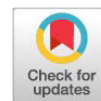


Research Article



The Effect of Acacia and Indigofera Leaves Combination in Haylage-Based Rations on Rumen Fermentability and Methane Gas Concentration

RISWANDI*, ASEP INDRA M. ALI, MUHAKKA, AREFAN ABRAR

Animal Science Department, Agriculture Faculty, Sriwijaya University, Indralaya, 30662, Indonesia.

Abstract | The main problem with traditional livestock farming in tropical regions is that the forage provided comes from natural pastures. The available forage varies with the season and is of low quality, leading to energy loss and increased methane emissions. The use of alternative feed derived from swamp forage haylage supplemented with tree legumes is a solution to meet the forage needs of ruminants, specifically during times of shortage. This study aimed to assess how combining swamp forage haylage-based diets with Acacia and Indigofera leaves affects digestibility, rumen fermentation, and methane levels. A completely randomized design was implemented, involving four treatments and replications. The treatments consisted of 70% swamp forage haylage and 30% concentrate (R0), 40% swamp forage haylage, 30% Acacia, and 30% concentrate (R1); 40% swamp forage haylage, 30% Indigofera, and 30% concentrate (R2); 40% swamp forage haylage, 15% Acacia, 15% Indigofera, and 30% concentrate (R3). Dry matter digestibility (DMD), organic matter digestibility (OMD), pH, total volatile fatty acids (T-VFA), N-ammonia (N-NH₃), partial VFA, methane gas, total bacteria, and protozoa were the observed parameters. The results showed that Acacia and Indigofera leaf combination increased ($p < 0.05$) DMD, OMD, T-VFA, N-NH₃, propionic, butyrate, and total bacteria, while the levels of protozoa, acetate-propionic ratio, and methane gas concentration decreased ($p < 0.05$). In conclusion, combining Acacia and Indigofera in the rations can enhance digestibility, improve rumen fermentation properties, and lower methane gas concentration.

Keywords | Haylage, Swamp forage, Methane gas, *In vitro*, Tree legumes

Received | April 07, 2026; **Accepted** | August 27, 2026; **Published** | September 19, 2026

***Correspondence** | Riswandi, Animal Science Department, Agriculture Faculty, Sriwijaya University, Indralaya, 30662, Indonesia; **Email:** riswandi@fp.unsri.ac.id

Citation | Riswandi, Ali AIM, Muhakka, Abrar A (2026). The effect of acacia and Indigofera leaves combination in haylage-based rations on rumen fermentability and methane gas concentration. J. Anim. Health Prod. 14(4): 1548-1556.

DOI | <https://dx.doi.org/10.17582/journal.jahp/2026/14.4.1548.1556>

ISSN (Online) | 2308-2801



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INTRODUCTION

Beef cattle productivity heavily depends on consistent access to quality and sufficient feed in terms of both amount and continuity. The feed provided should be nutritionally balanced, meeting the needs of rumen microbes and the cattle. Generally, cattle husbandry systems in Indonesia are still traditional, with feed provided from natural pastures and agricultural by-products. The feed provided often does not meet livestock needs due to fluctuating feed availability, seasonal variations, and low

forage quality (high fiber and low crude protein fractions), potentially increasing methane levels. Aragadvay-Yungán *et al.* (2021) reported that methane emissions reduced livestock nutrient absorption efficiency, as 12% of gross energy intake was lost as methane, impacting performance.

A solution needed to address the stated problem is to use the potential of local feed ingredients derived from swamp forage through haylage technology. McDonald *et al.* (2022) described haylage as a forage processed through anaerobic fermentation with a moisture content of 40-50%, targeted

at storing excess forage to increase production and allow provision to livestock during shortages. Swamp forage haylage alone may not provide sufficient nutrients to meet the nutritional requirements of livestock. Feed optimization and efficiency are improved through combination with tree legumes in rations based on swamp forage haylage.

Tree legumes are a widely available local feed, rich in protein, minerals, and phytochemical compounds such as flavonoids, tannins, and saponins. Research results show that phytobiotics can improve growth performance, livestock product quality and reduce enteric methane emissions and improves fatty acid profiles (Jayanegara *et al.*, 2019; Pudato *et al.*, 2025; Salim and Hariyono, 2025). Phytochemicals protect protein from rumen microbial degradation and are potential sources of bypass protein, also known as Rumen Undegradable Protein (RUP). Feeding more RUP can boost livestock growth by increasing amino acid (lysine and methionine) availability in the small intestine, enhancing the efficiency of protein metabolism (Rastgoo *et al.*, 2020).

Legume trees with potential as supplementary feed include Acacia and Indigofera. Acacia (*Acacia mangium*) is a plant containing high nutrients and various phytochemical compounds, including alkaloids, flavonoids, phenols, and saponins, as well as tannins. The Acacia species that have been evaluated show relatively high feed value with crude protein 5.84-28.98%, crude fiber 10.2-32.4%, Ether extract 1.2-4.8%, ash 4.5-12.3%, and nitrogen-free extract (NFE) 35.2-58.6% (Tegegne *et al.*, 2015). The use of acacia as a feed resource should consider several limiting factors, including high phytochemical compounds such as total tannins (3.32-6.03% DM) and condensed tannins (1.77-2.38% DM) in some species (Sujarnoko *et al.*, 2020). Riswandi *et al.* (2017) indicated that the influence of combining 7.5% acacia leaves and 7.5% water mimosa, in rations based on fermented kumpai grass (*Hymenachne acutigluma*) reduced dry matter digestibility (DMD) but increased N-ammonia (N-NH₃) and total VFA (T-VFA) levels.

