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RESEARCH ARTICLE

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Dietary supplementation with papaya (*Carica papaya* L.) leaf affects abundance of rumen methanogens, fermentation characteristics and blood plasma fatty acid composition in goats

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Abstract

The objective of this study was to test the effect of feeding papaya leaf (PL) on rumen microbial population, fermentation characteristics, blood fatty acid composition and antioxidant activity in goats. Three rumen fistulated male goats were assigned in a 3×3 latin square design using three levels of PL: no addition of PL in basal diet (control, CON, 50% concentrate + 50% alfalfa hay), 25% of alfalfa hay in basal diet replaced by PL (medium PL, MPL) and 50% of alfalfa hay in basal diet replaced by PL (high PL, HPL). Rumen fluid and blood plasma were sampled from the animals at the end of feeding trial (third week). Papaya leaf treatments (MPL and HPL) showed significant differences ($p<0.05$) in terms of rumen fermentation parameters as compared to the CON group at different times of measurement. The concentration of methanogenic archaea ($\log_{10}$cell/L) decreased ($p<0.05$) in both PL treatment groups as compared with CON group at all sampling times. The concentration of α-linolenic acid and total conjugated linoleic acid (CLA) were also higher ($p<0.05$) in the blood of goats fed PL especially HPL, compared to the CON. Blood plasma malondialdehyde concentration (mM/mL) of PL treatment groups showed a significant reduction as compared with CON. Supplementation of PL allowed the modulation of rumen characteristics which might represent a feeding strategy to reduce methane emission by directly inhibiting methanogens while improving the health benefits of ruminant products.

Additional keywords: blood; fatty acid; goat; papaya leaf; rumen.

Abbreviations used: CLA (conjugated linoleic acid); CON (control); CT (condensed tannin); FA (fatty acid); HDL (high density lipoprotein); HPL (high papaya leaf); LDL (low density lipoprotein); LNA (linolenic acid); MDA (malondialdehyde); MPL (medium papaya leaf); PL (papaya leaf); PUFA (polyunsaturated fatty acid); SA (stearic fatty acid); SFA (saturated fatty acid); TVFA (total volatile fatty acid); VFA (volatile fatty acid).

Authors' contributions: Conceived, designed and performed the experiments: SJ, YMG, MAR and ME. Analyzed the data; contributed reagents/materials/analysis tools: ME and MFJ. Wrote the paper: SJ. All authors read and approved the final manuscript.

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Introduction

The availability of feed sources for ruminant production in the tropics are turning to be challenging mostly because of increasing costs and limited supplies of conventional feedstuffs such as alfalfa, corn and other traditional feed ingredients (Jafari *et al.*, 2015). Tree leaves are an important source of supplementary protein, vitamins and minerals in developing countries that are widely used in the tropics and offer the opportunity to use as nitrogen and energy supplements to livestock where agricultural primary products are limited (Cherdthong *et al.*, 2010). Moreover, tree leaves contain different classes of bioactive compounds such as tannins, saponins, flavonoids and many other plant secondary metabolites which have been shown to potentially improve rumen microbial fermentation (Patra & Saxena, 2011). Tropical plants normally contain different spectrum of secondary compounds (Wanapat *et al.*, 2013). Papaya (*Carica papaya*) leaf

(PL) has high protein contents and is low in fiber (Jayanegara *et al.*, 2013), and can be found in all tropical countries and many subtropical regions of the world. Ayoola & Adeyeye (2010) indicated that PL served as a dual-purpose agent containing essential nutrients and broad spectrum of bioactive compounds such as tannins, saponins, cardiac glycoside and alkaloids. The highest amount of phenolic compound has also been exhibited in PL among other parts of papaya (*e.g.* unripe papaya fruit, ripe papaya fruit and seed) (Maisarah *et al.*, 2014). Improved rumen fermentation characteristics and reduced population of rumen methanogenic archea were observed after 24 h of *in vitro* incubation by using PL, PL methanolic extract and PL different solvent extracts in previous studies (Jafari *et al.*, 2016 a,b).

Vasta *et al.* (2009) and Buccioni *et al.* (2017) reported that tannin supplementation favored the accumulation of polyunsaturated fatty acid (PUFA) and reduced the accumulation of saturated fatty acid (SFA) in the blood of sheep. However, there are no previous reports on the effects of PL on rumen fermentation characteristics, microbial population and blood fatty acid (FA) parameters in an *in vivo* assay.

