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SUPPLEMENTAL EFFECT OF CONDENSED TANNINS FROM SENGON LEAVES (*ALBIZIA FALCATARIA*) ON *IN VITRO* GAS AND METHANE PRODUCTION

A. Afzalani,* R. A. Muthalib, R. Raguati, E. Syahputri, L. Suhaza and E. Musnandar

Faculty of Animal Science, University of Jambi, Jambi Indonesia 36361

*Corresponding Author: afzalani@unja.ac.id

ABSTRACT

The present study was conducted to evaluate the effect supplementation of sengon leaves (SL) with a condensed tannin (CT) content on the *in vitro* profile of cumulative gas production (GP), methane (CH_4) production, and rumen fermentation parameters. The SL containing 8.84% CT (88,4 mg per g SL) was supplemented at different levels of T0, T1, T2, T3 (0, 2, 4, and 6% CT per dry matter incubation of basal ration, respectively) during 48 h fermentation in 120 ml of serum bottles. The basal ration (BR) consisted of *Brachiaria mutica* grass and concentrate mix (60:40 ratio). Rumen fluid was collected from ruminally fistulated Bali cattle (*Bos sondaicus*). The profile of cumulative gas and methane production was fitted using $y= a+ b (1 – e^{-ct})$. The profile of cumulative gas and methane production was lower at T3 and T2 than at T1 and T0. Methane gas production stabilized after 24 h of incubation for T3 and T2. Meanwhile, the profile of methane GP was stabilized after 36 h for T1 (2% CT) and after 48 h for T0 (0% CT) (control). The fermentation parameters measured in this study showed that increased supplementation with CT-SL significantly ($P<0.05$) decreased the in vitro dry matter digestibility (IVDMD), in vitro organic matter digestibility (IVOMD), ammonia ($N-NH_3$), total volatile fatty acid (TVFA), total gas (TG, ml), total methane (ml) and methane percentage (CH_4 per TG). The study concluded that supplementation of CT-SL at T2 (4% CT equal to 0.09 g SL/0.2 g BR, or equal to supplementation of 45% SL per BR (DM basis) was more effective in controlling methane production and was still favorable in IVDMD, IVOMD, $N-NH_3$, and TVFA to maintain ruminal microbial activities and ruminant needs. The results suggest that evaluation under *in vivo* conditions is needed.

Key words: supplementation, sengon leaves, condensed tannins, methane, *in vitro*

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INTRODUCTION

Methane emitted from livestock primarily contributes to the total greenhouse gas (GHG) in the atmosphere. The methane emitted from ruminants can cause dietary gross energy loss up to 2-12% and represent globally 2,779 and 2,344 Mt CO_2-e/year in 2010 and 2020, respectively (Hristov *et al*., 2013; Subepang *et al*., 2019). In Indonesia, the agricultural sector was the third-highest emitting sector, with a total emission of 132 Mt CO_2-e in 2005, which is 13.6% of the national GHG emissions. It will grow up to 25 percent to 164 Mt CO_2-e in 2030 (NCCC, 2010). Methane emissions from the agriculture sector accounted for seventy-one percent of the emissions from rice fields, whereas livestock contributed to 16.5% of methane emissions from the agricultural sector. Emissions from the livestock sector are expected to increase from 25 Mt CO_2-e to 39 Mt CO_2-e. This condition is estimated to cause an increase of more than 50 percent in additional emissions (NCCC, 2010). The Indonesian government has planned to abate 105 Mt CO_2-e by 2030 from agriculture, including the livestock sector, by improving water management in rice cultivation, the restoration of degraded land and feed supplementation or feeding strategies for livestock (NCCC, 2010).

An approach to reduce methane production from ruminants has been conducted and shown to be effective using natural compounds in plants (Cottle *et al*., 2011; Jayanegara *et al*., 2014). In the tropics, as in Indonesia, there is a diversity of plants that, due to their good nutritive value, hold potential for ruminant feeding (Bhatta *et al*., 2013). Plants rich in condensed tannins can decrease bacterial and protozoal populations in the rumen as well as CH_4 emissions (Puchala *et al*., 2012). Some research on the mitigation of CH_4 production and improvement of ruminant performance has been done by supplementation of tree foliage with tannin content (Kennedy and Charmley 2012; Anas *et al*., 2015; Gemeda and Hassen, 2015; Min *et al*., 2015).

