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Determination of *in vitro* Gas Production Kinetics by Adding *Leucaena leucecophala* and Corn Oil to the Ration in Different Ratios

Research Article

C.T. Noviandi[1], K. Kustaantinah[1], A. Irawan[2*], B.P. Widyobroto[1] and A. Astuti[1]

[1] Faculty of Animal Science, Gadjah Mada University, Yogyakarta 55281, Indonesia
[2] Vocational School, Universitas Sebelas Maret, Surakarta 57126, Indonesia

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*Correspondence E-mail: a.irawan@staff.uns.ac.id

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ABSTRACT

This study was aimed to determine *in vitro* gas production kinetics and organic matter digestibility (IVOMD) of ration added by *Leucaena leucecophala* and corn oil (CO) at various ratios. Four levels of *Leucaena leucecophala* (0%, 25%, 50%, and 75%, DM basis) and three levels of corn oil (0%, 1%, and 2% of substrate) were arranged in a 4 × 3 factorial design. Hohenheim *in vitro* gas production procedure was employed to determine gas production kinetics, IVOMD, and partitioning factor (PF) value of the experiment. Supplementation of leucaena at 25% (L25) increased IVOMD (%), potential degradation fraction, cumulative gas production (GP) (mL), and metabolizable energy (ME) value (MJ/kg DM) of the ration ($P<0.01$). There was no effect on *in vitro* gas production kinetics when leucaena was given at higher levels in comparison with L25 ($P>0.05$). Besides, corn oil supplementation to the substrate did not negatively affect IVOMD and gas production kinetics. Instead, 2% of corn oil supplementation increased GP ($P<0.05$). Indicator for microbial efficiency as measured with PF value increased with leucaena and CO supplementation ($P<0.05$). The results indicated that incorporation of 25% leucaena and 2% of corn oil in the ration improved *in vitro* organic matter digestibility and gas production kinetics while a higher rate of supplementation did not give significant contribution in term of gas production on in vitro rumen fermentation system. Further study in chemical and biological treatment of leucaena or tannins sources and corn oil is needed to investigate specific mechanisms in modulating rumen fermentation *in vitro* and *in vivo*.

KEY WORDS corn oil, *in vitro* gas production parameters, *in vitro* organic matter digestibility, *Leucaena leucecophala*.

INTRODUCTION

Napier grass (*Pennisetum purpureum*) is considered one of the most economical feed resources for ruminant livestock in Indonesia. However, high crude fiber (CF) and low protein content (CP) contents can be a major problem to low voluntary intake thus often become the limitation in supporting ruminant production when fed alone (Phesatcha and Wanapat, 2016). *Leucaena leucocephala* is the most abundantly distributed legume in Indonesia that is characterized by a high concentration of protein, energy, and mineral, and also with high palatability (Barros-Rodríguez, 2014), thus potentially improve rumen microbial populations and nutrients utilization. Tannins contained in leucaena leaves have also been known to improve protein utilization efficiency and animal performance (Phesatcha and Wanapat *et al*. 2016). However, an excessive amount of leucaena in the ration was reported to have a detrimental effect on animal performance due to the presence of mimosine (α-amino, 3-hydroxy-4-pyridine- propanoic acid) as an anti-nutritive compound (Barros-Rodríguez, 2012; Soltan *et al*. 2013). For instance, Barros-Rodrıguez *et al*. (2012) reported lower

daily weight gain on sheep fed with a high density of leucaena compared with the control diet which did not contain leucaena. Some reports also explained an improvement in feed intake, digestibility, and metabolizable energy intake of ration that received 75% of leucaena (Tendonkeng *et al.* 2011; Soltan *et al.* 2013). For those explanations, responses vary due to different variety, form, pre-treatment, of leucaena, and type of animal and diet which indicating the need for further investigation.

Supplementing of oils into the diet has been acknowledged to improve energy efficiency and rumen fermentation due to their high energy density. Some advantages of the use of oils as energy and unsaturated fatty acids (UFA) sources are associated with suppressing CH_4 production and improving fatty acids profile in meat and milk (Martin *et al.* 2008; Wanapat *et al.* 2011). Large evidence showed a negative effect of oil supplementation on depressing dry matter intake (DMI), fiber digestibility, and rumen microbial population. Martin *et al.* (2008) suggested supplementation of fat oil rich in unsaturated fatty acid (UFA) content is a promising strategy to reduce CH_4 production and protozoal population. On the other hand, some studies also reported an increase in milk production resulting in feed efficiency improvement (Hristov *et al.* 2013). Corn oil (CO), rich in beneficial UFA, could be a candidate to modulate rumen fermentation (Girón *et al.* 2016).