Indigofera (*Indigofera zollingeriana*) has a fairly high biomass production with high nutrient content. This plant contains 22.66% dry matter (DM), 33.87% crude protein (CP), 16.37% crude fiber (CF), 7.02% ether extract (EE), 34.68% nitrogen-free extract (NFE), 0.27% tannins, and 4.25% saponins (Nadir *et al.*, 2024; Riswandi *et al.*, 2025). Indigofera has the potential to increase propionic acid, reduce acetate, and the acetate-propionate ratio. (Jayanegara *et al.*, 2014). Zain *et al.* (2020) stated that the effect of tannins and saponins on the legume *Gliricidia sepium* reduced methane gas emissions and increased rumen fermentation efficiency and feed digestibility. Based on previous research studies, so far there has been no research on the combination of Acacia and Indigofera tree legumes in haylage-based rations of swamp forage

on rumen characteristics and methane gas production. Therefore, this study aimed to evaluate the effects of Acacia and Indigofera leaf combination in swamp forage haylage-based diets on rumen fermentation characteristics and methane gas concentration.

MATERIALS AND METHODS

The swamp forage used for haylage production was Kumpai (*Hymenachne amplexicaulis*) and *Salvinia molesta* grasses obtained from swampland in Tanjung Senai Village, Ogan Ilit Regency, South Sumatra Province. The tree legumes used as feed supplements consisted of Acacia and Indigofera leaves, obtained from the collection garden of the Animal Husbandry Study Program at Sriwijaya University. Chemical and *in vitro* analyses were performed at the Animal Nutrition and Feed Laboratory, part of the Animal Husbandry Study Program at Sriwijaya University.

This study employed a Completely Randomized Design with four treatments and four replications. The treatments included R0 = haylage of swamp forage and concentrate (70%:30%); R1 = haylage of swamp forage and acacia, and concentrate (40%:30%:30%); R2 = haylage of swamp forage, Indigofera, as well as concentrate (40%:30%:30%); and R3 = haylage of swamp forage, acacia, Indigofera, and concentrate (40%:15%:15%:30%).

SWAMP FORAGE HAYLAGE PRODUCTION

The haylage used in this study consisted of Kumpai grass (80%) and *Salvinia molesta* grass (20%), which were cut into pieces of approximately 3 cm and hung for 24 h to decrease the moisture content to 40-50%. Fine bran was added to the haylage at 5% of the forage. The haylage was placed in plastic bags (silos), compacted, and tied to maintain anaerobic conditions. The filled plastic bags were kept in a space with a temperature of 26-28°C and stored for 21 days according to the procedure by Riswandi *et al.* (2021). The material, incubated for 21 days, was air-dried in an oven at 60°C for 24 h. The dried sample was ground and ready to be incorporated into the rations.

CHEMICAL QUALITY ANALYSIS OF FEED

Tree legumes consisting of acacia and Indigofera leaves were washed with running water, and the leaves served as study samples. Both materials were oven-dried for 24 hours, ground, and sieved through a 0.5 mm sieve. Methanol solvent of 10 mL was added to test tubes containing 0.5 g of plant material (Acacia and Indigofera leaves). The tubes were kept at room temperature for 20 minutes, and each sample was centrifuged (Thermo Scientific IEC Centra CL₂ Centrifuge, Fisher Scientific Pte Ltd., Singapore) at 3000 g and 4°C for 10 minutes. This process was carried out twice, then the supernatants were merged, and the levels

of tannin and saponin were determined (Jayanegara *et al.*, 2020; Nor *et al.*, 2018). All extractions were performed in triplicate, then the solvent type that produced the highest tannin and saponin concentrations was used for the *in vitro* rumen fermentation experiment. To accomplish this, the organic solvent was eliminated using a rotary evaporator (Buchi Rotavapor R-200, Germany), then freeze-dried for 24 hours to produce the dry extract. Distilled water was used to dissolve the dry extract, and the result was tested through *in vitro* rumen incubation, using the substrate and rumen buffer solution.

Table 1: Nutritional composition of swamp forage haylage (SFH), acacia leaves (AL), Indigofera leaves (IL), and concentrate (% DM).

Nutrient	SFH	AL	IL	CON
DM (%)	57.03	43.63	38.45	80.46
OM (%)	46.25	40.31	35.47	74.23
CP (%)	12.11	22.68	23.65	16.42
CF (%)	26.05	24.57	19.85	11.19
EE (%)	3.19	1.96	2.97	7.79
NFE (%)	47.87	47.46	50.56	58.35
TDN (%)	61.90	64.49	69.64	79.99
NDF (%)	67.69	49.62	48.85	37.19
ADF (%)	43.98	25.51	23.50	20.87
Hemicellulose (%)	23.71	24.11	25.35	16.32
Cellulose (%)	35.39	15.69	12.64	16.64
Lignin (%)	5.22	7.57	6.94	4.32
Tannin (%)	1.29	11.25	0.47	Nd
Saponin (%)	2.05	4.87	3.98	Nd

DM = Dry matter; OM = Organic matter; CP = Crude protein; CF = Crude fibre; EE = Ether extract; NFE = Nitrogen-free extract; TDN = Total digestible nutrients; NDF = Neutral detergent fibre; ADF = Acid detergent fibre; Nd = Not detected; SFH = Swamp forage haylage; AL = Acacia leaves; IL = Indigofera leaves; CON = Concentrate.

The rations were composed of swamp forage haylage flour, tree legumes (Acacia and Indigofera leaves), and concentrate. Tables 1 and 2 display the composition of feed components and the nutritional content of the rations. Sample preparation was carried out by grinding the feed ingredients consisting of swamp forage haylage, Acacia leaves, Indigofera, and concentrate with a ratio corresponding to the treatment. Swamp forage haylage flour and concentrate were mixed homogeneously at a formulation ratio of 70:30, then combined with tree legumes (Acacia and Indigofera leaves) according to the treatment. Nutritional content measurement was based on proximate analysis following AOAC procedures (AOAC, 2005) and fiber fractions (Van Soest, 1994), and rumen microbial characteristics, rumen fermentability, as well as *in vitro* digestibility of the rations.

Table 2: Ingredients and chemical compositions of the experimental diets (% DM).

