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AN *IN VITRO* STUDY ON THE ABILITY OF TANNIC ACID TO INHIBIT METHANOGENESIS AND BIOHYDROGENATION OF C18 PUFA IN THE RUMEN OF GOATS

Wisam S. Al-Jumaili[1, 2], Yong M. Goh[1♦], Saied Jafari[1], Mohamed A. Rajion[1], Mohamed F. Jahromi[3], Mahdi Ebrahimi[1]

[1]Department of Veterinary Preclinical Sciences, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia
[2]Department of Animal Resources, College of Agriculture, University of Al-Qassim Green, Babylon, 51013 Al-Qassim, Iraq
[3]Institute of Tropical Agriculture, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia
♦Corresponding author: ymgoh@upm.edu.my

Abstract

An *in vitro* gas production technique, using rumen fluid from four Kacang × Boer crossbred adult goats was used to study the effects of commercial tannic acid (TA, a hydrolysable tannin) on methanogenesis, fatty acid composition and biohydrogenation (BH) of C18 polyunsaturated fatty acids (PUFA) in the rumen. Treatments were control (CON, 50% alfalfa hay (AH) + 50% concentrate), 25 mg TA/250 mgDM (LTA, low TA) and 50 mg TA/250 mgDM (HTA, High TA), which were mixed with 30 mL of buffered rumen fluid and incubated for 24 h. The study revealed that TA supplementation had no negative effect on rumen fermentation parameters such as pH, NH_3N, acetic/propionic ratio and total volatile fatty acid (tVFA). Methane (CH_4) production (mL/250 mg DM) decreased ($P<0.05$) with increasing levels of TA. Greatest CH_4 reduction (%) was recorded for MTA (20.30%) and LTA (13.00%) compared with CON. Supplementation of the diet with TA did not affect the rate of rumen BH (%) of C18:1n-9 (oleic acid; OA), C18:2*n-6* (linoleic acid; LA), C18:3*n-3* (linolenic acid; LNA) and the concentration of fatty acids after 24 h of *in vitro* incubation. Based on this study, the addition of TA *in vitro* reduced rumen methanogenesis without negative effect of rumen fermentation characteristics, but *in vivo* studies need to be performed to determine if concentrations that inhibit methane are below toxic levels.

Key words: biohydrogenation, gas production, goat, methanogenesis, rumen, tannic acid

Methane (CH_4) production accounts for 2–12% loss of dietary gross energy in ruminants and is a potent greenhouse gas with a global warming potential 23 times greater than that of carbon dioxide (CO_2) in trapping the heat (Bhatta et al., 2013; IPCC, 2007). Therefore, reducing ruminal CH_4 not only improves the efficiency of

nutrient utilization but also helps to protect the environment from global warming. There is a need for identifying feed additives with the potential to modify rumen fermentation for enhancing the efficiency of utilization of feed energy while decreasing rumen methanogenesis (Bhatta et al., 2015).

The production of healthy food is an important issue in current ruminant nutrition research. The diet composition is the major factor influencing the fatty acid composition of ruminant's products (meat and milk) because the fatty acid of meat and milk are results of dietary origin as well as rumen microbial metabolism of dietary lipids (Buccioni et al., 2012). Rumen biohydrogenation (BH), the conversion of dietary polyunsaturated fatty acid (PUFA) to saturated fatty acid (SFA) by rumen microbes, is detrimental to human health because of increasing the risk of cardiovascular disease (Vasta et al., 2009). Transformation of PUFA to SFA results in increased accumulation of several hydrogenation intermediates in ruminant meat and milk, such as rumenic acid (*c9*, *t11* CLA) and vaccenic acid (C18:1*t-11*), which are well known for their anti-carcinogenic, anti-atherogenic and anti-oxidative health promoting properties (Durmic et al., 2008).