Therefore, we hypothesized that the addition of different dietary levels of PL would have modulatory effects on rumen fermentation characteristics, blood FA composition and inhibitory effects on the population of rumen methanogenic archaea and protozoa when tested *in vivo*.

Material and methods

Experimental animals and diets

The PL samples were collected from a papaya farm (1º34'57.7" N, 104º12'20.7" E) in Kota Tinggi, Johor, Malaysia. The PL samples at the phenological stage of recently matured and harvestable vegetative stage were air-dried for 2 days, followed by oven-drying overnight at 50ºC. After drying, the samples were chopped and stored in tightly closed plastic bags before the feeding trial. The experiment was conducted at the research farm of the Faculty of Veterinary Medicine, Universiti Putra Malaysia. Three Kacang crossbred male goats, each fitted with rumen fistulae, were used for the study. The body weight of goats was 39.0 ± 0.70 kg (mean ± SE). The goats were housed in individual pens with metal slotted flooring, raised above the ground. The care of the experimental goats was in accordance with the Animal Care and Use Committee of Universiti Putra Malaysia. Three experimental diets in a 3×3 latin square design were used for this study. Experiments were conducted for three periods, and each period lasted for 21 d. There was 7 days of wash-out interval between each period to minimize the carry over effect among treatments. The dietary treatments were: basal diet with no inclusion of PL (CON: 50% alfalfa hay + 50% concentrate), 25% of alfalfa hay in basal diet replaced by PL (medium PL: MPL) and 50% of alfalfa hay in basal diet replaced by PL (high PL: HPL). The diet was fed at a rate of 3% of body weight for maintenance requirements according to NRC (2007). Animals were fed twice a day at approximately 08:00 and 17:00. and had *ad libitum* access to fresh water.

Chemical composition of experimental diets

The experimental diets were dried at 60ºC for 48 h in an air oven, and ground in a Wiley Mill (A. H. Thomas Co., Philadelphia, PA, USA) through a 1-mm screen. Sample diets were analyzed according to AOAC (1990) for dry matter (DM), crude protein (CP), ether extract (EE) and ash.

Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were analyzed according to Van Soest *et al.* (1991). The analysis of NDF was conducted without sodium sulfite and with the use of a heat stable α-amylase. The chemical composition and fatty acid content of the experimental diets are shown in Table 1.

Rumen and blood sample collection and laboratory analysis

Rumen content samples (50 mL) were collected from different parts of the rumen at the last day of feeding trial (third week) at 0, 2, 4, 6 and 8 h post morning feeding. Rumen contents were sampled for determination of pH (Mettler-Toledo Ltd, England), NH_3N, total volatile fatty acids (VFAs) and for the quantification of microbial populations (methanogenic archea and protozoa). The rumen samples were strained through four layers of cheesecloth. One mL of strained sample was immediately snapped frozen at - 80ºC for microbial analyses.

The samples were then acidified with 25% metaphosphoric acid and centrifuged (10 min, 4ºC at 15,000 × g), filtered, and the filtrate was used for determination of NH_3N and VFAs. The concentration of NH_3N was determined using the colorimetric method as described by Jafari *et al.* (2016a). The total VFAs (TVFA) were determined using an Agilent 7890A gas-liquid chromatography (Agilent Technologies, Palo Alto, CA, USA) equipped with a flame ionization detector (FID).

An internal standard (4-methyl-n-valeric acid) was used for VFA determination. Blood samples were taken at 8 h post morning feeding for determination of blood

Table 1. Ingredients and chemical composition of the experimental diets: CON (50% concentrate + 50% AH), 25% of alfalfa hay in basal diet replaced by PL (MPL) and 50% of alfalfa hay in basal diet replaced by PL (HPL).