Ingredients	R0	R1	R2	R3
Swamp forage haylage (%)	70	40	40	40
Acacia leaves (%)	0	30	0	15
Indigofera leaves (%)	0	0	30	15
Concentrate (%)	30	30	30	30
TOTAL	100	100	100	100
DM (%)	64.06	60.04	58.49	59.26
OM (%)	54.64	52.86	51.41	52.14
CP (%)	13.40	16.57	16.87	16.72
CF (%)	21.89	21.32	19.90	20.61
EE (%)	4.57	4.20	4.50	4.35
NFE (%)	51.01	50.89	51.82	51.36
TDN (%)	67.32	68.10	69.65	68.87
NDF (%)	58.54	53.12	52.89	53.00
ADF (%)	37.04	31.50	30.89	31.20
Hemicellulose (%)	21.50	21.62	21.99	21.80
Cellulose (%)	29.77	23.86	22.94	23.40
Lignin (%)	4.95	5.66	5.47	5.56
Tannin (%)	0.90	3.89	0.66	2.27
Saponin (%)	1.44	2.29	2.02	2.15

Dry Matter (DM), Organic Matter (OM), Crude Protein (CP), Crude Fiber (CF), Ether extract (EE), Nitrogen Free Extract (NFE), Total Digestible Nutrient (TDN), Neutral Detergent Fiber (NDF), Acid Detergent Fiber (ADF)

CHEMICAL ANALYSIS AND *IN VITRO* FERMENTATION

This study was performed *in vitro* following the method outlined in a previous investigation (Theodorou *et al.*, 1994). Approximately 0.5 grams of substrate was added to each incubation bottle according to the treatment. The incubation medium for each bottle was 90 ml of buffer solution (pH 7.9) consisting of 86 ml of basal solution and 4 ml of reducing agent, as well as 10 ml of buffalo rumen fluid. Rumen fluid was collected from freshly slaughtered buffaloes at a licensed commercial abattoir immediately after evisceration. Only clinically healthy animals without visible abnormalities of the gastrointestinal tract were selected as rumen fluid donor. Because the donor animals originated from commercial farms prior to slaughter, detailed feeding histories were not available. To minimize variation, rumen fluid was collected only from apparently healthy animals slaughtered on the same day, and the inoculum from all donors was pooled before incubation. The rumen contents were transferred into pre-warmed insulated containers filled with CO₂ and transported to the laboratory within 30 min. Upon arrival, the rumen fluid was filtered through four layers of cheesecloth under anaerobic conditions and maintained at 39°C until inoculum preparation. Blank incubation bottles containing buffered rumen fluid without substrate were included in each incubation run to correct

gas production originating from the inoculum. Each incubation bottle was considered one experimental unit, and treatments were incubated in triplicate. Rumen fluid for *in vitro* fermentation was obtained from buffalo fed (70% swamp forage and 30% concentrate). The managed rumen fluid was added to the buffer solution (100 ml/900 ml of buffer solution). The incubation medium was saturated with CO₂ until anaerobic conditions were achieved, then 100 ml was added to each incubation bottle. Directly, the incubation bottle was closed and positioned in a water bath incubator (39 °C) and incubated for 48 hours. After the incubation time was complete, two drops of HgCl₂ were added to inhibit microbial activity. The sample and incubation media were centrifuged in a tube at 4000 rpm for 10 minutes to separate the supernatant and residue. The supernatant was collected to be analyzed for the concentration of N-NH₃, total volatile fatty acid (T-VFA), and partial VFA, and the total number of bacteria and protozoa. Analysis of N-NH₃ concentration was carried out utilizing the modified Conway procedure (General Laboratory Procedures, 1966). T-VFA and partial VFA were measured using gas chromatography (GC 8A, Shimadzu Corp., Japan). The total number of bacteria and protozoa was estimated following the procedure described by Ogimoto and Imai (1988). Moss *et al.* (2000) state that methane gas production can be calculated using partial VFA with the formula: CH₄ = 0.45 C₂ – 0.275 C₃ + 0.40 C₄.

DMD and OMD were assessed after 48 hours of incubation. The sinterglass was initially dried in an oven at 105°C for 24h, then weighed to select its weight. The incubation residue was dried in an oven at 105°C for 24h to calculate DMD and in a furnace at 550°C for 6h to calculate OMD.

DATA ANALYSIS

The data obtained consisted of DMD, OMD, rumen pH, N-NH₃, T-VFA, partial VFA, methane gas concentration, total number of bacteria and protozoa. Data were analyzed using one-way analysis of variance (ANOVA) with treatment as the fixed effect using SPSS version 13 (IBM Corp., Armonk, NY, USA). Prior to ANOVA, the assumptions of normality and homogeneity of variance

were evaluated using the Shapiro–Wilk and Levene’s tests, respectively. After confirming that the data met the assumptions of normality and homogeneity of variance ($p > 0.05$), treatment means were compared using Duncan’s multiple range test. Statistical significance was declared at $p < 0.05$.

RESULTS

RUMEN FERMENTABILITY CHARACTERISTICS

ANOVA results showed that the treatment increased DMD, OMD, N-NH₃, and T-VFA values ($p < 0.05$). However, there was no significant difference in pH values ($p > 0.05$), as shown in Table 3. The results showed that the DMD and OMD values of treatments R1, R2, and R3 were higher ($p < 0.05$) than in treatment R0 (control), R1 was lower than R2 and R3, while there was no significant difference between R2 and R3 ($p > 0.05$). The results showed that all treatments contributed to an increase ($p < 0.05$) in T-VFA levels in the rumen. The lowest content was in R0 (90.12 mM) and the highest was in R1. Treatment R1 facilitated the highest VFA production, reaching 108.28 mM. The results indicated that every treatment led to higher ammonia levels in comparison to the control. The lowest N-NH₃ content was observed in R0 (control) at 5.68 mM, while the highest was in R3 at 8.25 mM.

