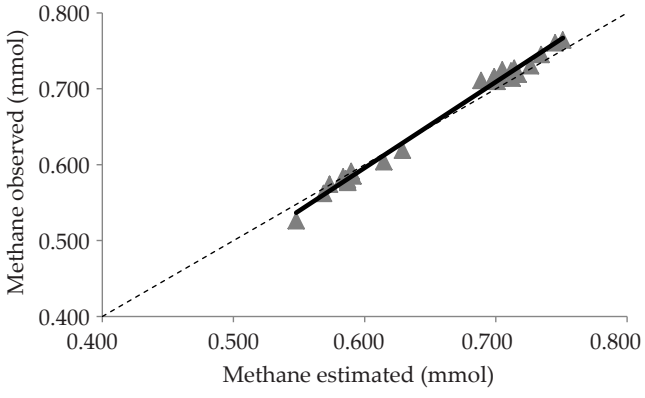

Figure 2. Plot between estimated (from volatile fatty acid profile) and observed (using infrared methane analyzer) methane emission. Regression equation: Methane observed= –0.084 + 1.133 × Methane estimated (P<0.001; R^2= 0.992; RMSPE= 1.75%). Dashed line indicates an ideal line where the estimated methane emission is equal to the observed methane emission.

CONCLUSION

Addition of purified hydrolysable and condensed tannins at 1 mg/ml into basal diet decreased methane emission by 20%-27% from control. No clear distinction was observed between the hydrolysable and the condensed tannins tested with regard to methane emission. Simultaneous addition of PEG with the tannins increased methane, confirming a specific effect of tannins in mitigating methane emission. Estimation of methane emission by using VFA profiles revealed an accurate result with a high coefficient of determination and a very low RMSPE, indicating the usefulness of such stoichiometric relationship when there is a lack of equipment.

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