Furthermore, the oil supplementation effect on rumen fermentation and nutrient digestibility will vary with the type of diet and its chemical composition. The information about optimal levels of leucaena on a different type of diet is also scarce. To our knowledge, the effect of CO on ruminal fermentation and organic matter digestibility has never been confirmed when added in different ratios of concentrate-leucaena combination diet. Thus, an interaction between levels of leucaena and corn oil (CO) may occur. The objective of this study was to determine *in vitro* gas production kinetics and organic matter digestibility (IVOMD) of ration added by *Leucaena leucecophala* and corn oil (CO) at different ratios.

MATERIALS AND METHODS

Sample preparation

The study was performed at the Faculty of Animal Science, Universitas Gadjah Mada, Yogyakarta, Indonesia. *Pennisetum purpureum* and *Leucaena leucocephala* leaves were collected from the pasture field of Faculty of Animal Science, Universitas Gadjah Mada, Yogyakarta (7˚ 46'07.3"S, 110˚ 23'10.6"E). *Pennisetum purpureum* were harvested from plants in the vegetative phase by cutting ± 20 cm above the soil while Leucaena leaves from randomly branch sampling of Leucaena trees.

Collected samples were cut 3-5 cm in length and packed in the bag then immediately dried at 55 ˚C for 72 h. The samples were then milled through a 1-mm screen for chemical analysis and *in vitro* assays. A 1 kg bottle of CO was purchased from a supermarket, Yogyakarta, Indonesia.

Experimental design

A 3 × 4 factorial design was arranged for two factorial treatment: levels of leucaena supplementation (0%, 25%, 50%, and 75%, DM basis) and corn oil supplementation (0%, 1%, 2% of the substrate, DM basis) on gas production kinetics by using hohenheim gas production technique (Menke and Steingass, 1988). Corn oil was added into the solution according to Wu *et al.* (2015), by mixing 6 and 12 mg of corn oil in 1 mL of ethanol and then added into the culture to provide 1 and 2% of corn oil for the substrate. The composition of concentrate was cassava flour, wheat bran, and macro minerals formula. The chemical composition of the rations is presented in Table 1.

Sample analysis

The dried and milled samples were analyzed for DM, OM, EE, and CP according to the Association of Official Analytical Chemists (AOAC, 2005). The neutral detergent fiber (NDF) and acid detergent fiber (ADF) fractions were analyzed according to Van Soest *et al.* (1991). Non fiber carbohydrate (NFC) content was calculated by using the formula given below:

NFC (g/ kg DM)= 100 - (NDF %+CP %+EE %+ash %)

The analysis of samples was conducted in duplicate. In addition, the total phenolic compounds (TP), total tannins (TT), and condensed tannin (CT) of leucaena leaves were quantified according to Makkar (2003). Hydrolyzable tannin was calculated by subtracting the TT to CT.

In vitro fermentation procedure

In vitro fermentation was conducted by Hohenheim gas production technique according to the protocol by Menke and Steingass (1988) using a 100 mL glass syringe (Fortune Models, Poultry, and Graft Gmbh). A sample of ± 300 mg DM was placed into a syringe for pre-incubation for 24 h at 39 ˚C. Two fistulated Bali cows weighed 250-300 kg were used as rumen fluid donor. The animals were adapted to diet for 7 days composed by 70:30 Napier grass and concentrate ratio with approximately 11% CP concentration and 60% estimated total digestible nutrients (TDN), respectively. Rumen fluid was collected at 6:30 am before morning feeding from the front ventral, middle ventral and cranial dorsal sac of the rumen with equal proportions into a pre-warmed thermos flask.

