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Research article

The effects of sappanwood *(Caesalpinia sappan)* addition on in vitro methane mitigation, gas production, and ruminal fermentation parameters

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Abstract

This research was designed to examine the methane-mitigating properties of sappanwood and its effects on ruminal fermentation parameters using the in vitro gas production method from Menke and Steingass (1988). The sappanwood was dried and pulverised to obtain the sappanwood powder, which was added to 300 mg of forage:concentrate substrate (60:40) at three different levels (2, 4, and 6% DM basis). The in vitro incubation followed a randomised block design and was incubated for 48 hours. The continuous data was then analysed using mixed models of the mixed procedure in SAS on Demand (SAS Institute, Inc. Carry). The results showed that all treatment levels did not affect total gas production, the gas fractions, total CO_2 productions, NH_3 levels, microbial proteins, and protozoal counts ($P>0.05$). The total CH_4, CH_4 and CO_2 productions per mg dry matter (DM) and organic matter (OM) degraded, digested dry matter (DDM), digested organic matter (DOM), pH values, total VFAs, acetic, propionic, and butyric acid productions differed significantly ($P<0.05$). The results emphasizing the 6% addition of sappanwood showed the best methane mitigating properties without hindering ruminal fermentation processes and ruminal microbes.

Keywords: *In vitro* fermentation, Methane, Plant secondary metabolites, Ruminants, Sappanwood

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INTRODUCTION

Ruminants are characterised by having large, multi-compartmentalised stomachs, and this unique stomach enables a rich variety of microorganisms to thrive, favouring the host (Pérez-Barbería, 2020). The microorganisms in a ruminant's stomach will digest plant cell walls and other feedstuffs, releasing fermentation products such as volatile fatty acids (VFAs), ammonia, H_2, and CO_2. H_2 will reduce CO_2 into CH_4 (methane) by methanogens through methanogenesis (Ku-Vera et al., 2020). Methane is a greenhouse gas that induces the greenhouse effect. Additionally, a high methane concentration indicates energy loss because of inefficient feed digestion. Decreasing methane production could reduce its negative environmental impact and increase feed digestion efficiency.

One of the attempts to reduce methane production is by manipulating rumen microbial fermentation using feed additives from local plants. Amidst plant secondary metabolites, flavonoids can be used to reduce methane production. Oskoueian et al. (2013) explained that flavonoids can lessen methane production, directly affect methanogens, and improve animals' health and production.

Caesalpinia sappan, or sappanwood, is a legume distributed in Southeast Asia. Sappanwood's heartwood contains various water-soluble flavonoid compounds, with brazilin, also known as its natural red colour, as the main constituent of its homoisoflavonoid content (Nirmal et al., 2015). Sommai et al. (2021) used flavonoid extracts from *Alternanthera sissoo* and found that methane production was suppressed and propionic acid production escalated as the flavonoid extract level increased. Oskoueian et al. (2013) used seven pure flavonoid compounds with ≥ 98% purity: rutin, catechin, flavone, quercetin, myricetin, kaempferol, and naringin. They found that, aside from catechin, all kinds of flavonoids suppressed methane production.

Many articles have shown the utilization of bioactive compounds from plants, as well as plant raw materials to reduce methane production from ruminants, but the utilization of sappanwood to reduce methane production from ruminants has not been done until present day. Hypothetically, the addition of sappanwood powder is capable of reducing methane production. Taking all this information into account, this experiment aimed to examine sappanwood's methane-mitigating properties and how it affects ruminal fermentation using the *in vitro* gas production technique.

MATERIALS AND METHODS

Ethical clearance

The Research Ethics Committee from the Faculty of Veterinary Medicine, Universitas Gadjah Mada, has approved the research procedure on 31 January 2023. The certificate's number is No. 017/EC-FKH/Eks./2023. The rumen fluid for this experiment was taken before the first feeding time in the morning at the Animal Science Faculty, Universitas Gadjah Mada, Indonesia.