Plant secondary metabolites such as hydrolysable tannins (HT) are particularly attractive as rumen modifiers as these compounds are natural products, which are generally accepted to be environmentally friendly and safe for food production systems (Frutos et al., 2004). Moreover, tannins (HT; polyesters of gallic acid and various individual sugars) are bioactive compounds able to interfere with protein and lipid metabolism in the rumen, by forming undegradable complexes with dietary proteins and by modulating several bacterial activities, including BH of PUFA (Buccioni et al., 2015). However, tannins in general concept are considered to have both adverse and beneficial effects on animals, depending on their concentration and nature, as well as other factors, such as animal species, physiological state of the animal, and composition of the diet (Jayanegara et al., 2011). Jayanegara et al. (2011) also suggested that phenols were able to modulate BH and methanogenesis processes simultaneously and both towards a desirable direction. However, there are not many reports in the literature on the effect of TA on rumen methanogenesis and BH.

Some *in vitro* studies have demonstrated that tannins (hydrolysable) are able to interfere with rumen fatty acid profile, according to the presence of hydrolysable tannin (Buccioni et al., 2015; Buccioni et al., 2011). One of the feed additives is tannic acid (TA), a hydrolysable tannin, which is used to reduce rumen methanogenesis (Bouchard, 2015; Tavendale et al., 2005). In addition to the beneficial effect of TA by increasing the efficiency of feed by reducing rumen methanogenesis (Patra and Saxena, 2011), TA showed an inhibitory effect on rumen BH of fatty acids rumen (Patra and Saxena, 2011). The results from *in vitro* and *in vivo* experiments often show conflicting results on the effect of hydrolysable tannins on the rumen BH (Buccioni et al., 2015). This is probably due to differences in the nature of tannin and the percentage inclusion in the diet.

Therefore, the objectives of the present study were to identify the effect of supplementation of different inclusion levels of TA on *in vitro* methanogenesis and BH of PUFA with the rumen liquid obtained from goats.

Material and methods

Animals and rumen liquor sampling

All animal management and sampling procedures were approved by the Universiti Putra Malaysia Animal Care and Use Committee. Four rumen-fistulated goats (Kacang × Boer crossbred) with an average body weight of 39±0.70 kg were used as rumen liquor donors. The diet was fed at a rate of 3% of body weight for maintenance requirements according to NRC (2007). The goats were fed twice daily with a diet containing a fixed amount of alfalfa hay (AH) and concentrate (50:50, w/w). Rumen liquor was sampled before the morning feeding at 08:30 h from four goats and placed immediately in warm (39°C) insulated flasks under anaerobic conditions. In the laboratory, samples were pooled in equal proportions and strained through four layers of cheesecloth under anaerobic conditions and used immediately. Chemical composition and fatty acid content of substrates used for the *in vitro* incubations are shown in Table 1.

Table 1. Chemical composition and fatty acid content of fermentation substrates used for the *in vitro* incubations

(g/kg DM)	AH	Concentrate
Corn	-	255.40
Soybean meal	-	190.0
Palm kernel cake	-	358.7
Rice bran	-	116.9
Palm kernel oil	-	50.00
Ammonium chloride	-	10.00
Vitamins and minerals	-	10.00
ME (Mcal/Kg)		2.51
DM	907.0	915.0
CP	203.0	167.0
NDF	517.0	244.0
ADF	334.0	117.0
EE	34.70	42.20
Fatty acid (g/100 g FA)		
C12:0	2.18	1.71
C14:0	1.61	1.63
C16:0	28.2	22.83
C16:1*n-7*	3.02	0.50
C18:0	7.44	4.72
C18:1*n-9*	5.40	16.40
C18:2*n-6*	42.59	43.92
C18:3*n-3*	9.56	8.29
Total SFA	39.43	30.89
Total MUFA	3.02	0.50
Total C18 PUFA	57.55	68.61

Alfalfa hay – AH; DM – dry matter; CP – crude protein; NDF – neutral detergent fiber; ADF – acid detergent fiber; EE – ether extract. Total SFA (saturated fatty acid) = sum of C12:0 + C14:0 + C16:0 + C18:0.

Total MUFA (monounsaturated fatty acid) = C16:1*n-7*.

Total C18 PUFA (polyunsaturated fatty acid) = sum of C18:1*n-9* + C18:2*n-6* + C18:3*n-9*.