	Experimental diets		
	CON	**MPL**	**HPL**
Ingredients (% of diet)			
Alfalfa hay	50.00	37.50	25.00
Papaya leaf	0	12.50	25.00
Corn, grain	10.00	10.00	10.00
Soybean meal	10.00	10.00	10.00
Palm kernel cake	20.00	20.00	20.00
Rice bran	5.00	5.00	5.00
Sunflower oil	2.00	2.00	2.00
Mineral premix	0.50	0.50	0.50
Vitamin premix	0.50	0.50	0.50
Ammonium chloride	1.00	1.00	1.00
Limestone	1.00	1.00	1.00
Chemical composition [1]			
DM (%)	85.20	87.00	88.10
OM (g/kg DM)	724.20	752.80	750.80
CP (g/kg DM)	208.30	218.50	225.00
EE (g/kg DM)	52.50	50.00	48.40
NDF (g/kg DM)	419.00	400.50	393.20
ADF (g/kg DM)	253.00	245.80	238.30
Fatty acid composition (g/100 g FA) [2]			
C14:0	5.82	3.66	1.46
C16:0	18.39	20.98	21.40
C16:1	1.25	1.47	1.52
C18:0	2.56	3.48	3.89
C18:1n-9	21.23	23.22	24.06
C18:2n-6	49.28	44.71	38.90
C18:3n-3	1.47	2.48	3.77
Total SFA	26.77	28.12	26.75
PUFA n-3	1.47	2.48	3.77
PUFA n-6	49.28	37.69	38.58
MUFA	22.48	24.69	30.58
C18 PUFA	50.75	47.19	42.67

[1]DM: dry matter. OM: organic matter. CP: crude protein. EE: ether extract. NDF: neutral detergent fiber. ADF: acid detergent fiber. [2]Total SFA= sum of C14:0 + C16:0 + C18:0. MUFA = (C16:1 + C18:1n-9). PUFAn-3 = C18:3n-3. PUFA n-6 = C18:2n-6. C18 PUFA= sum of (C18:2n-6 + C18:3n-3).

plasma FAs, biochemical analyses and antioxidant activity. Blood samples (about 10 mL) were collected from the jugular vein into tubes containing 12 mg of EDTA and plasma was separated by centrifugation at 500 × g for 10 min at 4°C and stored at −20◦C until analysis.

Fatty acid profile in experimental diets and blood plasma

The FAs in experimental diets and blood plasma (1 mL) were extracted based on the protocol of Ebrahimi *et al.* (2015) as described by Jafari *et al.* (2016a) using chloroform/methanol 2:1 (v/v). An internal standard, heneicosanoic acid (Sigma Chemical, St. Louis, MO, USA), was added to each sample before transmethylation to determine the individual FA concentration within the sample. Individual FAs were determined 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). The FAs concentrations were expressed as g/100 g of identified FA. Reference standards (mix C4 - C24 methyl esters; Sigma-Aldrich, Inc., St. Louis, MO, USA) and conjugated linoleic acid (CLA) standard mix (c9, t11 and t10, c12 CLA, Sigma-Aldrich, Inc., St. Louis, MO, USA) were used to determine recoveries and correction factors for determination of individual FA.

Rumen microbial quantification by real time polymerase chain reaction (PCR)

The DNA was extracted from the rumen fluid using QIAamp DNA Stool Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol. The extracted DNA was stored at -20˚C until used. Purity and concentration of genomic DNA in each sample were measured using a spectrophotometer and the number of copies of a template DNA per mL of elution buffer was calculated using the following formula (Genomics and Sequencing Center web-based calculator, http://cels.uri.edu/gsc/cndna.html).

$$\text{Number of copies} = \frac{\text{amount of DNA } (\frac{ng}{mL}) \times 6.022 \times 10^{23}}{\text{Length (bp)} \times 10^{9} \times 650}$$

Standard curves were constructed using amplification cycle threshold (CT) values that were obtained from a serial dilution of DNA of each bacterial group.

Primers used to quantify the population of the different groups of microorganisms were:

Total methanogen (F, TTCGGTGGATCDCARAGRGC; R, GBARGTCGWAWCCGTAGAATCC) (Zhang *et al.*, 2008) and total protozoa (F, ACCGCATAAGCGCACGGA; R, CGGGTCCATCTTGTACCGATAAAT) (Sylvester *et al.*, 2004). Real-time PCR was performed using the Bio-Rad CFX96 Touch (Bio-Rad Laboratories, Hercules, CA, USA) using optical grade plates. The PCR reaction was performed on a total volume of 25 µL using the iQTMSYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). Each reaction included 12.5 µL SYBR Green Super-

mix, 1 µL of each primer, 1 µL of DNA samples and 9.5 µL RNAse free water. To confirm the specificity of amplification, a melting curve analysis was carried out after the last cycle of each amplification.

Blood biochemical parameters and antioxidant activity

Blood plasma levels of glucose, urea N, uric acid, total protein, cholesterol, triglycerides, high density lipoprotein (HDL) and low density lipoprotein (LDL) were measured using analytical kits (Pointe Scientific Inc., MI, USA) and determined calorimetrically on a Hitachi 902 Automatic chemical analyzer (Roche International, Basel, Switzerland).