Chemical composition analyses

Elephant grass (*Pennisetum purpureum*), pollard bran, soybean meal, and sappanwood were analysed using the AOAC procedure (AOAC, 2005) to obtain the contents of dry matter (DM), crude protein (CP), ether extract (EE), crude fibre (CF), and organic matter (OM). Nutrient contents of P.purpureum, pollard bran, soybean meal, and sappanwood were shown in Table 1. Substrate compositions and nutrient contents were shown in Table 2.

Table 1 Feedstuffs used in the experiment

Nutrient contents (%)	Feedstuffs			
	Elephant grass	Bran Pollard	Soybean meal	Sappanwood
Dry matter	12.43	88.53	87.44	93.76
Ash	14.5	4.68	6.21	0.93
Organic matter	85.5	95.32	93.79	99.07
Crude protein	9.81	15.77	42.67	1.24
Crude fat	1.72	2.78	1.52	0.38
Crude fibre	32.08	8.51	4.51	56.31
Nitrogen-free extract	41.89	68.26	45.09	41.15
Total digestible nutrients	55.13	70.51	90.52	-

Table 2 Substrate composition used in the experiment

Feedstuffs (%)	Substrate composition (%)			
	0	2	4	6
Elephant grass	60	60	60	60
Bran Pollard	30	30	30	30
Soybean meal	10	10	10	10
Sappanwood	0	2	4	6
Nutrient contents (%)				
Dry matter	88.71	90.59	92.46	94.34
Ash	10.72	10.74	10.76	10.78
Organic matter	89.28	89.26	89.24	89.22
Crude protein	14.89	14.91	14.94	14.96
Crude fat	2.02	2.03	2.03	2.04
Crude fibre	22.25	23.38	24.51	25.63
Nitrogen-free extract	50.12	50.94	51.76	52.59
Total digestible nutrients	63.28	63.28	63.28	63.28

Sappanwood for this experiment was obtained from Magelang, Central Java, Indonesia. Sappanwood was ground into a fine powder and further analysed for its secondary metabolites: total phenolic compounds using gallic acids equivalent method and total flavonoids using quercetin equivalents method (Phuyal et al., 2020), total saponins using quillaja bark equivalent method (Grandón S et al., 2013), total tannins using tannic acid equivalent and total carotenoids using hexane method (Chanwitheesuk et al., 2005). Sappanwood's secondary metabolite contents were shown in Table 3.

In Vitro Gas Production Test Procedure

The experiment substrates were incubated using the in vitro gas production technique described by Menke and Steingass (1988). The substrate used in this experiment consisted of 60% *P. purpureum*, 30% pollard bran, and 10% soybean meal. Rumen fluid used in this experiment was procured from a fistulated Bali cattle, taken before morning's feeding time, filtered using 4 layers of gauze, and then mixed with buffer solution in a 1:2 ratio (v/v), respectively. The amount of substrate used in this experiment was 300 mg. Each substrate was placed in 100 mL glass syringes (Haberle Labortechnik, Lonsee, Germany). Subsequent to adding the substrate, each of the syringes was filled with 30 mL of buffered rumen fluid and then incubated at 39ºC for 48 hours. The experimental treatments were 0%, 2%, 4%, and 6% addition of sappanwood based on the substrate's DM contents, and each of the treatments was repeated three times. The incubation was executed in two replicates labelled as *week*. The total gas produced was monitored at 0, 2, 4, 8, 12, 24, and 48 hours of incubation by looking at the glass syringe's scale. In vitro gas production kinetics were fitted to an equation from Ørskov and McDonald (1979), which is $y = a + b (1 – e^{ct})$, where y is the gas produced at *t* time, a is the gas produced from easily-degraded soluble fraction, b is the gas produced from insoluble fraction, and c is the constant of gas production rate. Gas samples were taken from each glass syringe at 24 and 48 hours of incubation to measure the concentrations of methane and CO_2.

