Research Article

Effect of *Nigella sativa* L. as Saponin Sources on *In vitro* Rumen Fermentation, Enzyme Activity and Nutrients Digestibility

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Abstract | The effect of *Nigella sativa* L. as saponin sources on *in vitro* rumen fermentation, enzyme activity, and nutrients digestibility was investigated in this study. The diet consisted of 70% Napier Grass (*Pennisetum purpureum)* and 30% wheat pollard containing 0%, 0.2%, 0.4%, and 0.6% saponin, respectively. *In vitro* fermentation was conducted using Menke and Steingass gas production and two-stage Tilley and Terry. The acquired data were subjected to variance analysis and Duncan's Multiple Range Test (DMRT). The addition of *Nigella sativa* L. meal significantly decreases (P<0.05) methane (digested organic matter), protozoa population, and increased microbial protein but does not affect (P>0.05) pH, ammonia concentration, and volatile fatty acids (VFA) production. CMCase enzyme activity reduced (P<0.05) as saponin levels increased, but amylase and protein of enzyme were unaffected (P>0.05). Saponin reduced protein digestibility in the rumen, but there was no difference in dry matter (DM) or organic matter (OM) when compared to the control (P>0.05). Post-rumen nutrients digestibility did not affect by the saponin diet (P>0.05). In conclusion, saponins reduced protozoa population, CH_4 production, increased microbial protein, and improve rumen protein digestibility. Therefore, we recommend the use of 0.4% saponins *Nigella sativa* L. to reduce CH_4 production without affecting rumen fermentation.

Keywords | Fermentation, Methane, Rumen, Saponin, Ruminant

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INTRODUCTION

The livestock industry contributes to global climate change as a result of greenhouse gases (GHG). The main GHGs from the livestock sector are carbon dioxide (CO_2), methane (CH_4), ammonia (NH_3-N), and nitrous oxide (N_2O) (Dopelt et al., 2019; Grossi et al., 2019). Methane emissions are not only related to environmental problems but also represent a large part of the energy lost, consequently not utilized for livestock production processes. According to Johnson and Johnson (1995) and Thompson and Rowntree (2020) about 2 to 12% of the consumed feed energy is lost due to the formation of CH_4. Therefore, a mitigation strategy is needed to reduce the rate of GHG accumulation and energy loss that impacts ruminant performance.

An effort to reduce CH_4 emissions and improve ruminant productivity is to utilize the plant's secondary metabolites, one of which is saponins. Saponins decrease the population of rumen protozoa that contribute to CH_4 formation by lysing protozoa cells (Patra and Saxena, 2009; Ramos-Morales et al., 2017; Li et al., 2018). The use of saponins can increase the efficiency of microbial protein synthesis and protein flow into the duodenum (Ramaiyulis et al., 2018; Unnawong et al., 2021). In addition, a decrease in

the population of protozoa and methanogens enhancing the production of VFA, especially propionate (Jayanegara et al., 2014; Anantasook et al., 2016; Darabighane et al., 2021). Meanwhile, rumen defaunation by saponins affects the diversity of rumen microbes such as bacteria, protozoa and fungi, therefore it has an impact on rumen enzyme activity (Patra and Saxena, 2009).

Nigella sativa L. is one of the plant sources of saponins. *Nigella sativa* L. meal is by-product of extracting oil rich in nutrient such as crude protein (33.13%), fat (12.72%), all essential amino acids and also fatty acids. According to Abbas et al. (2013) and Michel et al. (2011) *Nigella sativa* L. contains 4.54% saponins, and other secondary metabolites such as flavonoids and alkaloids. In addition, *Nigella sativa* L. has antioxidant, antifungal and antibacterial properties (Niu et al., 2020).

Unnawong et al. (2021) reported that the use of saponins from *Sesbania grandiflora* pod meal significantly reduced the digestibility of protein, NH_3-N, protozoa, NH_4, while the total VFA increased when used in Thai Purebred Beef Cattle feed. The use of other plants containing saponins such as *Delonix regia* seed meal decreased CH_4 production and increased DM digestibility, but did not affect total VFA (Supapong et al., 2017). Anantasook et al. (2016) reported that the use of *Terminalia chebula* Retz. increased total VFA and decreased CH4, protozoa and acetate concentrations. However, the effect of using *Nigella sativa* L. meal containing saponins on the rumen of cattle has not yet been evaluated.

The hypothesis was made: *Nigella sativa* L. decreased CH_4 production and protozoa population in the rumen, but increased propionate concentration, enzyme activity and feed digestibility. Based on the description, this study aimed to determine the effect of adding *Nigella sativa* L. as a source of saponins to reduce CH_4 gas emissions on rumen fermentation and nutrient digestibility *in vitro*.

MATERIALS AND METHODS

Samples collection and preparation

Pennisetum purpureum grass was oven-dried at 55°C for three days subsequently ground to pass through a 1 mm sieve. Samples of grass, pollard, and *Nigella sativa* L. meal were analyzed proximately according to the AOAC (2005) method. Analysis of the saponin content in *Nigella sativa* L. using the spectrophotometric method according to Uematsu et al. (2000).

Animals and preparation of rumen inoculum

One Bali Cattle with body weight (BW) 400kg was fed 70% forage and 30% concentrate separately for feed adaptation. Feed was given twice a day with a portion of 60% in the morning and 40% in the afternoon for ten days before rumen fluid was used. Rumen liquor was obtained from Bali cattle before the morning feeding. The rumen fluid was filtered through cheesecloth into pre-warmed thermo flasks with water at 39°C and then transported to the laboratory.

In vitro fermentation and sample analysis

In vitro fermentation was carried out using two techniques, gas production according to Menke and Steingass (1988), to measure fermentation parameters and enzyme activity in the rumen and two steps Tilley and Terry (1963) to determine feed digestibility. For each treatment, three replications was prepared.

Menke and steingass

Menke and steingass gas production was carried out using 300 mg of substrate consisting of 70% *Pennisetum purpureum* grass, 30% pollard, and *Nigella sativa* L. meal as a source of saponins weight into 100 mL syringe. *Nigella sativa* L. was added until the feed saponin content was 0%, 0.2%, 0.4%, and 0.6%. Each treatment was replicated three times, and each replication was duplicated. The chemical composition of feed ingredient and proportion of dietary treatment was shown in Tables 1 and 2. Ruminal fluid was mixed with the artificial saliva solution of Menke and Steingass (1988) in a proportion 2:1 (v/v) at 39°C under continuous flushing with CO_2 and 30 mL of rumen inocula mixture were added into each syringe under CO_2 flushing. The syringe was sealed with rubber stoppers and aluminum caps and incubated at 39°C (48 h) for the in vitro gas test. A syringe without feed with 2 replications was used as a blank containing rumen fluid and a standard syringe filled with 300 mg of Pangola grass. Gas production was measured at 1, 2, 4, 6, 8, 12, 24, 36, and 48 hours. When the volume reached its maximum, the gas was removed and calibrated syringe.

Table 1: Chemical composition of feed ingredients.

Parameters (%)	*Nigella sativa* L.	Pennisetum purpureum	Pollard
Dry matter	91.55	90.75	88.08
Organic matter	85.13	76.86	74.45
Crude protein	5.45	33.4	9.05
Extract ether	3.13	1.9	9.7
Crude fiber	3.81	2.34	9.7

Table 2: The proportion of dietary treatment.