PARTIAL VFA, ACETATE-PROPIONIC RATIO, AND METHANE GAS CONCENTRATION

ANOVA results showed that the treatment had a significant effect ($p < 0.05$) on propionic acid (C₃), butyric acid (C₄), acetate-propionic ratio, and methane gas concentration values. Meanwhile, acetate (C₂) showed no significant effect ($p > 0.05$), as shown in Table 4. The results indicated that the proportion of acetic acid produced in treatments R0 to R3 was 64.17%, 51.97%, 53.71%, and 53.05%, while propionic acid was 26.54%, 38.48%, 38.75%, and 38.34%, respectively. The results showed that the combination of tree legume feed initiated a decrease ($p < 0.05$) in the acetate-propionic ratio in the rumen compared to the control. The acetate-propionic ratio for treatment R0 (2.36) was higher than for treatments R1 (1.34), R2 (1.50),

Table 3: Effect of Acacia and Indigofera leaves combination in haylage-based rations on DMD, OMD, pH, N-NH₃, and TVFA.

Treatment	DMD (%)	OMD (%)	pH	N-NH ₃ (mM)	T-VFA (mM)
Control	52.37±0.70 ^a	51.12±1.08 ^a	6.75±0.19	5.68±0.07 ^a	90.12±2.16 ^a
Acacia	55.18±0.32 ^b	53.28±1.20 ^b	6.76±0.24	7.01±0.56 ^b	108.98±4.76 ^c
Indigofera	56.02±0.98 ^c	55.52±0.98 ^c	6.78±0.16	8.19±0.44 ^c	104.65±2.28 ^b
Acacia + Indigofera	55.89±0.98 ^c	53.87±2.02 ^b	6.73±0.18	8.25±0.63 ^c	105.88±1.12 ^b

DMD = dry matter digestibility; OMD = organic matter digestibility; N-NH₃ = ammonia nitrogen; T-VFA = total volatile fatty acids. Different superscripts within the same column indicate significant differences ($P < 0.05$).

Table 4: Effect of Acacia and Indigofera leaves combination in haylage-based rations on acetate, propionate, butyrate, acetate-propionate ratio, and methane gas concentration.

Treatment	Acetate (mM)	Propionate (mM)	Butyrate (mM)	Acetate-propionate ratio	Methane gas concentration (mM)
Control	56.33±1.67	23.93±1.08 ^a	8.37±0.33 ^a	2.36±0.06 ^c	22.12±0.67 ^c
Acacia	56.12±3.30	41.95±2.77 ^c	10.41±0.54 ^c	1.34±0.12 ^a	17.88±1.78 ^a
Indigofera	59.20±2.16	39.51±1.24 ^b	8.95±0.64 ^b	1.50±0.06 ^b	19.35±1.02 ^b
Acacia + Indigofera	59.41±2.38	40.60±1.68 ^b	9.13±1.12 ^b	1.47±0.09 ^b	19.22±1.65 ^b

Different superscripts within the same column indicate significant differences ($P < 0.05$).

and R3 (1.47), while there was no significant difference between treatments R2 and R3. The findings indicated that using a combination of tree legumes significantly ($p < 0.05$) reduced methane gas concentration. The highest methane gas levels were found in R0 (22.12 mM), the lowest in R1 (17.88 mM), while there was no significant difference between treatments R2 (19.35 mM) and R3 (19.22 mM).

TOTAL BACTERIAL AND PROTOZOAN POPULATIONS

The findings indicated that including tree legumes in the rations led to a significant increase ($p < 0.05$) in the overall rumen bacteria population compared to the control, while protozoan populations decreased, as shown in Table 5. Treatment R1 (10.53 10^9 cfu) had a higher total bacterial count than treatments R0 (8.45 10^9 cfu), and R2 (9.63 10^9 cfu), and R3 (10.21 10^9 cfu), while there was no significant difference between treatments R1 and R3. The highest number of protozoa was found in R0 (9.61 10^5 cfu), and the lowest in R1 (5.76 10^5 cfu), while there was no significant difference between treatments R1 and R2 (6.49 10^5 cfu) and R3 (6.26 10^5 cfu).

Table 5: Effect of acacia and indigofera leave combination in rations on total bacterial and protozoan populations.

Treatment	Total bacteria (10^9 cfu)	Protozoa (10^5 cfu)
Control	8.45±0.18 ^a	9.61±0.83 ^b
Acacia	10.53±0.48 ^c	5.76±0.62 ^a
Indigofera	9.63±0.53 ^b	6.49±0.85 ^a
Acacia + Indigofera	10.21±0.76 ^c	6.26±0.96 ^a

Different superscripts in the same column indicate significant differences ($P < 0.05$).

DISCUSSION

This suggests that the provision of tree legumes as functional feed can increase the digestibility of DM and OM rations. The high DMD and OMD values in the combination of tree legumes are due to the availability of optimal nutritional and phytochemical content from the rations (Table 2) for rumen microbial growth. Chemical composition in the form of low fiber fraction and high crude protein content in the rations combined with tree

legumes have an impact on increasing the microbial population as well as DMD and OMD. Treatment R1 had lower DMD and OMD values than R2 and R3 due to the phytochemical effect of Acacia leaves (Table 1) in R1 such as tannins and saponins present in higher quantities. Phytochemicals protect proteins from rumen microbial degradation, serve as potential sources of bypass protein, and maintain protein availability in the post-rumen (small intestine). McDonald *et al.* (2022) reported that factors affecting digestibility were the chemical composition of the feed, the ratio between feed ingredients, the treatments, enzyme supplementation, and the level of administration. The findings align with Riswandi *et al.* (2017), stated that the combined effect of 7.5% acacia leaves and 7.5% water mimosa in rations based on fermented kumpai grass (*Hymenachne acutigluma*) reduced DMD but increased the levels of N-NH₃ and T-VFA. Zain *et al.* (2020) stated that the effect of tannins and saponins in the legume *Gliricidia sepium* increased feed digestibility and rumen fermentation efficiency. Suharlina *et al.* (2016) reported that the addition of 20% Indigofera legumes to concentrate feed showed the highest *in vitro* dry and organic matter digestibility, as well as crude protein.

The ideal pH value for rumen microbial growth ranged from 6.73 to 6.78. Therefore, microbial growth was not disrupted because fermentation products, such as T-VFA and N-NH₃, in this study were at optimal levels capable of maintaining normal rumen pH. McDonald *et al.* (2022) said that the balance of fermentation products maintained normal rumen pH (5.5–6.5). These results corresponded with the report of Sari *et al.* (2022), stating that the use of *T. diversifolia* and *L. leucocephala* legumes in the rations had no significant effect on pH, leading to a rumen pH ranging from 6.90 to 6.97.