Malonaldehyde (MDA) concentration was determined by the thiobarbituric acid reactive substance method as described by Tug *et al.* (2005). In brief, the proteins in 0.15 mL blood plasma were first precipitated by using sulphuric and phosphotungstic acid and then the levels of MDA in the samples were measured by using Shimadzu UV-1201V spectrophotometer (Shimadzu, Kyoto, Japan) at 532 nm. Different concentrations of tetraethoxypropane (1, 1, 3, 3-) were used as standard MDA to plot a standard curve.

Statistical analysis

Data of rumen fermentation characteristics, microbial population and blood biochemical parameters were analyzed using the GLM procedure of SAS (2003).

Sampling at different times (0, 2, 4, 6 and 8 h) was analyzed using repeated measures. The microbial data were normalized using the $\log_{10}$-transformation prior the statistical analysis. Multiple comparison of the means among treatments was performed using the Duncan's test. Values of $p<0.05$ were considered significant.

Results

Rumen fermentation parameters

The effects of PL supplementation on rumen fermentation parameters are shown in Table 2. In terms of pH and at different hours of measurement (0, 2, 4, 6 and 8 h), 0 h had the highest pH value among the treatments (6.85, 7.18 and 6.54 for CON, MPL and HPL, respectively) whilst 2 h post morning feeding showed the lowest values (6.19, 6.48 and 6.16 for CON, MPL and HPL, respectively). There were significant differences

Table 2. Effect of papaya leaf on rumen fermentation characteristics at different times of measurements.

	Experimental diets [1]			SEM	*p*-value
	CON	MPL	HPL		
pH					
0 h	6.85^{b}	7.18^{a}	6.54^{c}	0.077	0.0008
2 h	6.19	6.48	6.15	0.118	0.1588
4 h	6.42	6.60	6.33	0.084	0.1264
6 h	6.77^{a}	6.69^{a}	6.48^{b}	0.055	0.0129
8 h	6.81	6.75	6.47	0.373	0.0003
NH_3N (mg/dL)					
0 h	13.92^{a}	11.48^{ab}	9.79^{b}	0.8045	0.0169
2 h	13.07^{a}	9.07^{b}	10.93^{ab}	1.172	0.1058
4 h	12.83^{a}	8.92^{b}	12.98^{a}	0.948	0.231
6 h	10.74^{b}	10.61^{b}	14.60^{a}	1.139	0.0585
8 h	11.64	11.43	11.52	0.800	0.9829
TVFA (mmol/L)					
0 h	120.64^{b}	121.82^{ab}	123.93^{a}	2.050	0.0219
2 h	125.67	127.71	129.96	2.280	0.1800
4 h	134.45^{c}	137.27^{b}	141.31^{a}	3.180	0.0140
6 h	140.11^{b}	146.71^{ab}	147.80^{a}	3.520	0.0585
8 h	152.10^{c}	155.21^{b}	158.24^{a}	4.980	0.0201

[1]See diets in Table 1. Different superscript letters in each row denote significances ($p<0.05$). SEM=standard error of mean. TVFA= total volatile fatty acids.

(p<0. 05) in pH among the treatment being at 0 h the highest value for MPL (7.18), followed by CON (6.85) and HPL (6.54), respectively. The lowest pH value was recorded for HPL at 6 h (6.48) and 8 h (6.47) post morning feeding.

In regards to concentration of NH_3N, except for 8 h after feeding, there were significant differences for all sampling times among treatments. The highest concentration of NH_3N corresponded to CON at 0, 2, 4 and 6 h (13.92, 13.07, 12.83 and 14.60 mg/dL, respectively). Between the times of measurement for HPL, NH_3N concentration was highest at 4 h (12.98 mg/dL) and lowest at 0 h (9.79 mg/dL).

No reduction in the concentration of TVFA (mmol/L) in all PL treatment groups was observed as compared with CON at all times. The highest (p<0.05) concentration of TVFA was recorded for HPL as compared to CON at all times of measurements except at 2h of measurement which was not significantly different among the treatments (p>0.05).