***In vitro* gas production**

Gas production was measured according to Fievez et al. (2005) in which 30 mL of buffered (phosphate buffer contained 28.8 g Na_2HPO_4.12 H_2O, 6.1 g NaH_2PO_4. H_2O, 1.4 g NH_4Cl and bicarbonate buffer contained 39.2 g of $NaHCO_3$ per liter distilled water) rumen fluid solution (2:1; buffer: rumen fluid) were dispensed into 100 mL calibrated plastic syringes separately containing different concentrations of TA namely control with no addition of TA (CON), 25 mg/250 mg DM (low tannic acid; LTA) and 50 mg/250 mg DM (high tannic acid; HTA). Commercial tannic acid was purchased from Nacalai Tesque Co. Kyoto, Japan; 90% w/w HT. Linoleic acid (LA) (Sigma-Aldrich Chemical Company, USA) was also added to the incubations as a PUFA source. Six replicates per treatment were used within one run for *in vitro* incubation. Syringes were incubated at 39°C for 24 h. A buffered rumen liquor only incubated for 24 h was used as a blank to calibrate the *in vitro* gas production system. Total gas production at 2, 4, 6, 8, 10, 12 and 24 h of incubation was estimated by displacement of the syringe piston. The data of gas production have been obtained from the graduated body of syringes.

Post incubation sampling and measurements

After 24 h incubation, gas in the headspace was transferred into a syringe for analysis of CH_4 by a gas chromatograph (Agilent 5890 Series Gas Chromatograph, Wilmington, DE, USA) equipped with a thermal conductive detector (TCD). Calibration was completed using standard CH_4 prepared by Scotty Specialty Gases (Supelco, Bellefonte, PA, USA). The pH of the contents of the syringes was determined using a pH electrode (Mettler-Toledo Ltd., England). Then, 1 mL of metaphosphoric acid was added to four mL of the incubated samples and were centrifuged at 3000 × g for 10 min at 25°C. Then, 0.5 mL of clarified sample was added to 0.5 mL of 4-methyl-n-valeric acid (20mM) before determining the concentrations of VFA, namely acetic, propionic and butyric acids using gas chromatography with a Quadrex 007 Series (Quadrex Corporation, New Haven, CT 06525 USA) bonded phase fused silica capillary column (15m, 0.32 mm ID, 0.25 μm film thickness) in an Agilent 7890A gas-liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a flame ionization detector (FID). Concentration of NH_3N (mg/dL) was determined using the colorimetric method described by Solorzano (1969). A standard curve was prepared to determine whether a linear relationship existed between the varying concentrations of ammonium chloride (NH_4Cl) standard solution and the intensity of the color produced.

Fatty acid analysis and calculation of biohydrogenation

Total fatty acids were extracted from the whole syringe content after 24 h of incubation based on the method of Folch et al. (1957) as described by Ebrahimi et al. (2015) using chloroform/methanol 2:1 (v/v) containing butylated hydroxytoluene to prevent oxidation during sample preparation. After complete separation, the lower phase was collected in a round bottom flask and rotary evaporated (Laborota 4000-efficient; Heidolph, Germany) at 70°C. An internal standard, heneicosanoic acid (C21:0) (Sigma Chemical, St. Louis, MO, USA), was added to each sample be-

fore transmethylation to determine the individual FA concentration within the sample. Transmethylation of the extracted fatty acids to their fatty acid methyl esters (FAME) was carried out using KOH in methanol and 14% methanolic boron trifluoride (BF_3). The FAME were separated by gas chromatography (Agilent 7890A), using a Supelco SP 2560 capillary column of 100 m × 0.25 mm ID × 0.2 μm film thickness (Supelco, Bellefonte, PA, USA). One μL was injected by an auto sampler (Agilent Auto Analyzer 7683 B series, Agilent Technologies, Santa Clara, CA, USA) into the chromatograph equipped with a split/splitless injector and a FID. The carrier gas was nitrogen at a flow rate of 1.2 ml/min. The split ratio was 1:20 after injection of 1 μL of the FAME. The injector temperature was programmed at 250°C, and the detector temperature was 270°C. The column temperature program started to run at 150°C, for 2 min, warmed to 158°C at the 1ºC/min, held for 28 min, warmed to 220°C at the 1ºC/min and then held for 20 min to achieve satisfactory separation. The peaks of samples were identified, and concentrations calculated based on the retention time and peak area of known standards (Sigma Chemical). The fatty acid concentration is expressed as the g/100 g of total identified fatty acids.