Saponin levels	*Nigella sativa* L. (mg)	*Pennisetum purpureum* (mg)	Pollard (mg)
0.0	0	190	80
0.2	13.30	190	80
0.4	26.60	190	80
0.6	39.90	190	80

At the end of 48 hours of incubation, the fermented gas sample was used to analyze the levels of methane and carbon dioxide by gas chromatography method (Filípek and Dvořák, 2009). The fermented substrate was filtered, the residue was used to measure digestible dry matter and digested organic matter. The filtrate was used for pH measurement using pH meter (Hanna Ckecker 1 pH Taster, Hanna Instruments, Ann Arbor, Michigan, USA) and protozoa assays (Diaz et al., 1993). The other filtrate was centrifuged (3000 rpm, 10 min) to determine the ammonia content using the spectrophotometric method (Chaney and Marbach, 1962), and the remainder was centrifuged (10,000 rpm, 15 min). The precipitate was used for microbial protein assay by the Lowry method (Plummer, 1987), while the supernatant was used to determine VFA using gas chromatography method (Filípek and Dvořák, 2009) and the activity of CMCase and amylase enzymes (Halliwell, 1961).

Tilley and terry

Two steps Tilley and Terry's *in vitro* fermentation was incubated for 48 hours and 96 hours, respectively. The first step was digestibility in the rumen, a total 56 test tubes with a volume of 50 mL were filled with 250 feed material substrate and *Nigella sativa* L. with levels of 0, 0.2%, 0.4%, and 0.6%, rumen fluid and medium solution. The medium solution was mixed with rumen fluid in a 4:1 ratio. The medium solution used for testing the digestibility of DM and OM was 50 mL and 100 mL for the CP digestibility. Tubes without feed samples were used as blanks. The mixed solution is then flushing with CO_2 and sealed with a rubber stopper that has a valve to release the gas from the fermentation. The tubes were incubated for 48 hours at 39°C. The 28 tubes containing the fermented substrate were filtered using a glass wool crucible. Tilley and Terry 48 h *in vitro* fermentation residue was employed to determine the digestibility of crude protein (CP, OM, and DM in the rumen.

The second stage is post-rumen digestion. A total of 28 tubes containing substrate after being incubated for 48 hours were added with 3 ml of 20% HCl and 1 ml of 5% pepsin (3:1). The tubes that had been added with HCl and pepsin were incubated for another 48 hours at 39°C. After incubation, the fermented substrate was filtered, and the residue was analyzed for CP, OM, and DM to determine post-rumen nutrient digestibility.

Data analysis

Data were analyzed by one-way analysis of variance (ANOVA) using the Statistical Package for the Social Sciences (SPSS) program version 16.0. Differences between treatment means were further analyzed using Duncan's Multiple Range Test (DMRT) test (Gomez and Gomez, 1984).

RESULTS AND DISCUSSION

Effect of *Nigella sativa* L. on rumen fermentation and gas production parameters

The effect of using *Nigella sativa* L. meal as a source of saponins on rumen fermentation parameters and gas production is presented in Table 3. The addition of *Nigella sativa* L. meal with 0.6% saponin content did not affect ($P>0.05$) the rumen pH value. Rumen pH values in this study ranged from 6.72 to 6.81 and were still in the normal pH range of 6.3 to 7, according to Reis et al. (2014). A previous study showed that the use of 5% *Ageratum conyzoides* leaf extract and 5% *Zingiber officinale* in the diet did not affect pH, with a pH range of 6.83 to 6.95 (Hapsari et al., 2018). Another study showed that supplementation of 0.4 and 0.6% of *Sesbania grandiflora* pod meal did not affect rumen pH, ranging from 6.76-6.80 (Unnawong et al., 2021). Variations in rumen pH value are influenced by the feed consumed. Feeds containing grains caused a decrease in the pH to less than 5.0, while fibrous feeds increased the pH by more than 7.0 (Sung et al., 2007; Kim et al., 2018; Ogata et al., 2019). The fiber content of feed can affect rumen pH. According to Dijkstra et al. (2012), the content of VFA as a result of fiber degradation by cellulolytic microbes is closely related to the rumen pH value.

The addition of *Nigella sativa* L. meal with 0.6% saponin content did not affect ($P>0.05$) the rumen NH_3-N concentration. In previous studies, the use of 0.52% tea saponins did not affect the rumen NH_3-N content of lactating dairy cows (Guyader et al., 2017). In another study conducted by Aazami et al. (2013), the use of 0.02% saponins in Baluchi sheep feed and 0.054% in Saanen kids did not affect rumen NH_3-N concentrations. The concentration of NH_3 illustrates the degradation of protein by microbes. Microbes will degrade more than 60% of the protein in the rumen into amino acids, peptides, and NH_3-N (Kamalak et al., 2005; Liu et al., 2019a). The higher the protein content, the higher the concentration of NH_3-N produced (Griffin and Bradshaw, 2019). According to Putri *et al.* (2021), Rumen NH_3-N is utilized for microbial protein synthesis. Rumen NH_3-N concentrations ranged from 8.5 to 30 mg/100 mL (McDonald et al., 2012), excess NH_3-N more than 50 mg NH_3-N/L did not affect microbial protein synthesis and was excreted (Satter and Slyter, 1974; Neto et al., 2019).

When compared to the control, the usage of *Nigella sativa* L. meal containing saponins 0.4 and 0.6% enhanced ($P<0.05$) 8.8 and 24.44% microbial protein, respectively, while the 0.2% level had no effect. Previous studies showed

Table 3: Effect of *Nigella sativa* L. on rumen fermentation parameters.

Parameters	Saponin levels (%)			
	0	0.2	0.4	0.6
pH	6.81±0.03	6.77±0.08	6.74±0.02	6.72±0.01
NH_3-N (mg/100 mL)	74.70±5.03	71.24±7.40	66.14±3.02	72.21±1.36
Microbial protein (mg/mL)	0.45±0.04^{a}	0.46±0.03^{a}	0.49±0.01^{ab}	0.56±0.06^{b}
Protozoa (10^3 cells/mL)	1.46±0.08^{b}	1.77±0.10^{c}	1.17±0.04^{a}	1.15±0.13^{a}
CH_4 (ml)	11.12±0.13^{ab}	10.29±0.31^{a}	10.91±0.17^{a}	12.19±0.12^{b}
CH_4 (ml)/digested DM (mg)	0.07±0.06^{a}	0.06±0.03^{a}	0.06±0.07^{a}	0.10±0.04^{b}
CH_4 (ml)/digested OM (mg)	0.08±0.04^{c}	0.06±0.02^{a}	0.07±0.04^{b}	0.08±0.08^{c}
CO_2 (ml)	37.59±0.81^{a}	51.86±1.13^{bc}	50.38±1.13^{b}	53.64±0.57^{c}
VFA (mMol)				
Acetate (C2)	18.23±0.75	18.45±4.70	18.51±2.51	20.02±1.19
Propionate (C3)	5.44±0.28	6.02±0.24	5.96±0.46	5.98±0.35
Butyrate (C4)	2.61±0.50	2.65±1.06	2.85±0.91	2.51±0.75
Total VFA	27.28±1.44	27.12±5.50	26.80±3.84	28.51±1.59
C2:C3	3.55±0.10	3.09±0.89	3.08±0.26	3.40±0.39