Fatty acid composition in blood plasma

The FA profiles of blood plasma at 8 h of measurement are shown in Table 3. The concentration (g/100 g FA) of C18:0 was significantly lower (p<0.05) in HPL (14.8) compared to CON (17.8) at 8 h of measurement. Contrary, HPL (2.98) had higher (p<0.05) concentration of linolenic acid (LNA) compared to CON (1.03). The concentration (g/100 g FA) of c9t11 CLA was higher (p<0.05) for HPL (0.62) compared to CON (0.22). The concentration (g/100 g FA) of total CLA was also higher (p<0.05) for HPL (0.68) compared to CON (0.25) at 8 h of measurement. There were no differences (p>0.05)

Table 3. Effect of papaya leaf on blood plasma fatty acid composition of goats (g/100 g FA).

Fatty acid[1]	Experimental diets[2]			SEM	p- value
	CON	MPL	HPL		
C14:0	2.32	3.74	3.07	0.681	0.170
C16:0	24.01	20.38	20.79	1.491	0.122
C16:1n-7	2.69	2.46	2.49	0.451	0.849
C18:0	17.79^{a}	15.68^{ab}	14.81^{b}	0.673	0.056
C18:1n-9	35.45	35.76	35.91	1.126	0.742
C18:1t-11	0.53	0.68	0.81	0.012	0.587
C18:2n-6	10.66	11.61	11.86	1.125	0.553
c9t11 CLA	0.22^{b}	0.42^{ab}	0.62^{a}	0.015	0.032
t10c12 CLA	0.04	0.06	0.06	0.001	0.08
C18:3n-3	1.03^{c}	1.97^{b}	2.98^{a}	0.29	0.047
C20:4n-6	1.96	2.54	2.16	0.706	0.562
C20:5n-3	1.41	1.75	1.27	0.528	0.666
C22:5n-3	0.76	0.96	1.11	0.352	0.666
C22:6n-3	1.13	1.87	2.06	0.410	0.228
Total SFA	44.12	39.81	38.67	2.005	0.168
Total MUFA	38.15	38.32	38.40	1.187	0.810
PUFA n-6	12.62	14.16	14.02	1.634	0.511
PUFA n-3	4.33	6.56	7.42	0.985	0.140
n-6:n-3 ratio	3.03	2.23	2.09	0.417	0.210
Total CLA	0.25^{b}	0.48^{ab}	0.68^{a}	0.014	0.035
Total PUFA	16.95	20.71	21.44	1.762	0.158

[1]Total SFA= sum of C14:0 + C16:0 + C18:0. Total MUFA = sum of C16:1n-7+ C18:1n-9. Total PUFA n-3 = sum of C18:3n-3 + C20:5n-3 + C22:5 n-3 + C22:6n-3. Total PUFA n-6 = sum of 18:2n-6 + 20:4n-6. n-6: n-3 fatty acid ratio = (C18:2n-6 + C20:4n-6) / (C18:3n-3 + C20:5n-3 + C22:5n-3 + C22:6n-3). Total CLA: sum of c9,t11CLA+ t10,c12CLA. Total PUFA= sum of n-3 + n-6 (PUFA). [2]See diets in Table 1. SEM: standard error of mean. Different letters within a row denote significant difference at (p<0.05).

between treatments at 8 h of measurement in terms of other FA profiles in the blood.

Total methanogens and total protozoa population

The effect of PL on rumen methanogenic population is shown in Figure 1. The total number of methanogens (log_{10}cell/L) in the ruminal fluid was higher (p<0.05) in CON compared to the MPL and HPL at all times of measurement.

Except at 2 h of measurement, there was no significant difference (p>0.05) among the treatments in the total number of protozoa in the ruminal fluid as shown in Figure 2. In addition, total protozoa (log_{10}cell/mL) was highest for MPL (6.69) and HPL (7.78) at 2h of measurement and lowest at 4h of measurement; 5.91 and 5.24, respectively as compared to the other times of measurement.

Blood biochemical profile and antioxidant activity

The effects of PL on the blood biochemical parameters are shown in Table 4. The PL addition did not affect (p>0.05) blood biochemical parameters (glucose, urea N, total protein, uric acid cholesterol, triglycerides, HDL and LDL) of goats at 8 h.