Feeding condensed tannin-containing forages or feedstuffs to ruminants may be an effective natural practice for mitigating CH_4 emissions by ruminant livestock and increasing metabolizable energy intake (Naumann *et al*., 2017). The action of tannins in methane production directly prevents growth and methanogen activity (Tavendale *et al*., 2005) and indirectly creates complexes with polysaccharides and proteins by limiting

access to methanogens (Naumann *et al*., 2017). Nonetheless, the voluntary intake will decrease if the intake of condensed tannins is above 7% of the dry matter (DM). Moderate intake of condensed tannins (3-6% of DM ratio) may also lead to positive responses (Vázquez *et al*., 2016).

Sengon (*Albizia falcataria*) is a tree foliage of legumes planted to be used by the wood, paper, and paper tissue industries. Laboratory analysis has found that sengon leaves contain a high polyphenol compound as well as a condensed tannin content of 8.84% dry matter (DM).

Based on this finding, the purpose of the present study was to determine the supplementation effect of Sengon leaves (SL) (*Albizia falcataria*) as a condensed tannin source on *in vitro* gas and methane production.

MATERIALS AND METHODS

Sample Collection, Extraction and condensed tannin Analysis: Sengon leaves (SL) samples were obtained from the teaching and research farm of the University of Jambi, Faculty of Animal Science, Indonesia. The sample was air-dried indoors, oven dried for 24 hours at 60°C and ground to pass through a 1 mm sieve before condensed tannin (CT) analysis and *in vitro* incubation.

Extraction was carried out using maceration techniques with 95% methanol as a solvent. A total of 100 g of sample and 300 ml of methanol (1:3 w/v) were put into a glass beaker and soaked for 12 hours. The extract obtained was filtered with four-layer cloths. The filtrate obtained was then concentrated using a rotary evaporator and dried in an oven at 40°C to a constant weight. (Mayangsari *et al*., 2013).

To determine the condensed tannin (CT) fraction, the extract was treated with butanol-HCl in the presence of ferric ammonium sulfate, and CT was expressed as leucocyanidin equivalents as follows:

CT= (A550 nm×782.6)/sample weight (DM), where A550 nm is the absorbance value at 550 nm. Assuming that the effective E1 cm, 550 nm of leucocyanidin was 460 (Porter *et al*. 1986).

***In vitro* Rumen Fermentation and GP Measurement:** The basal rations (BR) used in this study were prepared with a protein content of ±13% and a TDN of ± 65% with *Brachiaria mutica* (BM) grass (60%) and concentrate mix (40%). The concentrates were formulated using 58% rice bran, 25% ground corn, 6% soybean meal, 9% coconut cake, 1% mineral mix, and 1% salt. The ration mixtures (DM basis) were dried at 60°C for 12 hours and ground with a 1 mm sieve. The nutrient composition of feeds and basal ration (BR) are presented in Table 1.

Table 1. Nutrient Composition of *Brachiaria mutica*, *Sengon Leaves*, Concentrate and Basal Ration

Nutrients	*Brachiaria mutica*	Sengon Leaves	Concentrate	Basal Ration
DM, %	92.25	92.02	91.48	91.94
CP, %	12.94	14.07	12.47	12.75
CF, %	27.67	14.64	8.81	20.13
EE, %	1.37	3.61	3.64	2.28
NFE, %	50.13	63.21	68.74	57.57
NDF, %	71.98	51.22	50.85	63.53
ADF, %	34.65	25.86	14.78	26,70
Ash, %	7.89	4.47	6.34	7.27
TDN, %	56.76	72.41	78.32	65.38
CT, %	-	8.84	-	-

DM; Dry matter, CP; Crude protein, CF; Crude fiber, EE; Ether extract, NFE; Nitrogen free extract, NDF; Neutral detergent fiber, ADF; Acid detergent fiber, CT; Condensed tannins