The incubation was stopped after 48 hours, the buffered rumen fluid was separated from the substrate by using glass wool and gooch crucible, and its pH value was gauged using a pH metre (HI 2210 pH metre, Hanna Instruments, US). The filtered substrate was measured for its degraded dry and organic matter content using the same method as dry and organic matter determination for chemical composition analysis from AOAC (2005).

The DDM was calculated using the equation:

$$(A - B)/A * 100\%$$

with A: substrate's DM weight, B: filtered substrate's DM weight

The DOM was calculated using the equation

$$(C - D)/C * 100\%$$

with C: substrate's OM weight, D: filtered substrate's OM weight

Aliquots of buffered rumen fluid are taken to determine the concentration of fermentation parameters. The NH_3 (Ammonia) concentration was measured using Chaney and Marbach's (1962) method. Volatile fatty acids (VFA) concentration was measured using Filipek and Dvorak's (2009) method. Microbial protein was gauged using the Lowry method (Plummer, 1987). Protozoal counts were counted using Diaz's (1993) method.

Statistical analysis

The experiment followed a randomised block design. The continuous data was analysed using the mixed models of the mixed procedure of SAS On Demand (SAS Institute Inc.). The models incorporated the treatment as fixed effects, the week as random effects, and the interaction of treatments and week as fixed effects. Significant results were further analysed using Tukey's HSD test. The value of $P \leq 0.05$ was set to consider statistical significance.

RESULTS

In vitro total gas and methane production

The results in Table 3 showed that sappanwood has several secondary metabolites. Those are phenols, tannins, saponins, flavonoids, and carotenoids.

Table 3 Secondary metabolites in sappanwood

Secondary metabolites	Concentration	Unit
Phenol	4.77	%
Tannins	10.83	%
Saponins	0.9	%
Flavonoids	0.93	%
Carotenoids	30.04	µg/g

The results in Table 4 showed no significant differences (P>0.05) in total gas production, all the fractions at 48 hours, and CO_2 produced after incubated for 48 hours among all the treatments. The fractions are easily degraded (a), potentially degraded (b), the b fraction's rate of gas production (c), and the potential gas production (a+b). The total amounts of methane produced after incubated for 48 hours are significantly different (P<0.05) with 4% sappanwood addition showing the lowest gas produced. The amounts of methane and CO_2 produced per mg of dry and organic matter degraded are also significantly different (P<0.05) with 6% sappanwood addition showing the lowest gas produced. The DDM and DOM dropped from the control treatment when adding 4% sappanwood, whereas 6% addition showed improvement from the control treatment (P<0.05).

Table 4 Total gas production (ml/300 mg DM) after 48 hours of incubation, gas fractions (a, b, a+b, and c), and methane emissions of four sappanwood addition levels

Gas Production	Sappanwood addition levels (%)				SEM	P-value
	0	2	4	6		
DDM (%)	50.31^{b}	59.62^{a}	46.01^{b}	59.67^{a}	3.21	0.001
DOM (%)	49.14^{ab}	59.67^{a}	44.04^{b}	58.54^{a}	3.75	0.002
Total gas production^{ns}	61.41	61.46	56.19	56.08	2.34	0.043
Gas fractions (ml/300 mg)						
a^{ns}	2.64	3.37	2.56	2.35	0.8	0.617
b^{ns}	61.74	62.25	56.97	57.21	2.07	0.032
a+b ^{ns}	64.39	65.61	59.53	59.56	2.62	0.064
c^{ns} (ml/hours)	0.061	0.055	0.063	0.058	0.005	0.419
CH_4 (mL/300 mg DM)	3.94^{a}	3.92^{a}	3.23^{b}	3.68^{ab}	0.22	0.015
CH_4 (mL/mg DDM)	0.029^{a}	0.024^{ab}	0.026^{ab}	0.022^{b}	0.002	0.013
CH_4 (mL/mg DOM)	0.034^{a}	0.027^{ab}	0.031^{ab}	0.025^{b}	0.003	0.025
CO_2^{ns} (ml/300 mg DM)	21.17	21.23	17.75	19.86	1.23	0.040
CO_2 (ml/mg DDM)	0.158^{a}	0.131^{ab}	0.144^{ab}	0.116^{b}	0.012	0.022
CO_2 (ml/mg DOM)	0.182^{a}	0.147^{ab}	0.169^{ab}	0.133^{b}	0.016	0.034