There was a significant decrease (p<0.05) in the plasma MDA concentration (mM/mL) of PL

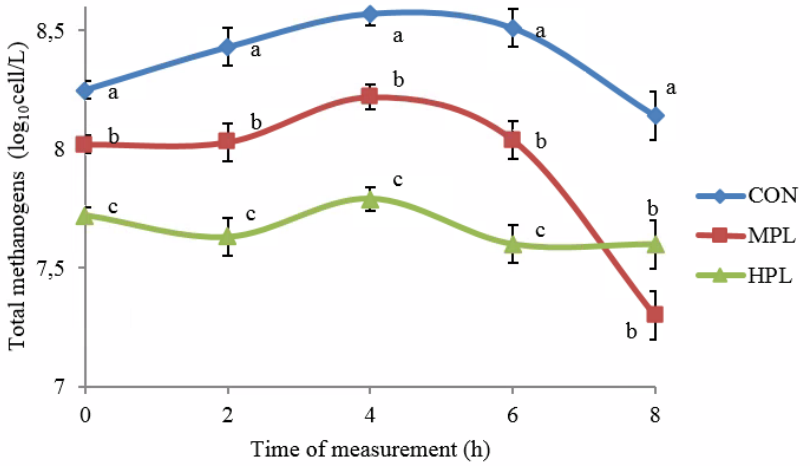

Figure 1. Effect of PL supplementation on rumen total methanogens. See diets in Table 1. Vertical bars are standard errors of mean. Different letters denote significant differences (p<0.05).

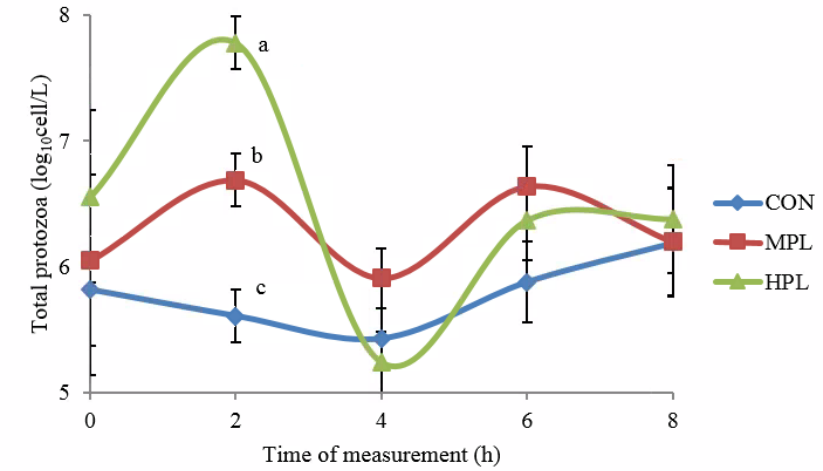

Figure 2. Effect of PL supplementation on rumen total protozoa. CON (50% concentrate + 50% AH), 25% of alfalfa hay in basal diet replaced by PL (MPL) and 50% of alfalfa hay in basal diet replaced by PL (HPL). Vertical bars are standard errors of mean. Different letters denote significant differences (p<0.05).

Table 4. Effect of PL on blood biochemical parameters of goats after 8 h of measurement.

Blood parameters[1]	Experimental diets[2]			SEM	*p*-value
	CON	MPL	HPL		
Glucose (mmol/L)	3.55	3.20	3.00	0.265	0.176
Urea N (mmol/L)	7.22	6.92	6.32	0.484	0.071
Total protein (g/L)	65.70	64.07	74.37	3.697	0.131
Uric acid (mmol/L)	6.25	6.00	5.80	0.568	0.589
Cholesterol (mmol/L)	1.18	2.07	2.36	0.133	0.478
Triglyceride (mmol/L)	0.21	0.34	0.31	0.024	0.145
HDL (mmol/L)	0.97	1.16	1.70	0.082	0.592
LDL (mmol/L)	0.14	0.83	0.68	0.047	0.592

[1]HDL: high density lipoprotein. LDL: low density lipoprotein.
[2]See diets in Table 1. SEM: standard error of mean.

treatment groups (2.82 and 1.33 for MPL and HPL, respectively) as compared with CON (3.33).

Discussion

Rumen fermentation parameters

Plants with bioactive compounds, which are well documented as antimicrobial agents, are considered as potential candidates to improve rumen fermentation characteristics (Thao *et al.*, 2015). It has been shown that tropical plants could impact rumen ecology and fermentation characteristics (Wanapat *et al.*, 2013). To the best of our knowledge, this is the first study investigating the effect of PL inclusion on rumen fermentation parameters *in vivo*.