The Theodorou and Brooks (1990) method was applied for the *in vitro* incubation. Approximately 0.2 g sample ration was scaled into a 120 ml bottle of serum to be kept in a 39°C incubator. Rumen fluid was obtained from fistulated Bali cattle in the morning before feeding time, which was then brought to the laboratory, strained using four layers of cheesecloth, and then mixed with McDougall buffer solution (1:4 v/v) according to the procedure in Tilley and Terry (1963). A total of 2000 ml of a mixture of rumen fluid and McDougall buffer solution was put into a 2500 ml dark bottle, saturated with CO_2, and then installed with an automatic dispenser pipette. Condensed tannins (CTs) were added and incubated in a serum bottle according to the following treatments: T0: basal ration (BR), T1: T0 + 2% CT (equal to 0.05 g SL/0.2 g BR, dry feed basis), T2: T0 + 4% (equal to 0.09 g SL/0.2 g BR, dry feed basis), and T3: T0 + 6% CT (equal to 0.14 g SL/0.2 g BR, dry feed basis). An amount of 40 ml rumen buffer solution was placed in each serum bottle. The bottles were sealed with rubber stoppers and crimp seal caps. A syringe was used to inject a small amount of gas into the serum bottle through the rubber stopper to initiate the point of incubation. A total of 51 serum bottles for 5 serum bottles of each sample treatment and 3 serum bottles as blanks (*i.e*.,

Rumen fluid only), were incubated in an incubator at 39°C for 48 h.

The volume of GP was recorded manually at 2, 4, 8, 16, 24, and 48 h after incubation by an inserted needle with a 10 ml gas syringe through the rubber stopper. GP was read by looking at changes in the scale of the gas syringe (***Fortune***®). Total gas value were corrected the blank. This study measured methane GP based on the Fievez *et al*. (2005) method. The total gas produced was flown into 4 M NaOH. carbon dioxide, which was the main gas produced during in vitro rumen fermentation, was then bound to NaOH. Another syringe connected to the system was used to read the methane volume. Methane gas production was corrected with a blank, and the methane concentration was determined as:

$$\frac{\text{Net methane production}}{\text{Net Gas production}} x\ 100$$

The methane reduction potential (MRP) was calculated by taking the net methane value for the control (basal ration without CT) as 100%:

$$\text{MRP} = \frac{\%\text{N m ii c} - \%\text{N m ii C st}}{\%N\ m\ ha\ i\ c} x\ 100$$

A total of 51 serum bottles for 2 serum bottles of each sample treatment and 3 serum bottles as blanks (*i.e*., Rumen fluid only) were incubated in an incubator at 39°C for 48 h. At the end of 48 h after fermentation, the pH was measured to determine the final pH using pH matter and then centrifuged at 6000×g for 15 min to obtain residues for the determination of in vitrodry matter digestibility (IVDMD) and in vitro organic matter digestibility (IVOMD). Meanwhile, the supernatant was used to determine total VFA and ammonia-nitrogen (N-NH_3). The IVDMD and IVOMD were measured using the procedures of AOAC (2007). The TVFA was determined using steam distillation and N-NH_3 with microdiffusion *Conway* according to the General Laboratory procedure (1966). The profile of cumulative gas and methane production was fitted to the model of Ørskov and McDonald (1979) as follows:

$$y = a + b\ (1 - e^{-ct})$$

where ***a*** is the gas and methane production from immediately soluble fraction, ***b*** the gas and methane production from the insoluble fraction, ***c*** the gas and methane production rate constant for insoluble fraction (***b***), t the incubation time, and y the gas and methane produced at time '***t***'.

Statistical Analysis: The experiment was conducted in a completely randomized design (CRD) that consisted of four treatments and five replicates. Each replicate was represented by two incubation bottles. Data obtained from the experiment were analyzed using analysis of variance (ANOVA). Comparisons among treatments were performed by applying Duncan's multiple range test with a significant difference at $P<0.05$. The data were analyzed using SPSS statistical software version 17.0.

RESULTS AND DISCUSSION

Profile of Gas Production (GP): The effect of supplementation of SL with CT content on the profile of GP is presented in Figure 1. It can shown (Figure 1) that the length of incubation time tends to increase the cumulative GP, where is because of the increased total substrate fermented by rumen microbes. However, supplementation of SL with a CT content had affected in reducing total GP. The average GP obtained in this experiment ranged from 1.06-1.55 ml per hour. The rate of GP was decreasing in line with the increased incubation time and level of CT supplementation. GP at 48 h varied between 50.17 - 73.47 ml, with the highest being for T0 (without CT) and lowest for T2 (4% CT) and T3 (6% CT).