$^{a-c}$Means within the same rows with varying superscripts differ significantly (P<0.05)
DDM = Digested dry matter, DOM = Digested organic matter

In vitro ruminal fermentation parameters

The results in Table 5 showed that the pH value differed significantly ($P<0.05$) with 4% sappanwood addition increased the pH value from the control and had the greatest value among all the treatments. The NH_3 level, microbial protein, and protozoal count values across all treatments did not differ significantly ($P>0.05$). In contrast the total VFAs, propionic acid, acetic acid, and butyric acid production were significantly different ($P<0.05$) with 4 and 6% sappanwood addition, showing lower concentrations than the control.

Table 5 Ruminal fermentation characteristics of four sappanwood addition levels over 48 hours

Ruminal fermentation characteristics	Sappanwood addition levels (%)				SEM	P-value
	0	2	4	6		
pH	7.03^{b}	7.05^{b}	7.16^{a}	7.14^{a}	0.03	0.003
NH_3^{ns} (mg/100 ml)	39.62	42.13	41.64	42.72	1.22	0.102
Microbial proteins^{ns} (mg/ml)	16.6	16.9	16.61	15.75	1.62	0.901
Protozoal counts^{ns} (*10^5/ml)	1.9	1.8	2	2.3	0.32	0.461
Total VFAs (mM)	121.24^{a}	115.86^{ab}	111.27^{b}	111.12^{b}	2.44	0.002
Acetic acid (mM)	94.20^{a}	89.83^{ab}	86.57^{b}	86.82^{b}	1.83	0.002
Propionic acid (mM)	16.00^{a}	15.42^{ab}	14.59^{b}	14.35^{b}	0.34	0.002
Butyric acid (mM)	8.51^{a}	8.20^{ab}	7.82^{b}	7.71^{b}	0.19	0.002

$^{a-c}$Means within the same row with varying superscripts differ significantly ($p < 0.05$)

DISCUSSION

Secondary metabolites are commonly found in plants for ecological interactions such as attracting pollinators, protecting itself from predators, and so forth. The secondary metabolites found in sappanwood used in this experiment were in line with Vij et al. (2023) who explained that sappanwood contains various bioactive compounds such as flavonoids, saponins, tanins, phenolic acids, alkaloids, steroids, triterpenoids, and anthraquinones.

The results indicated that the secondary metabolites contained in sappanwood powder up to 6% addition were insufficient to disrupt gas production by ruminal microorganisms. The total tannin contents in 6% addition were 1.95 mg and was the highest among all the treatments. Plant secondary metabolites such as flavonoids, saponins, tannins, and phenolic compounds are present in plants in their inactive form as glycoside molecules, and they need to be hydrolyzed by certain enzymes to activate them. The same result is reported by Gunun et al. (2018) who found no significant difference in gas production when supplementing rambutan peel powder (RPP) from 4-20 mg, which contained condensed tannins and saponins, suggesting that tannins from RPP had weak inhibition effects on gas production, the total tannin contents in 20 mg RPP addition were 2.2 mg.

The methane productions at 48 hours were suppressed when 4 and 6% sappanwood were added. However, the suppressed methane production was not followed by an escalation in propionic acid concentration and a reduction in microbial protein values. Sommai et al. (2021) explained that flavonoids may affect the methanogenesis pathway, making H_2 more available for propionic acid synthesis. Ku-Vera et al. (2020) explained that tannins could reduce the

availability of feed, thus impairing the microbial population in the rumen. Thus, these results indicated that the reduction in methane concentration was not caused by flavonoids and tannins in sappanwood.