The BH of PUFA (OA, LA and LNA) was calculated as the decrease of PUFA from the initial PUFA at time zero (0 h) of incubation as described by Jayanegara et al. (2012) using the formula:

$$BH(\%) = [PUFA(0H) - PUFA(24h) \div PUFA(0H)] \times 100$$

where:

PUFA (0 h) = concentration (g/100 g of FA) of PUFA at 0 h of incubation;

PUFA (24 h) = concentration (g/100 g of FA) of PUFA after 24 h incubation.

All experimental data were analyzed using SAS (version 9.1; SAS Institute Inc., Cary, NC) based on a completely randomized design. For statistical analysis of *in vitro* gas production, sampling at different times was added to the model and analyzed using repeated measures ANOVA. Multiple comparison of the means among times and treatments was performed using the Duncan's test. Polynomial contrasts (linear and quadratic effects) were also used. Values of $P<0.05$ were considered significant. The data were checked for normality using PROC UNIVARIATE of the SAS ver. 9.1.

Results

Addition of TA on gas and CH_4 production

The effect of tannic acid on total gas and CH_4 gas production after 24 h *in vitro* incubation is shown in Table 2. Total gas (mL/250 mg DM), rate of gas (mL/h) and CH_4 production (mL CH_4/250 mg DM) were significantly ($P<0.05$) affected by TA supplementation. MTA had 12, 11 and 20 (%) of reduction compared with CON in terms of total gas, rate of gas and CH_4 production, respectively. The effect of TA on

gas production at different times of incubation is shown in Figure 1. Except at 4 and 24 (h) of incubation, there were no significant differences (P>0.05) among the treatments in terms of gas production at different hours of incubation.

Table 2. Effect of tannic acid on total gas and CH_4 gas production after 24 h *in vitro* incubation

Parameters	Experimental diets			SEM	P-value	
	CON	LTA	HTA		linear	quadratic
Total gas (mL/250 mg DM)	43.08 a	41.00 ab	37.91 b	1.315	0.008	0.758
Rate (mL/h)	1.79 a	1.70 ab	1.58 b	0.054	0.009	0.763
CH_4 (mL CH_4/g DM)	66 a	57.44 b	52.60 b	1.968	0.0003	0.428
CH_4 (mL methane per L gas production)	74.79	64.85	59.62	1.82	0.002	0.528

CON – control (50% concentrate + 50% AH); CH_4 – methane, LTA – low tannic acid (25 mg of TA /250 mg DM); HTA – high tannic acid (50 mg of TA /250 mg DM); SEM – standard error of mean; a, b – different letters in each row denote significant difference (P<0.05); ns – not significant.

Methane production (mL/250 mg DM) decreased (P<0.05) with increasing levels of TA.

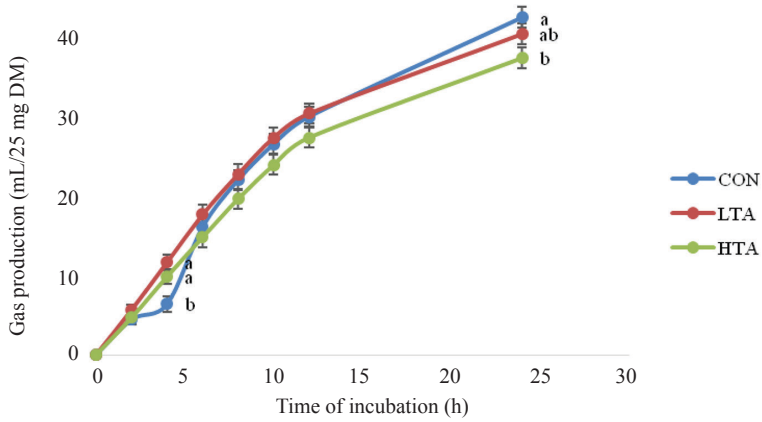

Figure 1. Cumulative gas production of different tannic acid levels at different times of incubation. CON: control (50% concentrate + 50% AH), LTA: low tannic acid (25 mg of TA /250 mg DM), HTA: high tannic acid (50 mg of TA /250 mg DM), DM: dry matter. Vertical bars are standard error

Addition of TA on rumen fermentation parameters

The effect of TA on the fermentation characteristics after 24 h *in vitro* incubation is shown in Table 3. In the present study, the use of TA did not significantly affect some rumen fermentation parameters such as pH, NH_3N, tVFA and acetic/propionic ratio compared to CON. Concentration of butyric acid was greatest (P<0.05) for CON (17.19 mM) and lowest for HTA (14.40 mM), respectively.