^{abc} Different superscripts on the same row are differed significantly (P<0.05).

that the use of 4% papaya (*Carica papaya* L.) leaf extract and powder increased 9.70% and 8.59% rumen microbial protein (Sairullah et al., 2016). Another study showed that the use of 0.6% *Flemingia macrophylla* silage as a source of saponins increased 43.64% of microbial protein synthesis (Viennasay and Wanapat, 2020). The main effect of saponins in the rumen appears to inhibit protozoa (defaunation), which might increase the efficiency of microbial protein synthesis and protein flow to the duodenum (Patra and Saxena, 2009). Protein fermentation in the rumen produces the final product of NH_3-N, which is essential for synthesizing microbial protein in the rumen. Saponins can lyse protozoa by forming complex bonds with sterols on the surface of the protozoa membrane (Hanim et al., 2009; Ramos-Morales et al., 2019). The use of *Nigella sativa* L. as a source of saponins aims to defaunate protozoa so that bacteria and rumen microbes. Defaunation reduces NH_3-N concentrations due to low levels of feed protein degradation (Harun and Sali, 2019). In this study, NH_3-N decreased but not significantly while microbial protein increased. The efficiency of microbial protein synthesis is influenced by several factors such as NH_3-N, availability of energy and carbon skeleton, mineral supply, consumption rate, and flow rate of feed particles in the rumen (Hackmann and Firkins, 2015; Harun and Sali, 2019).

The addition of *Nigella sativa* L. meal as a source of saponins significantly (P<0.05) increased 21.23% of the protozoa population at 0.2% levels but decreased the protozoa population to 19.86% at 0.4% levels compared to control. The 0.6% treatment had no significant effect when compared to the 0.4% level. Previous studies showed that the use of 0.50 g/L tea saponin powder reduced 9.64% of the rumen protozoa population (Guyader et al., 2017). Research conducted by Unnawong et al. (2021) showed that the use of 0.4% and 0.6% *Sesbania grandiflora* pod meal reduced the protozoa population from 9.22×10^5 cells/mL to 6.73×10^5 cells/mL. The decrease in protozoa with the addition of saponins was caused by the ability of saponins to lyse protozoa. Saponins are toxic to protozoa due to forming complexes with lipid membranes, which increase permeability, cause imbalance, and consequently promote cell lysis (Makkar et al., 1995; Fleck et al., 2019). In addition, saponins bind to sterols in the cell walls of protozoa, thereby changing the permeability of cell membranes (Patra et al., 2006; Belanche et al., 2016; Anggraeny et al., 2021). The decrease in the protozoa population led to increased total bacteria in the rumen but decreased bacterial methanogenesis (Ozutsumi et al., 2005; Li et al., 2018).

The addition of *Nigella sativa* L. saponins tended to decrease CH_4 production compared to control, but increased at 0.6% level. Expressed in mL per mg of digested DM, CH_4 production was similar but increased at the 0.6% level. In contrast, CH_4 production was reduced to a level of 0.4% but increased by 0.6% in mL per mg of ingested OM. The production of CO_2 gas increases with the addition of saponin levels. Supplementation of *Nigella sativa* L. reduced the protozoa population but was not accompanied by a decrease in methane gas production. Previous research showed that the use of *Sesbania sesban* leaves and Fenugreek seeds (*Trigonella foenum-graecum* L.) reduced the number of protozoa but did not reduce methane production (Goel et al., 2008a). Patra et al. (2006) showed that methanogenesis

is essentially unrelated to the density of protozoa. No inhibition of methane production with a decrease in methanogens could be due to (1) slow association between protozoa and methanogens due to the higher generation time of protozoa compared to methanogens, (2) increased metabolism of species-independent methanogenic microbes persisting after addition of saponins and/ or (3) by changes in the composition of the methanogenic community and increased efficiency of methane production (Machmüller et al., 2003). In addition, it can be said that at the inhibition of protozoa, species belonging to Methanobacteriaceae (living in association with protozoa) decreased with an increase in the number of free-living Methanobacteriales. Reduced levels of association of protozoa and methanogens may result in higher interspecies hydrogen transfer between an increased population of hydrogen-producing bacteria (*R. flavefaciens* and *F. succinogenes*) and free-living Methanobacteriales showing no effect on methane production (Goel and Makkar, 2012). Guyader et al. (2017) also reported that the use of tea saponins increased methane production in g/kg of digested DM and g/kg of digested OM lactating cows. In the study of Goel et al. (2008b), the saponin-rich fraction did not affect digestibility and a slightly higher tendency to produce gas, which may be due to the increase in saponin-mediated fiber-degrading bacteria. Therefore, it is possible to increase the production of CH_4 and CO_2. According to Ridla et al. (2021), the use of saponins in low levels (less than 0.5% DM) beneficially reduces enteric methane emissions and stimulates nutrient digestibility. The addition of high amounts of saponins appears to cause adverse effects on nutrient digestibility without further reduction in methane emissions.

The addition of *Nigella sativa* L. meal as a source of saponins to a level of 0.6% did not affect ($P > 0.05$) the production of acetate (C2), propionate (C3), butyrate (C4), and total VFA production. The same result was shown by Anggraeny et al. (2021) that the use of 4% *P. falcataria* and *S. saman* leaf meal did not affect the total VFA production and the proportion of C2, C3, and C4 in the rumen. Another study showed that 50% *Garcinia mangostana* L. as a source of saponins did not affect the total and proportion of goat rumen VFA (Shokryazdan et al., 2016). According to Gunun et al. (2018), the use of 20 mg rambutan peel powder did not affect the production and proportion of VFA and the ratio of C2:C3. The decrease in the number of protozoa led to an increase in amylolytic bacteria that digest starch and produce propionate as part of the VFA (Li et al., 2018; Park et al., 2019). Hess et al. (2003), Qin et al. (2012), and Nguyen et al. (2020) stated that decreased levels of protozoa were associated with an increase in propionate and a decrease in the acetate to propionate ratio. The formation of C2 will produce H_2, which is the precursor for the formation of CH_4, while the formation of C3 requires H_2. Increased C3 production will reduce CH_4 formation in rumen fermentation (Moss et al., 2000; Santos et al., 2016; Chen et al., 2020). The decrease in the number of protozoa in this study did not affect the proportion of rumen VFA.