Table 1 Diet composition of the treatment with the inclusion of different levels of *Leucaena leucocephala*

Diet composition (% DM)	Leucaena substitution levels (%)			
	L0	L25	L50	L75
Pennisetum purpureum	75	50	25	0
Leucaena leucocephala	0	25	50	75
Cassava bran	10	10	10	10
Pollard	13	13	13	13
Mineral mixed[1]	2	2	2	2
Total	100	100	100	100
Nutrient chemical composition (%)				
Dry matter (DM)	35.48	36.05	36.62	37.19
Organic matter (OM)	83.62	85.54	87.42	89.88
Ash	16.38	14.46	12.58	10.12
Crude protein (CP)	10.99	12.64	14.27	15.91
Neutral detergent fiber (NDF)[2]	54.2	46.95	39.88	32.81
Acid detergent fiber (ADF)[2]	31.29	27.46	23.63	19.8
Ether extract (EE)	3.29	3.8	4.31	4.82
Total phenolic (TP)	0.93	3.58	7.69	10.73
Total tannins (TT)	0.28	2.16	4.16	6.54
Hydrolyzable tannin (HT)	ND	1.27	2.59	4.09
Condensed tannin (CT)	ND	0.81	1.64	2.33

[1] 1 kg of mineral mixed comprised: Ca: 150 g; P: 50 g; Mn: 25 g; Co: 5 mg; Cu: 250 mg; Se: 1 % ; S: 50 g and Zn: 4 g.
[2] Calculated from individual NDF and ADF value of each feedstuff.
L0: forage without leucaena inclusion; L25: forage + 25% leucaena; L50: forage + 50% leucaena; L75: forage + 75% leucaena (DM basis).

The rumen fluid then immediately was transported to the laboratory, filtered through four layer polyester material (PeCAP, pore size 355 µm; B and SH Thompson, Ville Mont-Royal, QC, Canada) and mixed with a pre-warmed (39 ˚C) anaerobic buffer/ mineral solution (1:2 v/v). The supplementation of corn oil (CO) into the syringe was conducted by following the method as described by Wu *et al*. (2015).

For correction and adjustment purpose, 200 mg DM of standard hay (*Digitaria eriantha*) with known amounts of gas production and blank samples (only buffered-rumen fluid without substrate) were incubated in parallel time. The incubation was conducted for 48 h and gas production (GP) was recorded in 1, 2, 4, 6, 8, 12, 24, 36, and 48 h. This GP test was carried out in two runs, each run with three replications per sample. At the 24 h of incubation, subsamples were filtered through 2 layers of cheesecloth from half of the syringe (6 observations per treatment) for pH, VFA, NH_3-N, fatty acids profile, and methane production (data are not presented). Besides, the remaining syringes were continued for further incubation until 48 h and the residue was used for later determinations of 48 h DM and OM digestibility.

Calculations

The gas production profile from each syringe was fitted for estimation Gp kinetics with the non-linear equation (Ørskov and Mcdonald, 1979):

$$Gp = a + b\,[1 - e^{-ct)}] \qquad \text{[Equation 1]}$$

Where:
Gp: volume of gas production at time t (mL).
a: soluble fraction.
b: slowly degradable component (mL).
c: rate (% per hour) of GP from b fraction.

In vitro organic matter digestibility (IVOMD) and metabolizable energy (ME) were calculated according to Menke and Steingass (1988) with the following equations:

$$IVOMD = 148.8 + 8.893GP + 0.448CP + 0.651TA \qquad \text{[Equation 2]}$$

$$ME\ (MJ\ kg{-}1of\ DM) = 2.20 + 0.1357GP + 0.0057CP + 0.0002859EE \qquad \text{[Equation 3]}$$

Where the units are: IVOMD, mg/g; GP, mL; CP, g/kg DM; TA, g/kg DM; ME, MJ/kg; and EE, g /kg DM.

Furthermore, effective degradability (ED) was estimated as:

$$ED = a + [bc/(c+k)] \times e - k \times L \qquad \text{[Equation 4]}$$

Where:
k: particulate passage rate and was 0.025 per h for corn straw and 0.058 per h for concentrate mix.

The partitioning factor (PF) was also calculated at 24 h of incubation as the ratio between mg of TDOM (True digestible OM) and gas volume (mL) to evaluate the microbial degrading effectivity (Blümmel *et al.* 1997).

Statistical analysis

All data were subjected to ANOVA using General Linear Model (GLM) Procedure of SAS (2008) for completely randomized design following 4 × 3 factorial arrangement based on the model:

$$Y_{ijk} = \mu + A_i + L_j + M_k + (L \times M)_{jk} + e_{ijk}$$

Where:

Y_{ijk}: response variable (e.g. ME, PF, GP, fermentation kinetics parameters).

μ: overall mean.

Lj: leucaena effect.

Mk: CO supplementation effect.

$(L \times M)_{jk}$: interaction effect between leucaena × CO.

e_{ijk}: residual error.