Table 3. Effect of tannic acid on rumen fermentation characteristics after 24 h *in vitro* incubation

Parameters	Experimental diets			SEM	P-value	
	CON	LTA	HTA		linear	quadratic
NH_3N^{ns} (ppm)	0.172	0.132	0.108	0.204	0.043	0.742
Acetic^{ns} (mM)	53.16	50.59	49.39	1.223	0.356	0.657
Propionic^{ns} (mM)	32.68	32.35	35.15	1.232	0.044	0.626
Butyric (mM)	17.19 a	15.43 ab	14.40 b	0.629	0.027	0.928
Total VFA^{ns} (mM)	103.04	98.39	98.95	1.880	0.145	0.276
Acetic/Propionic ratio^{ns}	1.63	1.56	1.43	0.078	0.101	0.733

CON – control (50% concentrate + 50% AH); LTA – low tannic acid (25 mg of TA /250 mg DM); HTA – high tannic acid (50 mg of TA /250 mg DM); SEM – standard error of mean, different letters in each row denote significant difference (P<0.05); ns – not significant.

Addition of tannic acid on rumen fatty acid composition and biohydrogenation of PUFA

The results of rumen fatty acid concentration and BH of OA, LA and LNA are shown in Table 4 and Figure 2, respectively. Rumen fatty acid composition and BH of PUFA were not affected by the addition of TA.

Table 4. Effect of tannic acid on fatty acid concentration after 24 h *in vitro* incubation

Fatty acid (g/100 g FA)	Experimental diets			SEM	P-value	
	CON	LTA	HTA		linear	quadratic
C12:0	3.38	3.62	3.08	0.205	0.572	0.398
C14:0	7.38	7.49	6.79	0.787	0.776	0.822
C14:1	1.30	2.46	1.76	0.464	0.702	0.376
C16:0	20.87	18.23	21.32	0.690	0.778	0.054
C16:1	1.78	2.58	0.90	0.465	0.440	0.214
C18:0	38.52	36.71	34.33	2.392	0.505	0.958
C18:1*t-11*	10.58	10.87	11.14	0.593	0.720	0.993
C18:1*n-9*	10.12	10.43	11.16	0.759	0.599	0.903
C18:2*n-6*	3.00	4.08	4.80	0.519	0.169	0.869
C18:3*n-3*	1.19	1.26	1.47	0.089	0.209	0.736
c9, *t11* CLA	1.69	1.87	2.81	0.251	0.071	0.454
t10, *c12* CLA	0.21	0.41	0.44	0.049	0.052	0.398
Total SFA	70.15	66.04	65.52	1.016	0.658	0.558
Total MUFA	23.78	26.34	24.96	0.565	0.615	0.622
Total UFA	29.86	33.96	34.48	0.395	0.370	0.618
Total CLA	1.90	2.27	3.25	0.150	0.062	0.426

CON – control (50% concentrate + 50% AH); LTA – low tannic acid (25 mg of TA/250 mg DM); HTA – high tannic acid (50 mg of TA /250 mg DM); DM – dry matter; SEM: standard error of mean. Total SFA (saturated fatty acid) = sum of C12:0+C14:0+C16:0+C18:0; Total MUFA (monounsaturated fatty acid) = sum of C14:1+C16:1+C18:1 (*t-11*+ *n-9*).

Total UFA (unsaturated fatty acid) = sum of C14:1+C16:1+C18:1(*t-11* + *n-9*) +C18:2 (*c9*, *t11* + *t10*, *c12*+ *2n-6*).

Total CLA (conjugated fatty acid) = sum of *c9*, *t11* CLA + *t10*, *c12* CLA.