Inhibition of amino acid deamination has practical implications because it may increase ruminal escape of dietary protein and improve the efficiency of nitrogen use in the rumen (Jafari *et al.*, 2017). It is generally agreed that tannin containing feed materials reduce the rate of protein degradation in the rumen (Bhatta *et al.*, 2013). The bioactive compounds in PL contained 30.31 and 28.36 gallic acid equivalent per gram of total phenol and total tannins, respectively as described in our previous study (Jafari *et al.*, 2016a). In the current study, reduction in ruminal NH_3N concentrations with the use of PL in the diet of goat could probably have resulted from a greater concentration of tannins bound to proteins and decreased proteolysis of dietary protein (Bhatta *et al.*, 2013).

The concentration of TVFA was higher in the PL treatments as compared to CON. The VFAs are the end products of rumen microbial fermentation and represent the main supply of metabolizable energy for ruminants. Therefore, a reduction in their production would be nutritionally unfavorable for the animal. Moreover, increased TVFA can be interpreted as a result of improved digestion with PL diets compared to CON. Consistent to our results, Belanche *et al.* (2016) concluded that supplementing chitosan and ivy fruit saponins as natural feed additives shifted the fermentation pattern towards propionate production in an *in vitro* study. The lower pH and higher concentration of TVFA obtained in the current study also confirmed the results of Allen (1997) about the negative relationship of VFAs and pH.

Fatty acid composition of blood plasma

The need to lower the concentration of SFA in ruminant products due to their association with a higher risk of cardiovascular diseases is still important (Jafari *et al.*, 2017); however, the concentration of beneficial PUFA in rumen is low due to microbial biohydrogenation of PUFA (Wencelová *et al.*, 2015). In the current study, supplementation of PL, especially at higher concentration (HPL), decreased the concentration of stearic acid (SA) in the blood plasma by 17%, increased the concentration of LNA by 65% and total CLA by 63% compared to CON. The increased concentration of LNA in blood plasma in the current study may be due to an increased rumen passage rate, which led to a decreased biohydrogenation and an increased bypass of LNA (Adler *et al.*, 2013). However, rumen passage rate was not measured in the present experiment.

Decreased blood SA and increased LNA in our study is consistent with the results of Rana *et al.* (2012) supplementating polyphenol rich *Terminalia chebula* plant extract at different concentrations (1.06 g/kg and 3.18 g/kg of body weight). This suggested an influence on the rumen biohydrogenation and resultant higher absorption of the PUFA such as LNA as evidenced from the FA profile of blood. In our previous *in vivo* study, supplementation of PL at the current inclusion rate decreased the concentrations of rumen SFA and increased the concentration of rumen linoleic acid in goats (Jafari *et al.*, 2018). Vasta *et al.* (2009) reported that the FA composition of blood generally reflected the ruminal FA profile which was observed in our studies. Consistent to our study, Buccioni *et al.* (2017) showed that the FA profile of blood plasma regardless of the source of tannin, favored the accumulation of PUFA while reduced concentration of SA.

In the current study, HPL fed animals had higher concentration of c9t11 CLA (+64%) and total CLA (+63%) as compared with CON in the blood plasma at 8 h. The CLA refers to a group of positional and geometric isomers of linoleic acid in which the double bonds are conjugated, and many studies suggest that

CLA exhibits anti-carcinogenic, anti-adipogenic, anti-diabetogenic, anti-atherogenic and anti-inflammatory properties (Bauman *et al.*, 2000).

Total methanogen and total protozoa population

The MPL and HPL groups showed 2% and 6%, and 10% and 6%, of methanogenic archaea reduction compared with CON at 0 and 8 hours of measurement, respectively. It has been suggested that tannin-containing feed materials may have a direct inhibitory effect on the methanogens (Busquet *et al.*, 2006). The tannin content of 28.36 mg/g DM of PL has been reported in our previous study (Jafari *et al.*, 2016b). About 50-60% of the methane emissions globally are from the agricultural sector with the major source coming from ruminant animals (Bouchard *et al.*, 2015). Methane is one of the most important greenhouse gas that contribute to global warming through absorption of infrared radiation in the atmosphere (Wanapat *et al.*, 2013).

Methanogens produce methane for growth from a reaction that in the rumen uses mainly hydrogen and carbon dioxide as substrates. In an *in vivo* study, supplementation of beef cattle with sainfoin silage, containing 12 g condensed tannin (CT)/kg DM, the relative abundance of methanogenic archaea in rumen fluid was decreased (Bueno *et al.*, 2015). In an *in vivo* study, supplementation of rain tree pod meal (containing tannin and saponin) has shown to modulate rumen fermentation characteristics and reduce methane production through reduction in the number of rumen methanogens (Anantasook *et al.*, 2013). From the reduced methanogenic population in the PL treatment groups compared to CON, it could be hypothesized that PL might directly affect rumen methanogenesis, which could mainly be due to the presence of secondary metabolites in PL.