Treatment means were compared by Tukey's procedure for multiple comparisons means. The significant effect of treatments was declared when $P < 0.05$. Data results are reported as least square means and their associated standard errors of the mean (SEM).

RESULTS AND DISCUSSION

***In vitro* organic matter digestibility**

The IVOMD increased significantly as the proportion of leucaena increased by 25% (L25) level (Table 2). Conversely, at the level of 2%, CO decreased IVOMD ($P<0.01$). Both factors have a significant interaction effect on IVOMD ($P<0.01$; Table 2). The average data suggested that L50 did not differ between L25 but a significant decrease was observed in response to L75 compared to L25 and L50 ($P<0.01$; Table 2).

The increase of the IVOMD as a result of leucaena supplementation might be the result of changes in the chemical composition of the ration, primarily because linearly leucaena contributed to increasing CP concentration and NFC content. Consequently, it promotes a better balance of energy and protein than controls.

Barros-Rodríguez *et al.* (2014) suggested that leucaena is highly digestible in the rumen and supplementing leucaena to a forage-based diet improve nutrient digestibility. In addition, the legume is known to have lower lignin content rather than forage or roughage. Abd. El-Salam *et al.* (2011) reported an increase in IVOMD with leucaena supplementation.

The plant cell wall fraction in most forages has a negative correlation with ruminal digestibility (Jung and Allen, 1995) and the increased IVOMD in response to the increase of leucaena proportion could be associated with higher NFC proportion in the diet.

Tannins contained in legume also play an important role in improving nutrient digestibility. Jayanegara *et al.* (2012) suggested an adversely negative effect from tannins would be noticed when added more than 2%/kg DM. As confirmed in L25 and L50, tannins did not show a negative effect due to the low concentration in the ration. The highest CT concentration was at L75 (2.33% DM) whereas at this level allegedly disturbing some rumen microbial species, they also protect protein and carbohydrate fractions from rumen fermentation (Yonjalli *et al.* 2018). Other reason, it is also assumed that when the amount of supplemental nitrogen or nonprotein nitrogen (NPN) exceeds the maximum concentration, the rumen microbiome are inhibited and animal performance is decreased because of ammonia toxicity (Patra and Aschenbach, 2018).

Furthermore, the effect of corn oil on IVOMD was not detected in this experiment (Table 2). It is possible when oil supplementation is given at an appropriate level. Exceed the amount of oil supplementation is toxic because of polyunsaturated fatty acids (PUFA) containing oil have a detrimental effect on rumen microbes, resulting in an inhibition of nutrient degradation. Corn oil suppressed nutrients degradation in the rumen by direct and indirect mechanisms, which is by suppressing the population of fiber degrading bacteria and by protecting the fiber fraction (Wu *et al.* 2015). The toxic effects of polyunsaturated fatty acids (PUFA) on rumen digestibility and activity, according to (Cieslak *et al.* 2013), depending on the type of oil, the level of administration, the fatty acid composition, and the degree of saturation (Prieto-Manrique *et al.* 2018).

***In vitro* gas production parameters**

The easily degraded fraction, potentially degradable DM, and the degradation rate become an important indicator about nutrient digestibility and rumen microbial activity. The balance between high soluble nutrient fractions along with an increase in the proportion of N in diet can be a determinant of other variables in the fermentation process. Data on the effect of leucaena and grass balance with the addition of CO to the fraction a, fraction b, degradation potential (a+b), and the rate of particle degradation (*C*) are presented in Table 2.

As shown in Table 3, the increase of Leucaena level and CO had no effect on fraction a and *C* value ($P>0.05$). Meanwhile, leucaena and corn oil were significant to fraction b and a + b values, with varied responses affected ($P<0.05$).

Table 2 *In vitro* organic matter digestibility, gas production, and effective degradability as influenced by increasing levels of leucaena and corn oil