Effect of *Nigella sativa* L on rumen enzymes activity

The effect of *Nigella sativa* L. meal as the source of saponin on enzyme activity in the rumen is shown in Table 4. The addition of *Nigella Sativa* L. meal as a source of saponins significantly ($P<0.05$) decreased the activity of the CMCase enzyme in rumen fluid. The use of 0.2%, 0.4% and 0.6% of saponin caused a decrease in CMCase enzyme activity by 25.92%, 41.8% and 27.5%, respectively. However, the increase in saponins from 0.2% to 0.4% and 0.6% did not cause a decrease in the activity of the CMCase enzyme. Another study conducted by Belanche et al. (2016) showed that the use of 15% saponins from Ivy fruit (*Hedera helix*) did not affect the enzyme activity of the CMCase in the Rusitec system. Hristov et al. (2003) reported that the use of saponins from the extract of *Yucca schidigera* decreased the activity of CMCase, xylanase, and amylase *in vitro*. CMCase is a cellulase enzyme secreted by bacteria and fungi in the rumen (Kirn et al., 2018). Wang et al. (1998) noted a 30% reduction in cellulolytic bacteria when *Yucca schidigera* was added to the fermenter in a rumen simulation (Rusitec) device. According to Wina et al. (2005a) the use of *Sapindus rarak* saponins reduced the concentration of the bacteria *Ruminococcus albus*, *Ruminococcus flavefaciens* and anaerobic rumen fungi (*Chytridiomycetes*) causing a decrease in CMCase enzyme activity. However, some researchers revealed that decrease in xylanase or CMCase activity in the rumen seemed closely related to decreasing protozoa rather than decreasing cellulolytic microbes. There is a significant relationship between the number of protozoa and the activity of cellulolytic enzymes (Williams et al., 2020). Wina et al. (2005b) and Patel and Ambalam (2018) stated that protozoa also secrete cellulolytic enzymes and contribute 19-28% of the total rumen cellulolytic activity. In addition, Newbold et al. (2015) reported that based on meta-analysis, the absence of protozoa caused a decrease in the concentration of anaerobic fungi (–92%), *Ruminococcus albus* (–34%), and *Ruminococcus flavefaciens* (–22%), thereby affecting the activity of cellulolytic enzymes. Differences in substrates and bioactive components in plants are assumed to cause differences in research results.

The use of saponins as feed additives with levels of 0.2%, 0.4%, and 0.6% did not cause a significant difference ($P>0.05$) on the amylase enzyme activity in rumen fluid fermentation. Previous research showed that the use of 200 ppm *Hibiscus tiliaceus* leaf extract did not affect the amylase enzyme activity (Bata and Rahayu, 2016).

Table 4: Effect of *Nigella sativa* L. on rumen enzymes activity.

Parameters	Saponin levels (%)			
	0	0.2	0.4	0.6
Protein enzyme (U/g)	1.00±0.08	0.90±0.18	1.18±0.10	1.11±0.13
CMCase (U/g)	1.89±31.94[b]	1.40±24.91[a]	1.10±7.22[a]	1.37±14.60[a]
Amylase (U/g)	0.50±0.22	0.50±0.11	0.52±0.02	0.50±0.07

[abc] Different superscripts on the same row are differed significantly (P<0.05).

Table 5: Effects of *Nigella sativa* L. on *in vitro* rumen nutrient digestibility.

Parameters	Saponin levels (%)			
	0	0.2	0.4	0.6
Rumen				
Crude Protein (%)	42.77±2.77[ab]	45.07±3.07[b]	45.96±3.99[b]	37.56±3.18[a]
Organic Matter (%)	43.74±2.24	45.16±6.37	53.07±2.33	48.90±7.28
Dry Matter (%)	40.25±2.92	42.21±5.47	50.72±3.42	47.04±6.61
Post-rumen				
Crude Protein (%)	55.29±1.04	52.13±2.25	53.52±4.02	53.48±3.24
Organic Matter (%)	53.28±12.24	57.63±4.14	56.54±1.67	57.35±2.10
Dry Matter (%)	56.09±4.22	53.87±7.48	54.09±2.65	56.12±1.40

[abc] Different superscripts on the same row are differed significantly (P<0.05).

Another study conducted by Belanche et al. (2016) showed that 15% of saponins from Ivy fruit (*Hedera helix*) did not affect the amylase enzyme activity in the Rusitec system. Several studies have shown that saponins inhibit the activity of the amylase enzyme activity (Ali et al., 2006; Ercan and El, 2016; Hanh et al., 2016). It is conceivable that the lack of variation in amylase enzyme activity was due to saponins' lack of effect on amylolytic bacteria. *Yucca schidigera* extract saponins were found to have a direct negative effect on cellulolytic bacteria while being harmless to amylolytic bacteria (Wang et al., 2000; Patra and Saxena, 2009). Abdel-Raheem et al. (2019) stated that the use of saponins decreased NH_3-N levels, thereby reducing the population and activity of rumen fibrolytic bacteria (mostly Gram-positive) and increasing amylolytic bacteria (predominantly Gram-negative); therefore, amylase activity increased. Several studies have shown that saponins inhibit the activity of amylase enzyme activity (Hristov et al., 2003; Ercan and El, 2016; Hanh et al., 2016; Samtiya et al., 2020) by blocking the active site of the enzyme (Moein et al., 2017; Abu et al., 2020) and reduced amylolytic microbial populations (Castro-Montoya et al., 2011).

Effects of *Nigella sativa* L on *in vitro* rumen nutrient digestibility

The effect of giving *Nigella sativa* L. meal as saponin sources on nutrient digestibility in the rumen and post-rumen is presented in Table 5. The use of 0.6% *Nigella sativa* L. meal as a source of saponins significantly (P<0.05) decreased 12.18% rumen digestibility of CP but did not affect the digestibility of OM and DM. Previous research has shown that 80% *Tithonia diversifolia* flower extract can reduce the digestibility of CP (Jamarun et al., 2016). Hanim et al. (2017) showed that the addition of 0.2 mg of saponin *Hibiscus rosa-sinensis* L. leaves/100 ml of medium did not affect the digestibility of OM and DM. Another study conducted by Khoiriyah et al. (2016) showed that the addition of 4% papaya leaf powder and extract (*Carica papaya* L) did not affect the digestibility of OM and DM. Saponins reduce protein digestibility probably by forming difficulty digestible saponins–protein complexes, thereby inhibiting the microbial fermentation of protein (Potter et al., 1993; Francis et al., 2002; Das et al., 2012; Lakram et al., 2019). In some studies, there was a substantial decrease in the protozoa population and no compensatory increase in the bacterial population, and there was a decrease in digestibility. Differences in CP digestibility could be related to many factors, including supplement sources, sources' form, study dose, and diet composition (Jafari et al., 2019).

The effect of using 0.6% saponin *Nigella sativa* L. meal did not affect (P > 0.05) post-rumen digestibility of CP, OM, and DM. Although the activity of the CMCase enzyme decreased with 0.2% saponin administration (Table 4), this may not have caused a decrease in crude fiber digestibility, so the digestibility of OM and DM was not affected. The same result was shown by Aazami et al. (2013) that the use of 0.02% *Quillaja saponaria* powder did not affect the digestibility of CP, OM, and DM Baluchi sheep. Another study showed that the use of 0.6% *S. graniflora* pods meal

decreased the digestibility of CP but did not affect the digestibility of OM and DM Thai Purebred Beef Cattle (Unnawong et al., 2021). Kumar et al. (2017) showed that the use of 5.2% tea seed and 0.8% tea saponins did not affect the digestibility of CP, OM, and DM Gaddi kids. Makkar et al. (1993) stated that saponins might affect or have no effect on nutrient digestibility. The addition of saponins in the feed gave an inconsistent effect. These differences appear to be related to the chemical structure and dosage of saponins, diet composition, microbial community, and adaptation of the microbiota to saponins (Patra and Saxena, 2009; Singh and Kaur, 2020). The use of saponins has been reported to increase nutrient digestibility (CP, OM, DM, Acid detergent fiber (ADF), and Neutral detergent fiber (NDF)) (Wei et al., 2012; McMurphy et al., 2014; Liu et al., 2019b), but several studies have shown that saponins reduce nutrient digestibility (CP, OM, DM, and NDF) (Santoso et al., 2007; Jadhav et al., 2018; Dai and Faciola, 2019).