An *in vivo* study reported that protozoa concentrations in the rumen of animals fed olive leaf were lower than in animals fed alfalfa and commercial concentrate (Yañez-Ruiz *et al.*, 2004). The lack of reduction in rumen protozoa population among PL treatment groups as compared to CON could explain that the amount of bioactive compounds in PL was not high enough to affect rumen protozoa numbers or that they do not affect them as they do on methanogens.

Blood biochemical profile and antioxidant activity

The PL supplementation did not significantly (p>0.05) affect blood glucose, total protein, urea N, uric acid, cholesterol, triglyceride, HDL, and LDL concentrations at 8 h of measurement. Blood urea N concentrations in the present study ranged from 6.32 to 7.22 mmol/L among the treatments. According to Caldeira *et al.* (2007) blood urea N concentration is one of the best indicators of protein metabolism in ruminants. The lack of differences in plasma urea N concentrations among the treatment groups indicates that there was probably no amino acid deficiency or imbalance in the MPL and HPL groups compared to CON group. Consistent to our results, the addition of ensiled and sundried mulberry leaves (containing phenolic compounds) at 8% and 6.3% in the diet to crossbred steers did not show any significant differences on blood cholesterol, triglycerides, HDL and LDL concentration as compared to control (Zhou *et al.*, 2012). They also attributed their results to the low level of substitution of mulberry in the diet of their animals, which could not initiate obvious downstream effects in animal blood parameters.

Another study that disagree with our results showed that the addition of 15% of dried licorice (a tannin-containing plant, 4.1 g/kg DM of CT) leaves in the diet of growing lambs promoted a tendency to decrese plasma concentration of cholesterol compared to the control without the addition of licorice leaves (Zamiri *et al.*, 2015). They also attributed their results to the lipid binding capacity of tannin containing licorice leaves which led to hypo-cholesterolemia in animals fed these feed materials. Khalel *et al.* (2014) also found that blood glucose and total protein were higher for cows fed *Moringa oleifera* leaf at 20% and 40% in diet than those fed on *M. oleifera*-free diet. However, the results of our study showed no differences in the blood biochemical characteristics between the treatment groups which could be attributed to the supplementation of 12.5% and 25% of PL in the whole diet that probably could not trigger obvious downstream effects in animal physiological systems.

In the present study, PL treatment groups (MPL and HPL) showed decreased MDA concentrations in blood plasma compared to the CON at 8 h of measurement. A decreased MDA concentration is an indicator of an improved oxidative defense of animal tissues (Bueno *et al.*, 2015). As an end product of lipid peroxidation, the formation of MDA is accelerated by oxidative stress and thus detection of MDA can reflect the level of oxygen free radicals and the extent of lipid peroxidation (Riss *et al.*, 2007). It was shown that the addition of *Spirulina platensis* algae powder at 1 g/10 kg of body weight/day in fattening lambs could reduce the concentration of MDA in blood (EL-Sabagh *et al.*, 2014). They attributed their results to the presence of bioactive compounds which have potent antioxidant activities individually or in synergy on free radicals. It has also been shown that PL is a source of phytochemicals (phenolics) (Ikram *et al.*, 2015). The lower concentration of blood MDA

obtained in this study among PL treatment groups (MPL and HPL) compared to CON could also be due to the presence of these active compounds in PL. The PL has almost 80% antioxidant activity by β-carotene linoleate bleaching method (Ikram *et al.*, 2015). The ethanolic extracts of PL which contained flavonoids, terpenoids, saponins, tannins and reducing sugars, showed potent scavenging (antioxidant) activity by inhibition of 2,2-Diphenyl-1-picryl-hydrazyl radical (Vuong *et al.*, 2013).

In summary, the present study reveals that the use of PL in the diets of goats modulated the rumen fermentation characteristics, the abundance of rumen methanogenic archaea and also modified the biohydrogenation pattern. Based on the results obtained, PL supplementation could be introduced as a possible feeding alternative strategy in the tropics. However, the correct dietary concentration of PL should be carefully chosen to avoid negative effects on animal performance because of the presence of secondary compounds.

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