Treatment		Variables		
L	CO	IVOMD (%)	GP (mL)	ED (%)
	CO1	55.11^{de}	59.05^{de}	19.82
L0	CO2	55.31^{cde}	59.49^{cde}	20.93
	CO3	53.53^{e}	56.34^{e}	18.65
	CO1	60.06^{bc}	66.39^{bcd}	26.56
L25	CO2	59.97^{bcd}	66.82^{bcd}	24.31
	CO3	67.17^{a}	72.41^{a}	22.85
	CO1	61.02^{b}	67.30^{bc}	22.61
L50	CO2	60.86^{b}	68.17^{b}	21.42
	CO3	61.12^{b}	68.47^{b}	22.01
	CO1	61.11^{b}	66.01^{bc}	20.85
L75	CO2	63.69^{ab}	71.01^{b}	25.98
	CO3	62.70^{ab}	69.08^{b}	21.30
The average value of leucaena treatment				
LO		54.98^{r}	58.29^{r}	19.80
L25		59.40^{pq}	71.78^{p}	24.57
L50		63.00^{p}	67.98^{q}	22.01
L75		55.47^{r}	68.70^{pq}	22.71
Average value of corn oil treatment				
CO1		58.92	64.69^{y}	22.46
CO2		58.08	66.37^{xy}	23.16
CO3		57.63	69.07^{x}	21.20
SEM		0.972	1.614	1.727
P-value				
L		< 0.0001	< 0.0001	0.0209
CO		0.0444	0.0029	0.2858
L × CO		0.0008	< 0.0001	0.4262

L0: forage without leucaena substitution; L25: forage + 25% leucaena substitution; L50: forage + 50% leucaena substitution; L75: forage + 75% leucaena substitution (on DM basis); CO1: substrate without corn oil; CO2: substrate with 1% corn oil addition; CO3: substrate with 2% corn oil addition; IVOMD: *in vitro* organic matter digestibility; GP: cumulative gas production and ED: effective degradability.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

ANOVA test showed there were interactions between the two factors to the b value and a + b value (P<0.05), but the interaction did not occur in a fraction (P=0.187). The L75 significantly increased the fraction b compared to the control (L0) (P<0.05), which were 56.09 and 54.53% *vs.* 47.87%, respectively (Table 3).

The higher mean of fraction b reflects the higher potential of nutrient degradation in the rumen. The difference in the fraction a, fraction b, and a + b was influenced by the constituent components of the cell contents and the chemical composition of the feed ingredients which include CP, EE, cell wall content, and soluble minerals, as stated by Van Soest *et al.* (1991). The soluble organic matter is beneficial to stimulate rumen microbial growth in order to enhance their activity.

The fraction b degradability is strongly influenced by the composition of the cell wall, wherein the fiber structure (lignocellulose bond) could inhibit the penetration of microbial enzymes thus resulting in lower nutrient degradation. This is confirmed in the results that the fraction b in the L0 treatment was lower than the other treatments.

Table 1 showed that it has higher NDF content so that the degradability was lower. Potentially degradable DM fraction (A+B) ranged from 60.85-80.49% which is L25 and L50 was greater (P<0.05) than L0. The increase in A + B by increasing levels of leucaena at a maximum of 50% means there was an enhancement of the activity of the rumen fermentation. The highest CT concentration in L75 was 2.33% DM whereas it did not show a significant negative effect. This is consistent with Sofyan *et al.* (2016) where the add up to 2% DM of tannin extract from *Azadirachta indica* did not cause a decrease in fraction b, a + b, and c compared to the control.

Gas production, effective degradability, and metabolizable energy

Data on cumulative gas production (GP), effective degradability (ED), and metabolizable energy (ME) are summarized in Figure 1. A large variation in GP, ED, and ME was detected among treatments. Accordingly, L50 increased GP and ME (P<0.01) but CO_3 tended to decrease GP and ME (P<0.1). The ED was not affected by both factors (P>0.05).

Table 3 *In vitro* gas production parameters as influenced by increasing levels of leucaenaand corn oil

Treatment		Variables			
L	CO	a (%)	b (%)	a + b (%)	C (mL/h)
	CO1	14.43	47.87	62.29	0.06
L0	CO2	15.77	47.57	63.34	0.06
	CO3	13.74	47.11	60.85	0.05
	CO1	21.13	49.06	70.19	0.06
L25	CO2	18.53	52.15	70.68	0.06
	CO3	14.33	71.07	85.40	0.07
	CO1	16.53	54.53	71.06	0.06
L50	CO2	15.10	56.91	72.01	0.06
	CO3	15.78	56.93	72.70	0.06
	CO1	14.86	56.09	70.94	0.06
L75	CO2	20.04	56.44	76.49	0.06
	CO3	14.87	58.25	73.12	0.06
Average of Leucaena treatment					
LO		14.65	47.52^{q}	62.16^{r}	0.06
L25		18.00	57.43^{p}	75.42^{p}	0.06
L50		15.80	56.12^{p}	71.92^{q}	0.06
L75		16.59	56.92^{p}	73.52^{pq}	0.06
Average of corn oil treatment					
CO1		16.74	51.89^{y}	68.62^{y}	0.06
CO2		17.36	53.27^{x}	70.63^{xy}	0.06
CO3		14.68	58.34^{x}	58.34^{x}	0.06
SEM		1.726	1.355	1.395	0.0027
P-value					
L		0.1421	< 0.0001	< 0.0001	0.0445
CO		0.0916	< 0.0001	0.0007	0.5812
L × CO		0.1869	< 0.0001	< 0.0001	0.0945