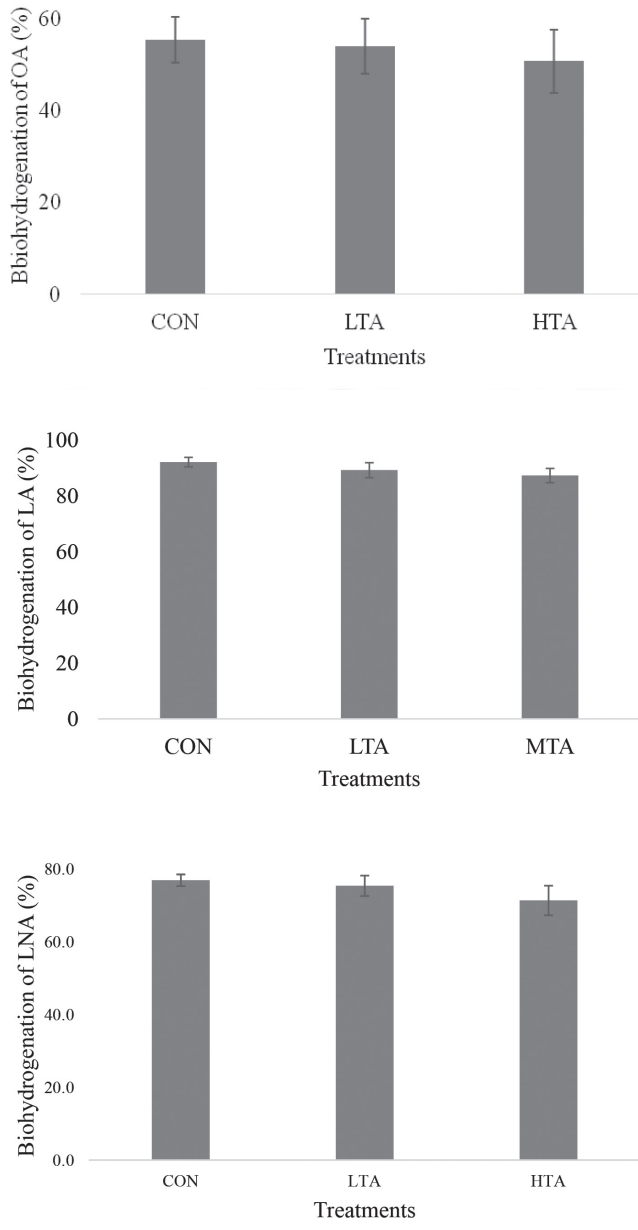

Figure 2. Effect of tannic acid on *in vitro* biohydrogenation (%) of oleic acid (OA), linoleic acid (LA) and linolenic acid (LNA)
CON – control (50% concentrate + 50% AH); LTA – low tannic acid (25 mg of TA /250mg DM); HTA – high tannic acid (50 mg of TA /250 mg DM). Vertical bars are standard error.
Increasing TA in syringes tended to increase CLA content of total fatty acids ($P<0.01$).

Discussion

The reduced total gas production in HTA (–12%) compared to CON in this study is consistent with the results reported by Bhatta et al. (2015) with the inclusion of *Oenothera lamarckiana* (containing a high quantity of HT) which produced less total gas than incubations of same substrate by adding PEG, a tannin binder in an *in vitro* condition.

Compared to the CON, the CH_4 reduction for LTA and HTA was 13% and 20%, respectively. Reduced rumen CH_4 production in this study could be linked to the role of tannins (e.g. hydrolyzed tannin) in which they could reduce CH_4 because of their inhibition of fiber degradation, reduction in protozoa and methanogenic archaea populations (Bhatta et al., 2012). Jayanegara et al. (2009) reported that inhibition of fiber degradation will shift fatty acid composition away from acetate and hence less production of hydrogen and less CH_4 production. Protozoa can synergistically provide hydrogen as a source of electrons to the methanogenic archaea, and hence, antiprotozoal effects of different tannins including hydrolysable and condensed tannin would be expected to decrease CH_4 production by methanogens attached to protozoa (Bhatta et al., 2013). Almost 36% CH_4 reduction was observed with the methanol extract of *Mangifera indica* at a concentration of 0.5 ml/30 ml of incubation medium in comparison to the control (medium with methanol only) in the study of Kumar et al. (2011) in which they attributed their results to the high quantity of HT content of the leaf samples. During the last decade, researchers have shown interest in using plant extracts containing secondary metabolites as feed additives to control CH_4 production due to their antimicrobial and antiprotozoal activities (Halim et al., 2011; Kamra et al., 2012; Wischer et al., 2013).