T-VFA is an energy source derived from carbohydrates in food, which is produced by microbes in the rumen. The composition of T-VFA formed is influenced by the fermented substrate, microbial population, and rumen ecology (McDonald *et al.*, 2022). This is due to the high levels of phytochemicals in R1 (Table 2), which can modify the rumen system, thereby providing nutrients for microbial growth and increasing microbial activity in fermenting

ration nutrients into T-VFA. The optimal T-VFA content needed to support rumen microbial growth is 70–150 mM (McDonald *et al.*, 2022). The results showed T-VFA content within the normal range to ensure rumen microbial activity and growth. This content corresponded to the study by Riswandi *et al.* (2017), reporting that supplementation of various legumes consisting of water mimosa (*Neptunia oleracea*), lamtoro leaves (*Leucaena leucocephala*), as well as acacia in fermented kumpai grass-based rations, increased T-VFA levels compared to the control, with values ranging from 70.02 to 158.84 mM.

N-NH₃ results from protein breakdown by rumen microbes, converting proteins into amino acids, which then undergo deamination to form NH₃, an essential component of microbial protein synthesis (McDonald *et al.*, 2022; Paswan *et al.*, 2022). R3 had a higher ammonia content than the other treatments, signifying low phytochemical levels (tannins and saponins) in the Indigofera legume (Table 1). The phytochemical effect of the acacia leaf combination treatment (R1) was more significant in protecting protein, as evidenced by lower ammonia levels compared to R2 and R3, due to the higher tannin and saponin present in R1 than in the other treatments (Table 1). Sujarnoko *et al.* (2020) stated that Acacia leaves had protective properties against proteins affecting rumen protein degradability due to the thick tannin content. Furthermore, Jayanegara *et al.* (2020) reported that tannin and saponin mechanisms differed in protecting proteins in the rumen. Tannins can bind proteins by slowing the deamination speed of amino acid degradation into ammonia. This study shows that ammonia levels meet the optimal N-NH₃ requirements for microbial protein synthesis. McDonald *et al.* (2022) report that the optimal N-NH₃ concentration in rumen fluid for supporting microbial growth and activity varies significantly, ranging from 4.72 to 16.67 mM.

Acetic acid (C2), propionic acid (C3), and butyric acid (C4) are the final products formed when microorganisms ferment both structural and non-structural carbohydrates in the rumen. Microbial fermentation of feed protein (McDonald *et al.*, 2022) also produces these partial VFAs. This indicates that combining tree legumes leads to a lower proportion of acetic acid as well as a higher proportion of propionic acid, thereby decreasing the acetate-propionate ratio. Acetic acid is produced at the end of fiber fermentation, whereas propionic acid is the final product of sugar and starch fermentation. The results showed that supplementation with a combination of tree legumes produced a proportion of acetic and propionic acid close to the report by Riswandi *et al.* (2025), stating that the Leucaena and Indigofera combination leaves in the rations produced a proportion of acetate ranging from 55.57 to 66.86% and propionate of 19.54 to 28.89%. Jayanegara *et al.*

(2020) found that adding different levels of tannin and saponin-rich plant extracts to the diet (comprising 70% Napier grass and 30% concentrate) resulted in a proportion of acetate levels ranging from 51.40% to 56.80% and propionate levels from 26.00% to 32.50%.

The low acetate-propionic ratio in the tree legume supplementation treatment was due to the higher nutrient and phytochemical content, which encouraged starch-degrading bacteria to produce higher propionic acid than acetic acid. In treatment R1 (without tree legumes) containing a high acetate-propionic ratio, the microbes included in fermentation were cellulolytic and hemicellulolytic bacteria, which facilitated the formation of acetic acid. The low acetate-propionic ratio in the addition of tree legumes compared to without tree legumes indicates that the addition of tree legumes is beneficial because the feed efficiency and energy use are relatively higher. McDonald *et al.* (2022) reported the acetate-propionic ratio to be influential in ruminology, as a low ratio would stimulate body fat formation (fattening). Propionic acid is glucogenic, a major precursor for blood glucose. Unnawong *et al.* (2021) reported that supplementation with the legume *S. grandiflora* resulted in a decrease in acetic acid levels and the acetate-propionic ratio and a significant increase in total volatile fatty acids (VFAs) and propionate concentrations. Riswandi *et al.* (2023) stated that the combination of different swamp forage types in Guinea grass-based rations reduced the acetate-propionic ratio.

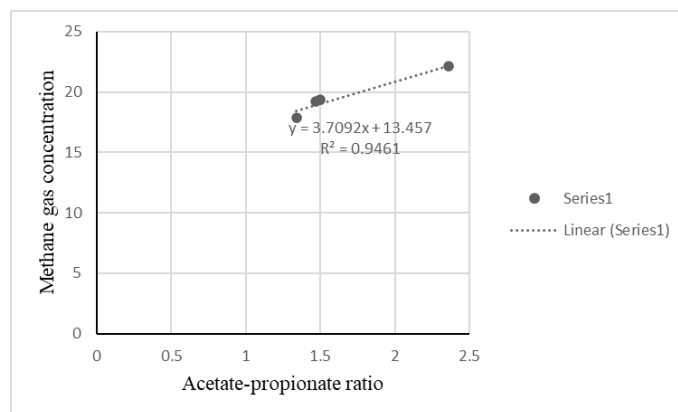


Figure 1: Correlation graph of acetate-propionate ratio with methane gas concentration.