L0: forage without leucaena substitution; L25: forage + 25% leucaena substitution; L50: forage + 50% leucaena substitution; L75: forage + 75% leucaena substitution (on DM basis); CO1: substrate without corn oil; CO2: substrate with 1% corn oil addition; CO3: substrate with 2% corn oil addition; a: soluble fraction; b: insoluble but potentially degradable DM; a + b: potential degradation and c: fermentation rate.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

This result was in contrast with other studies, where increased levels of tannin-containing plants tend to have a negative effect on gas production and other rumen fermentation parameters.

Jayanegara *et al*. (2009) reported that the addition of pure tannins of 0.5 mg/mL decreased gas production *in vitro*. The similar results were also confirmed by Sofyan *et al.* (2016), the addition of pure tannin lowers the production of VFA and gas production *in vitro*. The ability of tannins to interact with the nutrient polymer, enzymes, and rumen microbes lead to reduce gas production. The reduction of gas production only detected in L75 indicates that tannins concentration contained in L75 started to adversely affect GP.

Bhatta *et al.* (2012) and Soltan *et al*. (2013) supported where an increase in the proportion of leucaena at 60% was able to optimize the rumen fermentation. Soltan *et al.* (2013) reported that the content of mimosine in the leucaenacan inhibit CT activity, contributes to an increase in rumen fermentation activity as previously confirmed by Hart *et al.* (2008) that rumen microbes were able to degrade some secondary metabolites in the leucaena such as mimosine and use them as energy sources (Soltan *et al*. 2016). Allegedly, an increase in GP could be associated with the lower NDF content of leucaena as shown in Table 1, resulting in a higher the proportion of soluble carbohydrates. In addition, the high content of nitrogen and soluble carbohydrates spurred higher production of microbial biomass so that the fermentation activity is also higher. The higher ME was observed in L25, L50, and L75 (P<0.05). The mean value of ME tended to be higher with the addition of corn oil is presumably because oil has a high energy density (Hristov *et al*. 2013).

Partitioning factor

Partitioning factor (PF) is important to calculate in order to determine the efficiency of rumen microbes in degrading dry matter and organic matter material. This approach is needed as one of the important supporting factors in feed evaluation through gas production techniques. Therefore, the measurement of gas production and rumen fermentation characteristics individually would not sufficient in describing the effectiveness of rumen microbes in degrading feed substrates.

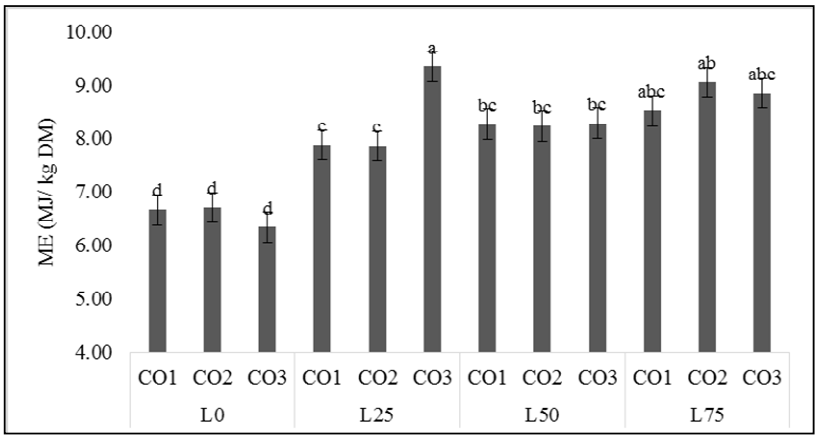

Figure 1 Metabolizable energy value (MJ/kg DM) as influenced by levels of leucaena and corn oil