The methane production decrease could be explained by looking at the methane and CO_2 production per mg DDM and DOM, 6% sappanwood addition depressed the production of both gases. These events could be the effect of saponins. Hess et al. (2003) compared the outcomes of adding three fruits that contain saponins using the rumen simulation technique (RUSITEC) on faunated and defaunated rumen fluid and found a reduction in daily methane production and weak interactions between diets and protozoal status (faunated and defaunated rumen fluid), on that account suggested that saponin could directly influence methanogen activity.

Hence, the 6% treatment showed the best result in mitigating methane production by also taking the methane and CO_2 produced per mg of DDM and DOM and the percentage of DDM and DOM into consideration. The methane and CO_2 produced per mg of DDM and DOM were suppressed to the lowest value when 6% sappanwood was added. However, the percentage of DDM and DOM improved to the highest value, meaning that the 6% sappanwood addition had the same amount of methane and CO_2 produced at 48 hours as the control treatment because it had greater DDM and DOM.

The results showed that sappanwood addition increased ruminal pH without negatively affecting ruminal protein degradation, microbial protein synthesis, and ruminal protozoa. Strobel and Russell (1986) explained that normal ruminal pH ranges from 6.5 to 7.5. The NH_3 value proved that the degradation of protein in the rumen was not affected, the microbial protein showed that microbial protein synthesis was not hindered, and the protozoal count showed that ruminal protozoa was not compromised.

The decrease in total VFA, propionic, acetic, and butyric acid concentrations, when linked to the ascended pH value, showed that sappanwood addition lightly affected cellulolytic microbes' performance. Phesatcha et al. (2022) explained that the optimal pH value for cellulolytic microbes is between 6.3 to 7. Nonetheless, the concentration of total VFAs in this experiment was still within normal levels, as explained by McDonald et al. (2010) who described the normal range for total VFA as 70-150 mmol/L.

No changes were found in the value of protozoal counts despite sappanwood containing both flavonoids and saponins. The cell membrane of the ruminal protozoa was damaged by the interactions of saponin with sterols which resulted in cell lysis. Non-affected protozoal counts could be described by some properties of saponins, according to Jayanegara et al. (2014), the anti-protozoal properties of saponins are source-dependent, relying on the nature, number, and sequence of the sugars in the saponin's structures. Ayemele et al. (2021) found that the pellicles of *Entodinium* were damaged and collapsed when assessing flavonoid-containing plants from Honghe, China, as feed additives for ruminants using *in vitro* technique. Cherdthong et al. (2019) explained that flavonoids might affect ruminal protozoa by inhibiting nucleic acid and cell wall synthesis. Oskoueian et al. (2013) found a drop in protozoal counts when flavonoid flavone, myricetin, naringin, quercetin, catechin, quercetin, and kaempferol were added.

No changes in microbial protein values were found despite the presence of tannin and carotene in sappanwood. Ku-Vera et al. (2020) explained that tannin could form tannin-protein complexes in the rumen, thus reducing substrate availability to rumen microorganisms and then impairing the rumen microbial population. Yan et al. (2007) found an escalation in microbial protein concentration (MCP) and a decrease in NH_3-N compared with the blank when supplemented with β-carotene, suggesting that β-carotene stimulates the growth of cellulolytic bacteria. These results indicated that secondary metabolites at all treatment levels were insufficient to cause adverse effects on protein microbial synthesis and protozoal count.

The microbial protein values aligned with NH_3 levels, emphasizing that all treatment levels did not affect substrate protein degradation and microbial protein synthesis.

CONCLUSIONS

Adding 6% sappanwood powder (DM basis) gives the best methane-mitigating effects without negatively impacting ruminal fermentation processes and microorganisms.

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AUTHOR CONTRIBUTIONS

Hilmy Abdurrasyid Ammar: Planned and executed the experiment, conducted laboratory analysis, analysed the data, and drafted the manuscript. Chusnul Hanim: Planned and supervised the experiment. Asih Kurniawati, Muhlisin, and Andriyani Astuti: supervised the experiment. All authors read and agreed on the final manuscript.

CONFLICT OF INTEREST

All authors declare that they have no competing interests.

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