The findings indicated that using a combination of tree legumes significantly ($P < 0.05$) reduced methane gas concentration. The production of methane can be estimated using a VFA profile consisting of acetic, propionic, and butyric acids. This method produces accurate results and a high measurement coefficient (Jayanegara *et al.*, 2015). The acetate-propionic ratio is strongly linked to methane production (Figure 1). The acetate-propionic ratio and methane are positively correlated, indicating

that the lower the acetate-propionic ratio, the lower the methane concentration (Jayanegara *et al.*, 2015; Walles *et al.*, 2017). Palangi *et al.* (2022) explain that the formation of acetate and butyrate generates H_2 , creating a chance for methanogenic bacteria to transform it into methane. Propionate formation requires H_2 , leading to the reduction of methane production. McDonald *et al.* (2022) reported that decreased methane gas production in the rumen could boost feed energy, providing a positive effect on livestock productivity. The present study demonstrated that Acacia supplementation resulted in the lowest methane production among all treatments. This finding agrees with previous studies reporting that condensed tannins suppress methane formation by directly inhibiting methanogenic archaea and indirectly decreasing protozoal populations that maintain symbiotic relationships with methanogens (Patra and Saxena, 2010; Jayanegara *et al.*, 2012). Condensed tannins may also reduce fiber degradation and hydrogen availability, thereby limiting methane synthesis (Min *et al.*, 2003). Surprisingly, combining Acacia with Indigofera did not further decrease methane production. One possible explanation is that the inclusion of Indigofera, a protein-rich legume containing lower concentrations of condensed tannins but appreciable levels of saponins, diluted the overall tannin concentration in the diet while increasing substrate fermentability. Enhanced fermentation may have generated additional hydrogen, partially offsetting the anti-methanogenic effect of condensed tannins. Similar non-additive responses among plant secondary metabolites have been reported previously, suggesting that the interaction between tannins, saponins, and nutrient composition is more complex than a simple synergistic relationship (Patra and Saxena, 2011; Beauchemin *et al.*, 2008). Higher methane gas production represents a lower feed efficiency. Methane gas productivity can be influenced by secondary metabolic compounds (phytochemicals) such as tannins and saponins. Ribeiro *et al.* (2016) reported that secondary metabolites such as tannins and saponins reduced CH_4 emissions by inhibiting rumen ciliate protozoa. Ramírez-Restrepo *et al.* (2016) suggested that the different impacts of tannins on methane gas could result from their chemical structure and concentration. Valenzuela Grijalva *et al.* (2017) reported that saponins affect methane gas by showing that tea saponins can inhibit protozoan activity through impacting cell membrane integrity. Jayanegara *et al.* (2020) found a positive correlation between methanogenesis and protozoa, utilizing the biological properties of saponins to reduce methane emissions. This suppression is achieved by decreasing the total population of rumen protozoa as well as modulating rumen fermentation patterns to inhibit methanogenic bacteria from producing methane.

The increased bacteria population was due to the lower

fiber fraction content of tree legumes compared to swamp forage haylage, as presented in Table 1, leading to a higher proportion of soluble carbohydrates. Furthermore, the high nitrogen (CP) and soluble carbohydrate content in tree legumes stimulated greater microbial biomass production, leading to higher fermentation activity. This was clear from the elevated DMD value, $N-NH_3$, and T-VFA levels within optimal limits, sufficiently supporting increased microbial protein synthesis. The observed increase in digestibility was consistent with increased microbial protein synthesis (Jayanegara *et al.*, 2020; McDonald *et al.*, 2022). The high total bacterial count in the tree legume treatment (R1 and R3) was due to the phytogenic feed activity stimulating the rumen system to function optimally, increasing the rumen microbial population. Additionally, a high overall population of rumen bacteria was observed alongside a low protozoan population. The described condition suggested that fewer rumen microorganisms were preyed on by protozoa, thereby increasing the total rumen microbial population. Found that protozoa contributed 10–40% of total rumen nitrogen. Reducing the protozoan population would reduce predation and bacterial lysis, leading to lower protein breakdown product release (McDonald *et al.*, 2022; Solomon *et al.*, 2022).

This study showed that protozoan populations decreased with the combination of tree legumes due to the presence of phytochemical compounds (saponins) with a defaunation effect, allowing protozoan cell lysis. A study by Ramírez-Restrepo *et al.* (2016) supported these results, showing that tea leave saponins had the potential to be used as antiprotozoals. Saponins react with cholesterol in protozoan membranes to increase the permeability of cell walls, inhibiting protozoan activity. Additionally, Riswandi *et al.* (2025) confirmed the results, stating that the combination of tree legumes (*Leucaena leucocephala* and *Indigofera*) as phytogenic feed sources in swamp forage-based rations could reduce protozoan populations. Jayanegara *et al.* (2014) stated that the presence of secondary compounds in Indigofera plants with tannin and saponin content of 2.9 g/kg and 2.6 mg/kg DM, respectively, had an antiprotozoal effect and was used as a defaunation agent, providing a reduction impact on protozoan populations.

CONCLUSION

In conclusion, the present study demonstrated that supplementation with Acacia and Indigofera leaves influenced *in vitro* rumen fermentation and methane production. While Acacia supplementation alone achieved the greatest reduction in methane production, the combination of Acacia and Indigofera improved nutrient digestibility and fermentation characteristics, indicating a

better overall nutritional response. Therefore, the combined treatment may represent the most suitable feeding strategy when both rumen fermentation efficiency and methane mitigation are considered. Further *in vivo* studies using swamp forage-based diets are required to confirm these findings under practical production conditions.

ACKNOWLEDGMENT

The authors are grateful to the Directorate General of Research and Community Service, Higher Education Ministry, Science, and Technology, for the support provided through the Fundamental Research Scheme in conformity with the Implementation Contract for the State University Operational Assistance Program for the 2025 Fiscal Year Research Program No: 109/C3/DT.05.00 /PL/2025.

NOVELTY STATEMENT

To the best of our knowledge, this is the first study in Indonesia investigating the effect of a combination of acacia and indigofera leaves in a haylage-based ration on rumen fermentability and methane gas concentration.

AUTHOR'S CONTRIBUTION

Riswandi contributed to formulating the research problem, the initial conceptualization and research design. Asep Indra M. Ali and Muhakka helped with the experiments, reviewed the experimental design, and analyzed the data. Riswandi and Arfan Abrar participated in data analysis review and interpretation, and manuscript revision. All authors have read and approved the final version of the manuscript. The authors declare that they have no competing interests concerning the publication of this manuscript.