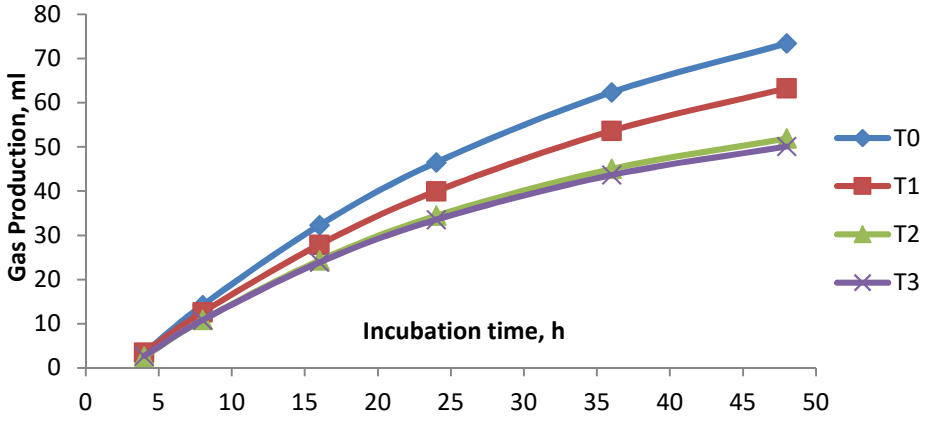

Figure 1. The Effect supplementation of sengon leaves with condensed tannin content on cumulative gas production (ml).

GP after fermentation greatly indicates the number of available carbohydrates as the energy source for rumen microorganisms (Ushlu *et al*., 2018). The extent of GP depends on the amount of fermentable carbohydrates, but the presence of secondary metabolite substances such as tannins may also influence it (Jayanegara *et al*., 2014; Kondo *et al*., 2014; Bueno *et al*., 2020). Tannins are also found to decrease GP in the *in vitro* system by forming a tannin-macromolecule complex, which inhibits the activities of the microbial fibrolytic enzyme. CT is able to reduce the fermentation and digestibility of organic matter found in the rumen by altering the proportion of VFAs, particularly the acetate:propionate ratio. Under *in vitro* conditions, it increases the molar proportion of propionate without unaffecting the molar proportion of acetate (Jayanegara *et al*., 2012; Hassanat and Benchaar, 2013; Ningrat *et al*., 2017; Bueno *et al*., 2020).

Profile of Gas Methane: The effect of supplementation of SL with a CT content on the profile of methane GP is presented in Figure 2.

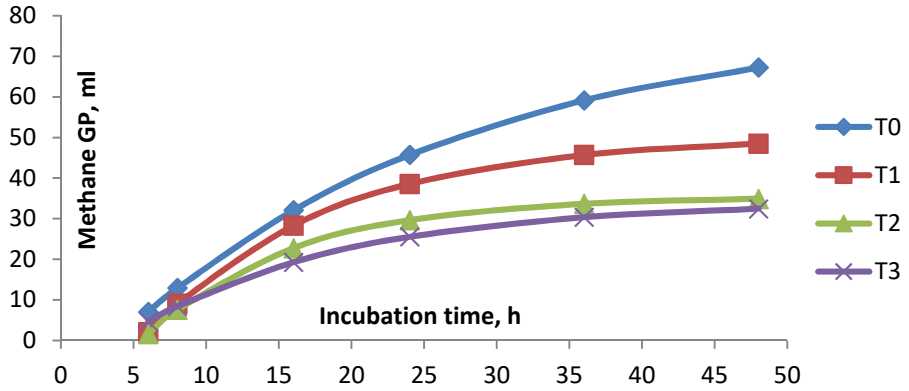

Figure 2. The effect supplementation of sengon leaves with condensed tannin content on methane gas production (ml)

In general, from figure 2 above, all treatments increased methane production during early incubation at 6-16 h and then began a decline after 16 h, as shown by the slope of the curve and stabilized after 24 h, especially for the T2 (4% CT) and T3 (6% CT) treatments. Meanwhile, the methane GP profile was stabilized after 36 h for T1 (2% CT) and after 48 h for T0 (0% CT) (control). This shows that methanogens are more active at the early incubation stage but less active as the fermentation substrate is reduced. However, it appears that supplementation of feed containing CT tends to reduce methane GP. The specific effect of CT on the reduction in methane emissions is unknown (Piñeiro-Vázquez, *et al*., 2015), but other studies have pointed out that they form complexes with dietary proteins and cabohydrates in the rumen, thus decrease the DMD and OMD and indirectly affect the release of H_2 (Jayanegara *et al*., 2011; Bueno *et al*., 2020).

Rumen Fermentation Parameters: The fermentation parameters (pH, IVDMD, IVOMD, N-NH_3, total VFA, total gas, total methane and methane reduction potency) are presented in Table 2.