CONCLUSIONS AND RECOMMENDATION

The addition of *Nigella sativa* L. meal as a source of saponins decreased protozoa population and CH_4 production. As saponin levels increased, microbial protein increased and rumen protein digestibility was improved but CMCase enzyme activity was reduced. The authors recommend the addition of 0.4% *Nigella sativa* L. saponin to reduce CH_4 production without affecting rumen fermentation.

AUTHOR'S CONTRIBUTION

Satyaning Widyarini and Faradista Sekar Nagari performed the experiment, collected the sample, and wrote the manuscript. Chusnul Hanim designed and supervised the experiment. Zaenal Bachruddin supervised the study and revised the manuscript. Muhlisin analyzed the data and supervised the experiment. Lies Mira Yusiati designed and supervised the experiment and revised the manuscript. All authors read and approved the final manuscript for publication.

Conflict of interest

The authors have declared no conflict of interest.

REFERENCES

- Aazami MH, Tahmasbi AM, Ghaffari MH, Naserian AA, Valizadeh R, Ghaffari AH (2013). Effects of saponins on rumen fermentation, nutrients digestibility, performance, and plasma metabolites in sheep and goat kids. Annu. Rev. Res. Biol. 3: 596–607.
- Abbas M, Shahid M, Iqbal M, Anjum F, Sharif S, Pirzada T (2013). Antitermitic activity and phytochemical analysis of fifteen medicinal plant seeds. J. Med. Plants Res., 7: 1608–1617.
- Abdel-Raheem SM, Farghaly MM, Hassan EH (2019). Effect of dietary supplementation with Yucca schidigera powder on nutrient digestibility, rumen fermentation, rumenal enzyme activities and growth performance of buffalo calves. Biol. Rhythm Res., 2: 1–13. https://doi.org/10.1080/09291016.2019.1691832
- Abu OD, Onoagbe IO, Osemwenoyenmwen O (2020). Alpha amylase and alpha glucosidase inhibitory activities of isolated total saponins and tannins of Dialium guineense stem bark. J. Cell. Mol. Biol. Res., 1: 1–3.
- Ali H, Houghton PJ, Soumyanath A (2006). α-Amylase inhibitory activity of some Malaysian plants used to treat diabetes; with particular reference to Phyllanthus amarus. J. Ethnopharmacol., 107: 449–455. https://doi.org/10.1016/j.jep.2006.04.004
- Anantasook N, Wanapat M, Gunun P, Cherdthong A (2016). Reducing methane production by supplementation of Terminalia chebula RETZ. containing tannins and saponins. Anim. Sci. J., 87: 783–790. https://doi.org/10.1111/asj.12494
- Anggraeny YN, Pamungkas D, Mariyono, Krishna NH, Antari R, Putri AS, Apriliza MN (2021). Evaluation of the use of plant organic components and probiotics on ruminal characteristics and as a decrease of methane. IOP Conf. Ser. Earth Environ. Sci., 648: 012191. https://doi.org/10.1088/1755-1315/648/1/012191
- AOAC (2005). Official Methods of Analysis. 20th ed. Association of Official Analytical Chemists, Washington DC, USA.
- Bata M, Rahayu S (2016). Study of hibiscus tiliaceus leaf extract carrier as additive in the diets for fattening of local cattle (*In vitro*). Pakistan J. Nutr., 15: 969–974. https://doi.org/10.3923/pjn.2016.969.974
- Belanche A, Pinloche E, Newbold CJ, Preskett D (2016). Effects and mode of action of chitosan and ivy fruit saponins on the microbiome, fermentation and methanogenesis in the rumen simulation technique. FEMS Microbiol. Ecol., 92: fiv 160. https://doi.org/10.1093/femsec/fiv160
- Castro-Montoya JM, Makkar HPS, Becker K (2011). Chemical composition of rumen microbial fraction and fermentation parameters as affected by tannins and saponins using an *in vitro* rumen fermentation system. Can. J. Anim. Sci., 91: 433–448. https://doi.org/10.4141/cjas2010-028
- Chaney AL, Marbach EP (1962). Modified reagents for determination of urea and ammonia. Clin. Chem., 8: 130–132. https://doi.org/10.1093/clinchem/8.2.130
- Chen J, Harstad OM, McAllister T, Dörsch P, Holo H (2020). Propionic acid bacteria enhance ruminal feed degradation and reduce methane production *in vitro*. Acta Agric. Scand. Anim. Sci., 69: 169–175. https://doi.org/10.1080/09064702.2020.1737215
- Dai X, Faciola AP (2019). Evaluating strategies to reduce ruminal protozoa and their impacts on nutrient utilization and animal performance in ruminants. A meta-analysis. Front. Microbiol., 10: 2648. https://doi.org/10.3389/fmicb.2019.02648
- Darabighane B, Mahdavi A, Aghjehgheshlagh FM, Navidshad B, Yousefi MH, Lee MRF (2021). The effects of dietary saponins on ruminal methane production and fermentation parameters in sheep: A meta analysis. Iran. J. Appl. Anim. Sci., 11: 15–21.