From the current results obtained about the tVFA, it could be speculated that the addition of TA at concentration of 25 and 50 (mg/250 mg DM) was not toxic to the ruminal microbes. Moreover, VFA are the end products of rumen microbial fermentation and represent the main supply of metabolizable energy for ruminants. Therefore, their reduced production would be nutritionally unfavorable for the ruminants (Busquet et al., 2006). In this study, the concentration of NH_3N was not affected by TA supplementation compared to CON. Conversely, Bhatta et al. (2013) reported that addition of *Oenothera lamarckiana* (containing high quantity of HT) to the *in vitro* incubation decreased NH_3N concentration. They also attributed their results to the formation of complexes between HT and proteins. Inhibition of amino acid deamination and less production of NH_3N has practical implications because it may increase ruminal escape of dietary protein and improve the efficiency of nitrogen use in the rumen (Busquet et al., 2006). It is known that NH_3N is regarded to be the most important nitrogen source for microbial protein synthesis in the rumen (Wanapat et al., 2008). Wei et al. (2012) reported that the addition of three plant extracts of tea saponin, mulberry leaf extract and ecdysterone did not show any significant difference on fermentation parameters such as pH, NH_3N, total VFA, individual VFA and the ratio of acetic/propionic acid compared to CON compared with inclusion of no extracts at 28 h of incubation. From the results obtained in this study and results of Wei et al. (2012) on rumen fermentation characteristics, it could be inferred that

the source, type and the level of TA used in this study prevented negative effects on rumen fermentation characteristics. According to McSweeney et al. (2001) HT can be degraded by some microbial species in the rumen, the toxicity appears to be due to absorption of degraded products of HT and high amount of phenols in the bloodstream, which is more the capability of liver to detoxify phenols (Makkar et al., 2007). Thus, a proper strategy to prevent the toxicity of HT while obtaining their potential beneficial effects on ruminants is to administer them at low to moderate amount (Reed, 1995). Several experiments have shown that feeding of HT at less than 20 g/kg DM did not cause any adverse effects on production parameters of ruminants (Śliwinski et al., 2002; Krueger et al., 2010; Toral et al., 2011). Nevertheless, pure tannic acid has to be investigated *in vivo* for their potential to decrease methane production or biohydrogenation and to determine toxicity, if any (Jayanegara et al., 2015). Present research should be shifted towards some potent HT since the nature and the toxicity of HT are quite diverse.

Increasing PUFA and CLA contents in ruminant products through nutritionally controlling BH has attracted much attention in recent years. Among recommended nutritional strategies, supplementing with phenolic compounds seems to play a major role in this respect (Jayanegara et al., 2012; Vasta et al., 2009). In the current study, BH of PUFA was not affected by TA supplementation. Contrary to our results, in an *in vitro* study conducted by Jayanegara et al. (2012) it was shown that phenols were able to modulate rumen BH *in vitro*. The CLA content has shown an increase with increasing the level of TA in the diet but not significantly different. Since CLA in animal products has beneficial effects for human consumption, then increasing levels of TA should be investigated. Another study concerning incubation of 27 different tropical plant species in *in vitro* conditions showed that hydrolysable tannin and condensed tannin contributed to the inhibition of fatty acid BH but at different steps (Jayanegara et al., 2011). The lack of significant differences in the biohydrogenation results among the treatments might be due to the level of tannic acid and it can be affected on biohydrogenation of unsaturated fatty acids using higher levels of tannic acid.

Based on the data obtained from the present *in vitro* study, it could be concluded that supplementation of TA does not result in significant changes in fermentation characteristics and fatty acid composition *in vitro*. Interestingly, TA supplementation reduced rumen methanogenesis at 24 h of incubation *in vitro*. However, more comprehensive *in vivo* studies with animal hosts need to be carried out to evaluate the sustainability of TA supplementation to mitigate CH_4 production without detrimental effects on the animal as a whole.

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