Tabel.2. Effect of supplementation sengon leaves with condensed tannin content on rumen fermentation parameters.

Parameters	Treatments				SEM	p value
	T0	T1	T2	T3		
pH	6.92	6.92	6.90	6.86	0.033	<0.487
IVDMD (%)	62.738[a]	56.08[b]	50.28[c]	39.68[d]	1.736	<0.001
IVOMD (%)	62.86[a]	47.41[b]	44.450[b]	36.382[c]	1.285	<0.001
N-NH_3 (mM)	6.45[a]	3.93[b]	3.59[c]	3.26[c]	0.173	<0.001
TVFA (mM)	178.00[a]	161.00[b]	150.00[c]	123.00[d]	3.122	<0.001
TG (ml)	74.45[a]	64.30[b]	52.98[c]	51.10[c]	0.933	<0.001
CH_4 (ml)	66.47[a]	47.982[b]	33.53[c]	31.03[c]	0.947	<0.001
CH_4 (% TG)	89.29[a]	74.63[b]	63.29[c]	60.63[d]	0.821	<0.001
MRP (%)	-	27.82	49.56	53.32	-	-

[a, b, c, d] Different superscript letters in each row indicate significant differences at p<0.05.

T0 to T3 were treatments supplemented with 0, 2, 4, and 6% condensed tannin-sengon leaves from dry matter of feeds. IVDMD = in vitro dry matter digestibility, IVOMD= in vitro organic matter digestibility, N-NH_3= N-ammonia, TVFA= total volatile fatty acids, TG= total gas, CH_4= gas methane, MRP= methane reduction potency.

Ruminal pH is a fermentation parameter that quantifies the state of acidity and alkalinity of the rumen. The results in Table 2 indicate that supplementation of SL with a CT content did not affect (P> 0.05) rumen pH. The obtained rumen pH is approximately 6.86-6.92. This pH value is still in the optimal range for rumen microbial fermentation activity, where the normal rumen pH for rumen microbial activity is 6.0-7.0 (Grant and Martens, 1992). Meanwhile, for optimal rumen function, Kamra (2005) and Ososanya *et al*. (2013) state that rumen pH must range between 6.0-6.8.

The present study showed that increasing the supplementation of SL with a CT content linearly (P<0.05) decreased IVDMD and IVOMD. This result is in line with that obtained by Kondo *et al*. (2014) and Yuliani *et al*. (2014), where supplementation of tannins from lerak fruit (*Sapindus rarak*) and tea byproducts caused a decrease in IVDMD and IVOMD. Another study found that adding 2 mg/ml tannin during the 48 h incubation period significantly reduced IVDMD and IVOMD (P<0.05) (Yugianto *et al*., 2014). The decrease in IVDMD and IVOMD with increased supplementation of SL with a CT content is due to the role of tannins in forming complex bonds with carbohydrates and proteins. Stergiadis and Harvey (2017) state that tannins can bind to protein and polysaccharides so that the feed becomes difficult to degrade by rumen microbes and can cause a decrease in the value of feed digestibility. Bueno *et al*. (2020) found that tannins inhibited DMD and OMD through their interactions with proteins and polysaccharides. The reduction of DMD and OMD will positively impact an increase in protein and nonstructural carbohydrate flow to the small intestine. Min *et al*. (2006) state that formation complexes of CT with protein and polysaccharides thus increase the amount of protein with low rumen degradability that flows to the small intestine, favoring an increase in daily live weight gain and milk production.

The concentration of ammonia ($N\text{-}NH_3$) in the rumen is influenced by the protein content and amino acids of the feed. Ammonia is formed from the process of deamination of amino acids by microbial activity so that the concentration is influenced by the digestible protein content in the feed. Most of the ammonia absorbed through the rumen wall will be used directly by rumen microbes to meet nitrogen needs (Yugianto *et al*., 2014). $N\text{-}NH_3$ that was obtained in the present study ranged from 3.26 - 6.45 mM (5.54 - 10.97 mg/dl), and this value is still in the optimal range to meet the needs of N microbes in the rumen. According to Satter and Slyter (1974), a minimum $N\text{-}NH_3$ concentration of 5 mg/dl is needed to support optimal rumen microbial growth. Increasing the supplementation of SL with a CT content linearly decreased (P<0.05) the ruminal $N\text{-}NH_3$ concentration. The results of this study are in line with those reported by several researchers in which tannin administration significantly decreased $N\text{-}NH_3$ concentrations (Sharifi *et al*., 2013; Bhatta *et al*., 2013; Yuliana *et al*., 2014; Jolazadeh *et al*., 2017). According to this study, CT from SL has the ability to protect dietary protein from ruminal degradation by forming tannin-protein complexes and could be used to increase bypass protein and to improve ruminant performance. When the sheep's diet was switched from perennial grass without the tannin content into *Lotus corniculatus* with CT (32 g of CT/kg of DM), the proteolytic bacteria *Butyrivibrio fibrosolvens* population decreased (Bhatta *et al*., 2009).