GENERATIVE AI AND AI ASSISTED TECHNOLOGY STATEMENT

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

REFERENCES

- AOAC (2005). Official Methods of Analysis of AOAC International. 18th ed. AOAC International: Gaithersburg, MD.
- Aragadway-Yungán RG, Barros-Rodríguez M, Ortiz, Carro MD, Navarro Marcos C, Elghandour MMY, Salem AZM (2021). Mitigation of ruminal methane production with enhancing the fermentation by supplementation of different

- tropical forage legumes. Environ. Sci. Pollut. Res., 29: 3438–3445. <https://doi.org/10.1007/s11356-021-15749-7>
- Beauchemin KA, Kreuzer M, O'Mara F and McAllister TA (2008). Nutritional management for enteric methane abatement: A review. Austral. J. Exp. Agric., 48(2): 21–27. <https://doi.org/10.1071/EA07199>
- General Laboratory Procedures (1966). Department of dairy science. University of Wisconsin, Madison.
- Jayanegara A, Leiber F, Kreuzer M (2012). Meta-analysis of the relationship between dietary tannin level and methane formation in ruminants from *in vivo* and *in vitro* experiments. J. Anim. Physiol. Anim. Nutr., 96(3): 365–375. <https://doi.org/10.1111/j.1439-0396.2011.01172.x>
- Jayanegara A, Makkar HPS, Becker K (2015). Addition of purified tannin sources and polyethylene glycol treatment on methane emission and rumen fermentation *in vitro*. Media. Petern., 38: 57–63. <https://doi.org/10.5398/medpet.2015.38.1.57>
- Jayanegara A, Sujarnoko TUP, Ridla M, Kondo M, Kreuzer M (2019). Silage quality as influenced by concentration and type of tannins present in the material ensiled: A meta analysis. J. Anim. Physiol. Anim. Nutr., 103: 456–465. <https://doi.org/10.1111/jpn.13050>
- 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-Australas. J. Anim. Sci., 27(10): 1426–1435. <https://doi.org/10.5713/ajas.2014.14086>
- Jayanegara A, Yogianto Y, Wina E, Sudarman A, Kondo M, Obitsu T, Kreuzer M (2020). Combination effects of plant extracts rich in tannins and saponins as feed additives for mitigating *in vitro* ruminal methane and ammonia formation. Animals, 10(1531): 1–14. <https://doi.org/10.3390/ani10091531>
- McDonald P, Edwards R, Greenhalgh J, Morgan C, Sinclair L, Wilkinson R (2022). Animal nutrition. Eight Ed. England: Pearson Education Limited.
- Min BR, Barry TN, Attwood GT, McNabb WC (2003). The effect of condensed tannins on the nutrition and health of ruminants fed fresh temperate forages: A review. Anim. Feed Sci. Technol., 106(1–4): 3–19. [https://doi.org/10.1016/S0377-8401\(03\)00041-5](https://doi.org/10.1016/S0377-8401(03)00041-5)
- Moss AR, Jouany JP, Newbold J (2000). Methane production by ruminants: Its contribution to global warming. Ann. Zoot., 49: 231–253. <https://hal.science/hal-00889894v1/document> <https://doi.org/10.1051/animres:2000119>
- Nadir M, Syahrir S, Munasirah AL, Warni I, Sari N, Satriani, Sulistiawati A, Anugrah AP (2024). Exploration of nutritional value of Indigofera shoot leaves based on different ages. Int. J. Chem. Biochem. Sci., 25(19): 773–778. <https://doi.org/10.62877/91-IJCBS-24-25-19-91>
- Noer S, Pratiwi RD, Gresinta E (2018). Penetapan kadar senyawa fitokimia (tanin, saponin dan flavonoid sebagai kuersetin) pada ekstrak daun inggu (*Ruta angustifolia* L.). Eksakta J. Ilmu-Ilmu MIPA, 8(1): 19–29. <https://doi.org/10.20885/eksakta.vol18.iss1.art3>
- Ogimoto K, Imai S (1988). Atlas of ruminant microbiology. Tokyo: Japan Sci Press, Japan.
- Palangi V, Taghizadeh A, Abachi S and Lackner M (2022). Strategies to mitigate enteric methane emissions in ruminants: A review. Sustainability, 14(20): 1–15. <https://doi.org/10.3390/su142013229>
- Paswan VK, Kumar K, Shehata AM (2022). Rumen microbiology and microbial degradation of feedstuffs BT- animal manure:

- Agricultural and biotechnological applications. In: Mahajan, S., Varma, A., Eds., Springer International Publishing: Cham, pp. 45–60. https://doi.org/10.1007/978-3-030-97291-2_4
- Patra AK, Saxena J (2010). A new perspective on the use of plant secondary metabolites to inhibit methanogenesis in the rumen. *Phytochemistry*, 71(11–12): 1198–1222. <https://doi.org/10.1016/j.phytochem.2010.05.010>
- Patra AK, Saxena J (2011). Exploitation of dietary tannins to improve rumen metabolism and ruminant nutrition. *J. Sci. Food Agric.*, 91(1): 24–37. <https://doi.org/10.1002/jsfa.4152>
- Pudota BA, Tambireddy N, Chennu R, Chethurajupalli L, Shaik H, Nadella RK, Chatterjee NS, Paturi AP (2025). Exploring the perspectives of phytobiotics and their role in aquaculture: Present status and future trends. *Microbe*, 8: 100496. <https://doi.org/10.1016/j.microb.2025.100496>
- Ramírez-Restrepo CA, Tan C, O'Neill CJ, López-Villalobos N, Padmanabha J, Wang J, and McSweeney CS (2016). Methane production, fermentation characteristics, and microbial profiles in the rumen of tropical cattle fed tea seed saponin supplementation. *Anim. Feed Sci. Technol.*, 216: 58–67. <https://doi.org/10.1016/j.anifeedsci.2016.03.005>
- Rastgoo M, Kazemi-Bonchenari M, Hossein Yazdi M, and Mirzaei M (2020). Effects of corn grain processing method (ground versus steam-flaked) with rumen undegradable to degradable protein ratio on growth performance, ruminal fermentation, and microbial protein yield in Holstein dairy calves. *Anim. Feed Sci. Technol.*, 269: 114646. <https://doi.org/10.1016/j.anifeedsci.2020.114646>
- Ribeiro RS, Terry SA, Sacramento JP, Silveira SRE, Bento CBP, Da Silva EF, Mantovani H C, Da Gama MAS, Pereira LGR, Tomich TR, Maurício RM and Chaves AV (2016). *Tithonia diversifolia* as a supplementary feed for dairy cows. *PLoS One*, 11(12): 1–18. <https://doi.org/10.1371/journal.pone.0165751>
- Riswandi R, AIM, A, Muhakka M, Imsya A, and Wijaya A (2023). The effect of swamp forages combination in rations on rumen fermentability characteristics and *in vitro* methane production. *Adv. Anim. Vet. Sci.*, 11(4): 672–678. <https://doi.org/10.17582/journal.aavs/2023/11.4.672.678>
- Riswandi R, Ali AIM, Imsya A (2021). Effects of *saccharomyces cerevisiae* and *Aspergillus oryzae* supplementation in swamp roughage haylage based rations on *in vitro* rumen fermentation characteristics and methane gas emission. *Adv. Anim. Vet. Sci.*, 9(8): 1143–1149. <https://doi.org/10.17582/journal.aavs/2021/9.8.1143.1149>
- Riswandi R, Priyanto L, Imsya A, Nopiyanti M (2017). *In vitro* digestibility of fermented *Hymenacne acutigluma*-based rations supplemented with different legumes. *J. Vet.*, 18(2): 303–311. <https://doi.org/10.19087/jveteriner.2017.18.2.303>
- Riswandi, Ali AIM, Sandi S, Muhakka, Pratama ANT (2025). Effect of combining leucaena and *Indigofera* leaves in swamp grass-based diets on digestibility, rumen fermentability and methanogenesis. *Iraqi J. Agric. Sci.*, 56(1): 391–401. <https://doi.org/10.36103/kk0g8487>
- Salim M A, Hariyono MAS (2025). The beneficial impact of legume supplementation on nutrient intake, digestibility, growth and reproductive performances of goats: A brief review. *J. Adv. Vet. Res.*, 15(1): 148–152. <https://advetresearch.com/index.php/AVR/article/view/2074>
- Sari RM, Zain M, Jamarun N, Ningrat RWS, Elihasridas and Putri EM (2022). Improving rumen fermentation characteristics and nutrient digestibility by increasing rumen degradable protein in ruminant feed using *Tithonia diversifolia* and *Leucaena leucocephala*. *Int. J. Vet. Sci.*, 11(3): 353–360. <https://doi.org/10.47278/journal.ijvs/2021.121>
- Solomon R, Wein T, Levy B, Eshed S, Dror R, Reiss V, Zehavi T, Furman O, Mizrahi I, and Jami E (2022). Protozoa populations are ecosystem engineers that shape prokaryotic community structure and function of the rumen microbial ecosystem. *Int. Soc. Microbial Ecol.*, 16(4): 1187–1197. <https://doi.org/10.1038/s41396-021-01170-y>
- Suharlina, Astuti DA, Nahrowi, Jayanegara A, Abdullah L (2016). *In vitro* evaluation of concentrate feed containing *Indigofera zollingeriana* in goat. *J. Indones. Trop. Anim. Agric.*, 41(4): 196–203. <https://creativecommons.org/licenses/by-sa/4.0/> <https://doi.org/10.14710/jitaa.41.4.196-203>
- Sujarnoko TUP, Ridwan R, Nahrowi, Jayanegara A (2020). Extraction of tannin from acacia (*Acacia mangium*) bark and its use as a feed additive for protecting *in vitro* ruminal degradation of tofu dregs. *Adv. Anim. Vet. Sci.*, 8(7): 761–765. <https://doi.org/10.17582/journal.aavs/2020/8.7.761.765>
- Tegegne F, Kitaw G, Tileye M (2015). Review on the nutritive value of some selected acacia species for livestock production in dryland areas. *J. Nutr. Food Sci.*, 3(2): 1–5.
- Theodorou MK, Williams BA, Dhanoa MS, McAllan AB, France J (1994). A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. *Anim. Feed Sci. Technol.*, 48(3–4): 185–197. [https://doi.org/10.1016/0377-8401\(94\)90171-6](https://doi.org/10.1016/0377-8401(94)90171-6)
- 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(2): 35. <https://doi.org/10.3390/vetsci8020035>
- Valenzuela-Grijalva NV, Pinelli-Saavedra A, Muhlia-Almazan A, Domínguez-Díaz D, Gonz alez-Ríos H (2017). Dietary inclusion effects of phytochemicals as growth promoters in animal production. *J. Anim. Sci. Technol.*, 59: 8. <https://doi.org/10.1186/s40781-017-0133-9>
- Van Soest PJ (1994). Nutritional ecology of the ruminant. 2nd ed. Comstock Publishing Associates. A. Division of Cornell University Press. Ithaca and London. <https://doi.org/10.7591/9781501732355>
- Wallace RJ, Snelling TJ, McCartney CA, Tapio I, Strozzi F (2017). Application of metaomics techniques to understand greenhouse gas emissions originating from ruminal metabolism. *Genet. Select. Evol.*, 49(1): 27. <https://doi.org/10.1186/s12711-017-0285-6>
- Zain M, Putri EM, Rusmana WSN, Erpomen, Makmur M (2020). Effects of supplementing *Gliricidia sepium* on ration based ammoniated rice straw in ruminant feed to decrease methane gas production and to improve nutrient digestibility (*in-vitro*). *Int. J. Adv. Sci. Eng. Inf. Technol.*, 10(2): 724–729. <https://doi.org/10.18517/ijaseit.10.2.11242>