- Das TK, Banerjee D, Chakraborty D, Pakhira MC, Shrivastava B, Kuhad RC (2012). Saponin: Role in animal system. Vet. World, 5: 248–254. https://doi.org/10.5455/vetworld.2012.248-254
- Diaz A, Avendano M, Escobar A (1993). Evaluation of Sapindus saponaria as a defaunating agent and its effects on different ruminal digestion parameters. Livest. Res. Rural Dev., 5: 1–6.
- Dijkstra J, Ellis JL, Kebreab E, Strathe AB, López S, France J, Bannink A (2012). Ruminal pH regulation and nutritional consequences of low pH. Anim. Feed Sci. Technol., 172: 22–33. https://doi.org/10.1016/j.anifeedsci.2011.12.005
- Dopelt K, Radon P, Davidovitch N (2019). Environmental effects of the livestock industry: The relationship between knowledge, attitudes, and behavior among students in Israel. Int. J. Environ. Res. Publ. Health, 16: 1359. https://doi.org/10.3390/ijerph16081359
- Ercan P, El SN (2016). Inhibitory effects of chickpea and Tribulus terrestris on lipase, α-amylase and α-glucosidase. Food Chem., 205: 163–169. https://doi.org/10.1016/j.foodchem.2016.03.012
- Filípek J, Dvořák R (2009). Determination of the volatile fatty acid content in the rumen liquid: Comparison of gas chromatography and capillary isotachophoresis. Acta Vet. Brno, 78: 627–633. https://doi.org/10.2754/avb200978040627
- Fleck JD, Betti AH, Pereira da Silva F, Troian EA, Olivaro C, Ferreira F, Verza SG (2019). Saponins from Quillaja saponaria and Quillaja brasiliensis: Particular chemical characteristics and biological activities. Rev. Mol., 24: 171. https://doi.org/10.3390/molecules24010171
- Francis G, Kerem Z, Makkar HPS, Becker K (2002). The biological action of saponins in animal systems: A review. Br. J. Nutr., 88: 587–605. https://doi.org/10.1079/BJN2002725
- Goel G, Makkar HPS (2012). Methane mitigation from ruminants using tannins and saponins. Trop. Anim. Health Prod., 44: 729–739. https://doi.org/10.1007/s11250-011-9966-2
- Goel G, Makkar HPS, Becker K (2008a). Effects of Sesbania sesban and Carduus pycnocephalus leaves and Fenugreek (*Trigonella foenum-graecum* L.) seeds and their extracts on partitioning of nutrients from roughage- and concentrate-based feeds to methane. Anim. Feed Sci. Technol., 147: 72–89. https://doi.org/10.1016/j.anifeedsci.2007.09.010
- Goel G, Makkar HPS, Becker K (2008b). Changes in microbial community structure, methanogenesis and rumen fermentation in response to saponin-rich fractions from different plant materials. J. Appl. Microbiol., 105: 770–777. https://doi.org/10.1111/j.1365-2672.2008.03818.x
- Gomez KA, Gomez AA (1984). Statistical procedure for agricultural research. John Wiley and Sons, USA.
- Griffin JWD, Bradshaw PC (2019). Effects of a high protein diet and liver disease in an in silico model of human ammonia metabolism. Theor. Biol. Med. Model, 16: 11. https://doi.org/10.1186/s12976-019-0109-1
- Grossi G, Goglio P, Vitali A, Williams AG (2019). Livestock and climate change: Impact of livestock on climate and mitigation strategies. Anim. Front., 9: 69–76. https://doi.org/10.1093/af/vfy034
- Gunun P, Gunun N, Cherdthong A, Wanapat M, Polyorach S, Sirilaophaisan S, Wachirapakorn C, Kang S (2018). *In vitro* rumen fermentation and methane production as affected by rambutan pee powder. J. Appl. Anim. Res., 46: 626–631. https://doi.org/10.1080/09712119.2017.1371608
- Guyader J, Eugène M, DoreauM, Morgavi DP, Gérard C, Martin C (2017). Tea saponin reduced methanogenesis in vitro but increased methane yield in lactating dairy cows. J. Dairy Sci., 100: 1845–1855. https://doi.org/10.3168/jds.2016-11644
- Hackmann TJ, Firkins JL (2015). Maximizing efficiency of rumen microbial protein production. Front. Microbiol., 6: 465. https://doi.org/10.3389/fmicb.2015.00465
- Halliwell G (1961). The action of cellulolytic enzymes from Myrothecium verrucaria. Biochem. J., 79: 185–192. https://doi.org/10.1042/bj0790185
- Hanh TTH, Dang NH, Dat NT (2016). α-amylase and α-glucosidase inhibitory saponins from Polyscias fruticosa leaves. J. Chem., 2016: 2082946. https://doi.org/10.1155/2016/2082946
- Hanim C, Kurniawati A, Al-Huda TI (2017). The effect of Hibiscus Rosa-sinensis L. leaves as defaunating agent on in vitro gas production of feed. Proc. 5th Int. Semin. Anim. Nutr. Feed Sci. pp. 324–330.
- Hanim C, Yusiati LM, Alim S (2009). Effect of saponin as defaunating agent on *in vitro* ruminal fermentation of forage and concentrate. J. Indones. Trop. Anim. Agric., 34: 231–235. https://doi.org/10.14710/jitaa.34.4.231-235
- Hapsari NS, Harjanti DW, Muktiani A (2018). Feed fermentability with babadotan (Ageratum conyzoides) and ginger (Zingiber officinale) leaf extracts in *in vitro* dairy cattle. J. Agripet, 18: 1–9. https://doi.org/10.17969/agripet.v18i1.9672
- Harun AY, Sali K (2019). Factors affecting rumen microbial protein synthesis: A review. Vet. Med., 4: 27–35. https://doi.org/10.17140/VMOJ-4-133
- Hess HD, Kreuzer M, Díaz TE, Lascano CE, Carulla JE, Soliva CR, Machmüller A (2003). Saponin rich tropical fruits affect fermentation and methanogenesis in faunated and defaunated rumen fluid. Anim. Feed Sci. Technol., 109: 79–94. https://doi.org/10.1016/S0377-8401(03)00212-8
- Hristov AN, Ivan M, Neill L, McAllister TA (2003). Evaluation of several potential bioactive agents for reducing protozoal activity in vitro. Anim. Feed Sci. Technol., 105: 163–184. https://doi.org/10.1016/S0377-8401(03)00060-9
- Jadhav RV, Kannana A, Bhar R, Sharma OP, Gulati A, Rajkumar K, Mal G, Singh B, Verma MR (2018). Effect of tea (Camellia sinensis) seed saponins on in vitro rumen fermentation, methane production and true digestibility at different forage to concentrate ratios. J. Appl. Anim. Res., 46: 118–124. https://doi.org/10.1080/09712119.2016.1270823
- Jafari S, Ebrahimi M, Goh YM, Rajion MA, Jahromi MF, Al-Jumaili WS (2019). Manipulation of rumen fermentation and methane gas production by plant secondary metabolites (saponin, tannin and essential oil). A review of ten-year studies. Ann. Anim. Sci., 19: 3–29. https://doi.org/10.2478/aoas-2018-0037
- Jamarun N, Elihasridas, Pazla R, Fitriyani (2016). *In vitro* nutrients digestibility of the combinationtitonia (Tithonia diversifolia) and napier grass (Pennisetum purpureum). International Seminar on Tropical Animal Production (ISTAP): Contribution of Livestock Production on Food Sovereignty in Tropical Countries. Yogyakarta. Pages 122–127.
- Jayanegara A, Wina E, Takahashi J (2014). Meta-analysis on methane mitigating properties of saponin-rich sources in