TVFA concentrations and TG values are presented in Table 2. TVFA and TG decreased (P < 0.05) with increasing the supplementation of SL with a CT content. TVFA obtained in this study varied from 123-178 mM. The TVFA value obtained is still optimal to meet the rumen microbial energy needs. Van Soest (1982) states that the concentration of TVFA needed to support optimal microbial growth ranges from 80 - 160 mM. Hungate (1966) stated that a TVFA concentration of 111 mM was the minimum value needed for rumen microbial growth. CT supplementation up to T3 treatment (6% CT) still did not disrupt the energy supply needed by rumen microbes. Bhatta *et al*. (2014) reported that tannins per se do not cause substantial inhibition of TVFA concentrations, which generally does not indicate specific inhibition of rumen fermentation activity. Although the inclusion of tannin plants affects the degradability of OM and NDF, the total and individual VFAs are relatively similar between feeds. This is consistent with the findings of several previous researchers (Hariadi and Santoso., 2010; Jayanegara *et al*., 2011; Bhatta *et al*., 2014; Pineiro-Vazquez *et al*., 2018).

Increasing the level of CT supplementation from SL effectively reduced GT production. The GT produced in this study varied from 51.1 to 74.45 ml/200 mg DM, where GT production was higher at T0 (P <0.05) than at T1, T2, and T3. Meanwhile, T1 treatment was higher (P <0.05) compared to T2, T3, and then between T2 and T3 were not different (P> 0.05) (Tabel 2, Figure 1). The decrease in gas production obtained in this study was due to decreased microbial enzyme activity, especially cellulase enzymes. Cellulase enzymes play a role in degrading the fraction of fibers that contribute to the production of fermented gas in the rumen. Bueno *et al*. (2020) found that tannins can decrease cumulative gas production by forming a tannin-macromolecule complex bond that inhibits microbial enzyme activities. Another study stated that a high tannin concentration in the diet may lead to a reduction in microbial enzyme activities, such as cellulase (Tabbaco *et al*., 2006).

The production of methane gas and the percentage of methane gas from total gas decreased significantly (P <0.05) with increasing levels of CT-SL supplementation. The total methane gas and methane gas percentages vary from 31.03 - 66.47 ml/200 mg DM and

60.63 - 89.29%, respectively. The methane reduction potential (MRP) of T3 (53.32%) and T2 (49.56%) was higher than that of T1 (27.82%). The methane production of T0 and T1 was higher ($P < 0.05$) than that of T2 and T3, while that of T2 and T3 was not different ($P > 0.05$). Hariadi and Santoso (2010) also reported that CH_4 production decreased with increasing concentrations of total tannins in plants. Cieslak *et al*. (2016) confirmed the same where increasing CT decreases TG and methane production. This study showed that CT from SL could act as an antimetanogenic compound by directly inhibiting methane production. On the other hand, CT was also decreasing in OMD and TVFA. This study corresponds to the findings of Jayanegara *et al*. (2009), Jayanegara and Palupi (2010), and Bueno *et al*. (2020) that tannins have a direct influence on methanogens, cellulolytic bacteria, and proteolytic bacteria as well as an indirect influence on decreasing OMD and TVFA production by inhibiting the hydrogen supply for methane production.

Conclusion: Sengon leaves (SL) with condensed tannin (CT) content could be used as a feed supplement to decrease methane production. Supplementation SL with CT content was more effective in decreasing total gas and methane production with a CT concentration of 4% (equal to 0.09 g SL/0.2 g BR or equal to supplementation of 45% SL from a dry feed basis) without a negative effect and was still favorable for IVDMD, IVOMD, N-NH_3, and TVFA to ruminal microbial activities and ruminant animal needs. The results suggest that evaluation of *in vivo* conditions **is needed.**

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