L0: forage without leucaena substitution; L25: forage + 25% leucaena substitution; L50: forage + 50% leucaena substitution; L75: forage + 75% leucaena substitution (on DM basis); CO1: substrate without corn oil; CO2: substrate with 1% corn oil addition; CO3: substrate with 2% corn oil addition

The means within the same column with at least one common letter, do not have significant difference (P>0.05)

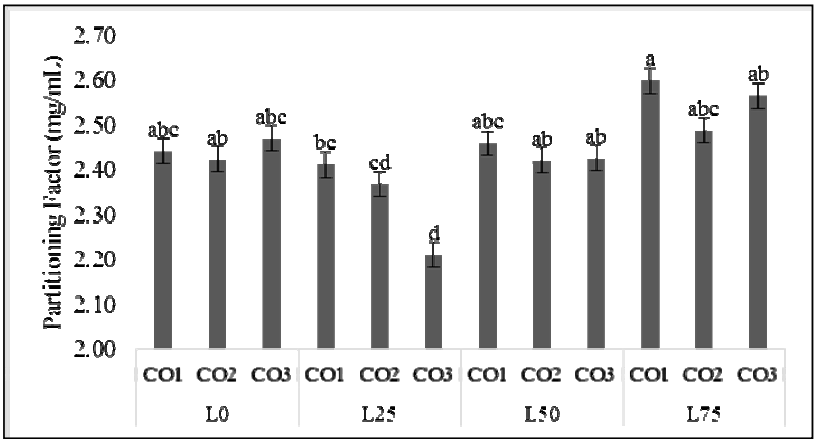

Figure 2 Partitioning factor (mg/mL) as influenced by levels of leucaena and corn oil

L0: forage without leucaena substitution; L25: forage + 25% leucaena substitution; L50: forage + 50% leucaena substitution; L75: forage + 75% leucaena substitution (on DM basis); CO1: substrate without corn oil; CO2: substrate with 1% corn oil addition; CO3: substrate with 2% corn oil addition

The means within the same column with at least one common letter, do not have significant difference (P>0.05)

Blümmel *et al.* (1997) defined the value of PF as mg of degraded substrate per mL of gas produced for 24 h incubation. The PF values in the present study are presented in Figure 2.

The results showed an increase in PF value along with an increase in the proportion of leucaena (P<0.01). The mean value of L75 was higher than control (2.55 *vs.* 2.44, P<0.01).

There was no difference between L25 and L50 compare to control. It was possible that the CT level does not have a significant effect on the effectiveness of degradation. These results simultaneously confirmed that low levels of CT, particularly in the L25 and L50, are less effective in affecting nutrient digestibility and rumen fermentation.

When associated with methane gas production, CT at that level predominantly has a direct effect on methanogenic bacteria and protozoa rather than indirect effect on nutrient digestibility.

The higher PF indicates the higher biomass and the ability of rumen microbes to degrade nutrients component. In another word, it can be explained the higher efficiency of microbial protein synthesis occurred.

Forage with high PF is known to be positively correlated with DM intake and microbial protein synthesis shown by purine derivatives *in vivo* (Blümmel, 2000). In addition, the value of PF also has a relationship with the production of methane gas, where the higher the PF value the lower the production of methane produced (Blümmel, 2000).

These relationships provide important information in feed evaluation to know its potency in reducing methane emission while improving rumen fermentation efficiency. Jayanegara *et al*. (2015) reported that the introduction of CT linearly improved the PF which was also confirmed in suppressing methane production. Furthermore, PF value decreased with CO addition as occurred on CO3 ($P<0.01$). The average PF value suggested that the PF in CO_3 was lower than CO1 or control (2.41 *vs*. 2.48, $P<0.01$). These results indicate that PUFA from CO negatively affected gas production and nutrient digestibility. PF value also decreased as observed in the L25CO2 and L25CO2 which might be due to the low production of microbial biomass as a result of the unbalanced metabolism of nitrogen and energy in the rumen.

CONCLUSION

In conclusion, this study demonstrated that incorporation of 25% leucaena into the ration improved *in vitro* organic matter digestibility and gas production kinetics while a higher rate of supplementation did not give a significant contribution in terms of gas production on *in vitro* rumen fermentation system. Supplementation of corn oil up to 2% did not show a detrimental effect on *in vitro* rumen digestibility and fermentation. To know other beneficial effects and specific mechanisms in modulating rumen fermentation, further study is needed *in vitro* and *in vivo*.

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