the Rumen: Influence of addition levels and plant sources. Asian-Aust. J. Anim. Sci., 27: 1426–1435. https://doi.org/10.5713/ajas.2014.14086
- Johnson KA, Johnson DE (1995). Methane emissions from cattle. J. Anim. Sci., 73: 2483–2492. https://doi.org/10.2527/1995.7382483x
- Kamalak A, Canbolat Ö, Gürbüz Y, Özay O (2005). Protected protein and amino acids in ruminant nutrition. J. Sci. Eng., 8: 84–87.
- Khoiriyah M, Chuzaemi S, Sudarwati H (2016). Effect of flour and papaya leaf extract (*Carica papaya* L) addition to feed on gas production, digestibility and energy values *in vitro*. J. Ternak Trop., 17: 74–85. https://doi.org/10.21776/ub.jtapro.2016.017.02.10
- Kim YH, NagataR, Ohkubo A, Ohtani N, Kushibiki S, Ichijo T, Sato S (2018). Changes in ruminal and reticular pH and bacterial communities in Holstein cattle fed a high-grain diet. BMC Vet. Res., 14: 310. https://doi.org/10.1186/s12917-018-1637-3
- Kirn F, Siddiqa A, Noreen S, Khalid AM, Irshad M (2018). Optimized production of cellulase (CMCase). Int. J. Appl. Biol. Forensics, 2: 194–202.
- Kumar M, Kannan A, Bhar R, Gulati A, Gaurav A, Sharma VK (2017). Nutrient intake, digestibility and performance of Gaddi kids supplemented with tea seed or tea seed saponin extract. Asian-Aust. J. Anim. Sci., 30: 486–494. https://doi.org/10.5713/ajas.16.0451
- Lakram N, En-Nahli Y, Zouhair FZ, Moutik S, Kabbour R, El-Maadoudi EH, Bendaou M, El-Housni A, Naciri M (2019). The impact of optimizing the detoxification of Argane (Argania spinosa) press cake on nutritional quality and saponin levels. Iran. J. Appl. Anim. Sci., 9: 235–246.
- Li Z, Deng Q, Liu Y, Yan T, Li F, Cao Y, Yao J (2018). Dynamics of methanogenesis, ruminal fermentation and fiber digestibility in ruminants following elimination of protozoa: A meta-analysis. J. Anim. Sci. Biotechnol., 9: 89. https://doi.org/10.1186/s40104-018-0305-6
- Liu C, Li D, Chen W, Li Y, Wu H, Meng Q, Zhou Z (2019a). Estimating ruminal crude protein degradation from beef cattle feedstuff. Sci. Rep., 9: 1–8. https://doi.org/10.1038/s41598-019-47768-3
- Liu Y, Ma T, Chen D, Zhang N, Si B, Deng K, Tu Y, Diao Q (2019b). Effects of tea saponin supplementation on nutrient digestibility, methanogenesis, and ruminal microbial flora in dorper crossbred ewe. Animals, 9: 29. https://doi.org/10.3390/ani9010029
- Machmüller A, Soliva CR, Kreuzer M (2003). Effect of coconut oil and defaunation treatment on methanogenesis in sheep. Reprod. Nutr. Dev., 43: 41–55. https://doi.org/10.1051/rnd:2003005
- Makkar HPS, Blümmel M, Becker K (1995). *In vitro* effects of and interactions between tannins and saponins and fate of tannins in the rumen. J. Sci. Food Agric., 69: 481–493. https://doi.org/10.1002/jsfa.2740690413
- Makkar HPS, Blümmel M, Borowy NK, Becker K (1993). Gravimetric determination of tannins and their correlations with chemical and protein precipitation methods. J. Sci. Food Agric., 61: 161–165. https://doi.org/10.1002/jsfa.2740610205
- McDonald P, Edwards RA, Greenhalgh JFD, Morgan CA, Sinclair LA, Wilkinson RG (2012). Animal Nutrition. Seventh ed. Prentice Hall, Harlow, London.
- McMurphy CP, Sexten AJ, Mourer GL, Sharman ED, Trojan SJ, Rincker MJ, Coblentz WK, Lalman DL (2014). Effects of including saponins (Micro-Aid®) on intake, rumen fermentation and digestibility in steers fed low-quality prairie hay. Anim. Feed Sci. Technol., 190: 47–58. https://doi.org/10.1016/j.anifeedsci.2014.01.007
- Menke KH, Steingass H (1988). Estimation of energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. Anim. Res. Dev., 28: 7–55.
- Michel CG, El-Sayed NS, Moustafa SF, Ezzat SM, Nesseem DI, El-Alfy TS (2011). Phytochemical and biological investigation of the extracts of *Nigella sativa* L. seed waste. Drug Test. Anal., 3: 245–254. https://doi.org/10.1002/dta.225
- Moein S, Pimoradloo E, Moein M, Vessal M (2017). Evaluation of antioxidant potentials and α-amylase inhibition of different fractions of labiatae plants extracts: As a model of antidiabetic compounds properties. Biomed. Res. Int., 2017: 7319504. https://doi.org/10.1155/2017/7319504
- Moss AR, Jouany JP, Newbold J (2000). Methane production by ruminants: Its contribution to global warming. Ann. Zootech., 49: 231–253. https://doi.org/10.1051/animres:2000119
- Neto JAS, Oliveira JS, Oliveira CJB, Santos EM, Costa ECB, Saraiva CAS, Pinho RMA (2019). Ammonia levels on in vitro degradation of fibrous carbohydrates from buffel grass. South Afr. J. Anim. Sci., 49: 585–597. https://doi.org/10.4314/sajas.v49i3.19
- Newbold CJ, De la Fuente G, Belanche A, Ramos-Morales E, McEwan NR (2015). The role of ciliate protozoa in the rumen. Front. Microbiol., 6: 1313. https://doi.org/10.3389/fmicb.2015.01313
- Nguyen SH, Nguyen HDT, Hegarty RS (2020). Defaunation and its impacts on ruminal fermentation, enteric methane production and animal productivity. Livest. Res. Rural Dev., 32: Article 60.
- Niu Y, Zhou L, Meng L, Chen S, Ma C, Liu Z, Kang W (2020). Recent progress on chemical constituents and pharmacological effects of the genus Nigella. Evidence-based Complement. Altern. Med., 2020: 6756835. https://doi.org/10.1155/2020/6756835
- Ogata T, Kim YH, Masaki T, Iwamoto E, Ohtani Y, Orihashi T, Ichijo T, Sato S (2019). Effects of an increased concentrate diet on rumen pH and the bacterial community in Japanese black beef cattle at different fattening stages. J. Vet. Med. Sci., 81: 968–974. https://doi.org/10.1292/jvms.19-0077
- Ozutsumi Y, Tajima K, Takenaka A, Itabashi H (2005). The effect of protozoa on the composition of rumen bacteria in cattle using 16S rRNA gene clone libraries. Biosci. Biotechnol. Biochem., 69: 499–506. https://doi.org/10.1271/bbb.69.499
- Park T, Mao H, Yu Z (2019). Inhibition of rumen protozoa by specific inhibitors of lysozyme and peptidases *in vitro*. Front. Microbiol., 10: 2822. https://doi.org/10.3389/fmicb.2019.02822
- Patel S, Ambalam P (2018). Role of rumen protozoa: Metabolic and fibrolytic. Adv. Biotechnol. Microbiol., 10: 79–84. https://doi.org/10.19080/AIBM.2018.10.555793
- Patra AK, Kamra DN, Agarwal N (2006). Effect of plant extracts on in vitro methanogenesis, enzyme activities and fermentation of feed in rumen liquor of buffalo. Anim. Feed Sci. Technol., 128: 276–291. https://doi.org/10.1016/j.anifeedsci.2005.11.001
- Patra AK, Saxena J (2009). The effect and mode of action of saponins on the microbial populations and fermentation in

the rumen and ruminant production. Nutr. Res. Rev., 22: 204–219. https://doi.org/10.1017/S0954422409990163
- Plummer DT (1987). An introduction to practical biochemistry. Third. Mc. Graw-Hill Book Company, New Delhi.
- Potter SM, Jimenez-Flores R, Pollack J, Lone TA, Berber-Jimenez MD (1993). Protein-Saponin interaction and its influence on blood lipids. J. Agric. Food Chem., 41: 1287–1291. https://doi.org/10.1021/jf00032a023
- Putri EM, Zain M, Warly L, Hermon H (2021). Effects of rumen-degradable-to-undegradable protein ratio in ruminant diet on *in vitro* digestibility, rumen fermentation, and microbial protein synthesis. Vet. World, 14: 640–648. https://doi.org/10.14202/vetworld.2021.640-648
- Qin WZ, Li CY, Kim JK, Ju JG, Song MK (2012). Effects of defaunation on fermentation characteristics and methane production by rumen microbes in vitro when incubated with starchy feed sources. Asian-Aust. J. Anim. Sci., 25: 1381–1388. https://doi.org/10.5713/ajas.2012.12240
- Ramaiyulis RWS, Ningrat, Zain M, Warly L (2018). Optimization of rumen microbial protein synthesis by addition of Gambier leaf residue to cattle feed supplement. Pak. J. Nutr., 18: 12–19. https://doi.org/10.3923/pjn.2019.12.19
- Ramos-Morales E, de la Fuente G, Duval S, Wehrli C, Bouillon M, Lahmann M, Preskett D, Braganca R, Newbold CJ (2017). Antiprotozoal effect of saponins in the rumen can be enhanced by chemical modifications in their structure. Front. Microbiol., 8: 399. https://doi.org/10.3389/fmicb.2017.00399
- Ramos-Morales E, Lyons L, De La Fuente G, Braganca R, Newbold CJ (2019). Not all saponins have a greater antiprotozoal activity than their related sapogenins. FEMS Microbiol. Lett., 366: 1–7. https://doi.org/10.1093/femsle/fnz144
- Reis LF, Minervino AHH, Araújo CASC, Sousa RS, Oliveira FLC, Rodrigues FAML, Meira-Júnior EBS, Barrêto-Júnior RA, Mori CS, Ortolani EL (2014). Comparison of rumen fluid pH by continuous telemetry system and bench pH meter in sheep with different ranges of ruminal pH. Sci. World J., 2014: 195782. https://doi.org/10.1155/2014/195782
- Ridla M, Laconi EB, Nahrowi, Jayanegara A (2021). Effects of saponin on enteric methane emission and nutrient digestibility of ruminants: An *in vivo* meta-analysis. IOP Conf. Ser. Earth Environ. Sci. 788: 012028. https://doi.org/10.1088/1755-1315/788/1/012028
- Sairullah P, Chuzaemi S, Sudarwati H (2016). Effect of flour and papaya leaf extract (*Carica papaya* L.) in feed to ammonia concentration, volatile fatty acids and microbial protein synthesis in vitro. J. Ternak Trop., 17: 66–73. https://doi.org/10.21776/ub.jtapro.2016.017.02.9
- Samtiya M, Aluko RE, Dhewa T (2020). Plant food anti-nutritional factors and their reduction strategies: An overview. Food Prod. Process. Nutr., 2: 6. https://doi.org/10.1186/s43014-020-0020-5
- Santos NW, Zeoula LM, Yoshimura EH, Machado E, Macheboeuf D, Cornu A (2016). Brazilian propolis extract used as an additive to decrease methane emissions from the rumen microbial population *in vitro*. Trop. Anim. Health Prod., 48: 1051–1056. https://doi.org/10.1007/s11250-016-1062-1
- Santoso B, Kilmaskossu A, Sambodo P (2007). Effects of saponin from Biophytum petersianum Klotzsch on ruminal fermentation, microbial protein synthesis and nitrogen utilization in goats. Anim. Feed Sci. Technol., 137: 58–68. https://doi.org/10.1016/j.anifeedsci.2006.10.005
- Satter LD, Slyter LL (1974). Effect of ammonia concentration on rumen microbial protein production in vitro. Br. J. Nutr., 32: 199–208. https://doi.org/10.1079/BJN19740073
- Shokryazdan P, Rajion MA, Goh YM, Ishak I, Ramlee MF, FJahromi MF, Ebrahimi M (2016). Mangosteen peel can reduce methane production and rumen biohydrogenation *in vitro*. South Afr. J. Anim. Sci., 46: 419–431. https://doi.org/10.4314/sajas.v46i4.10
- Singh AS, Kaur P (2020). Effect of saponins mitigation of methane. A review. Int. J. Curr. Microbiol. Appl. Sci., 9: 3310–3324. https://doi.org/10.20546/ijcmas.2020.906.394
- Sung HG, Kobayashi Y, Chang J, Ha A, Hwang IH, Ha JK (2007). Low ruminal pH reduces dietary fiber digestion via reduced microbial attachment. Asian-Aust. J. Anim. Sci., 20: 200–207. https://doi.org/10.5713/ajas.2007.200
- Supapong C, CherdthongA, Seankamsorn A, Khonkhaeng B, Wanapat M, Uriyapongson S, Gunun N, Gunun P, Chanjula P, Polyorach S (2017). In vitro fermentation, digestibility and methane production as influenced by Delonix regia seed meal containing tannins and saponins. J. Anim. Feed Sci., 26: 123–130. https://doi.org/10.22358/jafs/73890/2017
- Thompson LR, Rowntree JE (2020). Invited review: Methane sources, quantification, and mitigation in grazing beef systems. Appl. Anim. Sci., 36: 556–573. https://doi.org/10.15232/aas.2019-01951
- Tilley JMA, Terry RA (1963). A two-stage technique for the in vitro digestion of forage crops. Grass Forage Sci., 18: 104–111. https://doi.org/10.1111/j.1365-2494.1963.tb00335.x
- Uematsu Y, Hirata K, Saito K, Kudo I (2000). Spectrophotometric determination of saponin in Yucca extract used as food additive. J. AOAC Int., 83: 1451–1454. https://doi.org/10.1093/jaoac/83.6.1451
- Unnawong N, Cherdthong A, So S (2021). Influence of supplementing Sesbania grandiflora pod meal at two dietary crude protein levels on feed intake, fermentation characteristics, and methane mitigation in Thai Purebred Beef Cattle. Vet. Sci., 8: 35. https://doi.org/10.3390/vetsci8020035
- Viennasay B, Wanapat M (2020). Strategic supplementation of Flemingia silage to enhance rumen fermentation efficiency, microbial protein synthesis and methane mitigation in beef cattle. BMC Vet. Res., 16: 480. https://doi.org/10.1186/s12917-020-02703-x
- Wang Y, McAllister TA, Newbold CJ, Rode LM, Cheeke PR, Cheng KJ (1998). Effects of Yucca schidigera extract on fermentation and degradation of steroidal saponins in the rumen simulation technique (RUSITEC). Anim. Feed Sci. Technol., 74: 143–153. https://doi.org/10.1016/S0377-8401(98)00137-0
- Wang Y, McAllister TA, Yanke LJ, Cheeke PR (2000). Effect of steroidal saponin from Yucca schidigera extract on ruminal microbes. J. Appl. Microbiol., 88: 887–896. https://doi.org/10.1046/j.1365-2672.2000.01054.x
- Wei M, Ren L, Zhou Z, Meng Q (2012). Effect of addition three plant extracts on gas production, ruminal fermentation, methane production and ruminal digestibility based on an in vitro technique. J. Anim. Vet. Adv., 11: 4304–4309.
- Williams CL, Thomas BJ, McEwan NR, Stevens PR, Creevey CJ, Huws SA (2020). Rumen protozoa play a significant role in fungal predation and plant carbohydrate breakdown. Front. Microbiol., 11: 720. https://doi.org/10.3389/

fmicb.2020.00720
•Wina E, Muetzel S, Becker K (2005a). The dynamics of major fibrolytic microbes and enzyme activity in the rumen in response to short- and long-term feeding of Sapindus rarak saponins. J. Appl. Microbiol., 100: 114–122. https://doi.org/10.1111/j.1365-2672.2005.02746.x
•Wina E, Muetzel S, Becker K (2005b). The impact of saponins or saponin-containing plant materials on ruminant production. A review. J. Agric. Food Chem., 53: 8093–8105. https://doi.org/10.1021/jf048053d