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Chemical composition, in vitro rumen profile and methane emission of fermented and non-fermented grass-legume mixtures

VITA APRIANI[1], YULIANRI RIZKI YANZA[2], WULANSIH DWI ASTUTI[3], VINCENT NIDERKORN[4], RIMA S. H. MARTIN[1], JIDAN RAMADANI[1], WELAS SRI MULYATI[1], ANURAGA JAYANEGARA[1,♥]

[1]Department of Nutrition and Feed Technology, Faculty of Animal Science, Institut Pertanian Bogor. Jl. Agatis, Kampus IPB Darmaga, Bogor 16680, West Java, Indonesia. Tel.: +62-251-8626213, ♥email: anuraga.jayanegara@gmail.com
[2]Faculty of Animal Science, Universitas Padjajaran. Jl. Ir. Soekarno Km. 21 Jatinangor, Sumedang 45363, West Java, Indonesia
[3]Research Center for Applied Zoology, National Research and Innovation Agency. Jl. Raya Bogor KM. 46 Cibinong, Bogor 16911, West Java, Indonesia
[4]INRAE, VetAgro Sup, UMR Herbivores, Université Clermont Auvergne. 63122 Saint Genès-Champanelle, France

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Abstract. *Apriani V, Yanza YR, Astuti WD, Niderkorn V, Martin RSH, Ramadani J, Mulyati WS, Jayanegara A. 2025. Chemical composition,* in vitro *rumen profile and methane emission of fermented and non-fermented grass-legume mixtures. Biodiversitas 26: 869-878.* This study aimed to evaluate the impact of several grass-legume mixtures, either fermented or non-fermented, on chemical composition, in vitro rumen fermentation profiles, and gas and methane production kinetics. Seven tropical leguminous plants were investigated, i.e., *Indigofera zollingeriana*, *Calliandra calothyrsus*, *Clitoria ternatea*, *Centrosema pubescens*, *Leucaena leucocephala*, *Bauhinia purpurea*, and *Arachis pintoi*. Each legume was combined with *Pennisetum purpureum* (elephant grass) in a 50:50 ratio, prepared in both fresh (non-fermented) and fermented (silage) forms. A completely randomized factorial design was employed, with legume type as the primary factor and fermentation status (non-fermented vs fermented) as the secondary factor (N = 5 replicates per treatment). Results revealed that legume type significantly influenced ($P<0.05$) In Vitro Dry Matter Digestibility (IVDMD) and In Vitro Organic Matter Digestibility (IVOMD), with *I. zollingeriana* demonstrating the highest digestibility values. A significant interaction between fermentation and legume type was observed in reducing methane gas production ($P<0.05$). Both legume inclusion and fermentation effectively lowered methane emissions ($P<0.05$), with the lowest levels recorded for *C. calothyrsus* and *B. purpurea*. The study concluded that incorporating tropical legumes, in both fermented and non-fermented forms, into elephant grass can improve digestibility and mitigate methane emissions, with *Indigofera* and *Calliandra* showing the most promising results, respectively.

Keywords: Grass, legume, methane, rumen, silage

INTRODUCTION

Forage serves as the primary feed source for ruminants and plays a pivotal role in the livestock sector. In Indonesia, various types of forage are available, including grasses, legumes, and tree leaves. However, a significant limitation of grasses in Indonesia is their low nutritional quality, characterized by high fiber and low protein content (Ridla et al. 2023). This necessitates supplementation with high-quality forage sources. Legumes, in particular, contribute substantial protein level to the diets of ruminants (Voisin et al. 2014). Beyond their protein content, many legume species provide essential nutrients such as vitamins and minerals and are rich in bioactive compounds, including tannins, saponins, and phenolics (Sońta et al. 2020). Secondary metabolites like tannins can influence feed degradation by binding to proteins and protecting them from rumen degradation (Jayanegara et al. 2020). These bioactive compounds are recognized for their beneficial effects on livestock performance and their potential to mitigate environmental impacts, such as reducing enteric methane emissions through the inhibition of methanogenesis in the rumen of ruminants (Cieslak et al. 2013; Jayanegara et al. 2015; Niderkorn and Jayanegara 2021) and decreasing nitrogen pollution (Schuba et al. 2017).

Provision of grasses and legumes to ruminants may be performed either in the form of fresh, dried or fermented (silage) ingredients. Silage is a feed product produced through fermentation mediated by microorganisms, which preserves the quality of forage and extends its shelf life (Franco and Rinne 2023). The combination of grasses and legumes as silage ingredients holds promise for ensuring year-round forage availability as a preserved feed for ruminants (Kondo et al. 2014). The inclusion of legumes in grass silage can prevent protein degradation into ammonia during the ensiling process, thereby improving silage quality (Ineichen et al. 2023). In addition, the ensiling process activates polyphenol oxidase, which converts phenolic compounds into quinones. Quinones, in turn, protect proteins during ensiling by facilitating microbial protein synthesis and reducing nitrogen degradation (Lee 2014). A study of Xue et al. (2020) demonstrated that combining orchardgrass with alfalfa legume had been found to enhance nutrient digestibility and absorption efficiency in livestock due to the action of polyphenol oxidase, which mitigates protein degradation during both ensiling and rumen fermentation.

Although a number of studies have been performed concerning the evaluation of grass-legume silage mixtures on their chemical composition, fermentation quality and nutrient utilization (in vitro or in vivo), such studies were

mainly conducted under temperate conditions. Limited studies are available for tropical conditions, including those from Indonesia. Indonesia has a mega biodiversity for various plant species, both for grasses and legumes. This study therefore aimed to evaluate the impact of several grass-legume mixtures, either fermented or non-fermented, on chemical composition, in vitro rumen fermentation profiles, and gas and methane production kinetics.

MATERIALS AND METHODS

Grass and legume species

The forages used in the study are commonly found and utilized in Indonesia as feed ingredients for ruminants. Seven legume species were selected and utilized in the present study, i.e., *Indigofera zollingeriana* Miq., *Calliandra calothyrsus* Meisn., *Clitoria ternatea* L., *Centrosema pubescens* Benth., *Leucaena leucocephala* (Lam.) de Wit, *Bauhinia purpurea* L., and *Arachis pintoi* Krapov. & W.C.Greg., and a grass species namely *Pennisetum purpureum* (elephant grass) as the control. These forage species are shown in Figure 1.

These forages were allocated into a factorial experimental design (8 × 2). The first factor was forage type (8 treatments), which included EG = 100% elephant grass; IZ = 50% elephant grass + 50% *I. zollingeriana*; CC = 50% elephant grass + 50% *C. calothyrsus*; CT = 50% elephant grass + 50% *C. ternatea*; CP = 50% elephant grass + 50% *C. pubescens*; LL = 50% elephant grass + 50% *L. leucocephala*; BP = 50% elephant grass + 50% *B. purpurea*; AP = 50% elephant grass + 50% *A. pintoi*. The EG was considered as the control. Meanwhile, the secondary factor was ensiling process, consisted of T1 = non-ensiling (non-fermented) and T2 = ensiling (fermented). Data collection was carried out with five replicates (N = 5).

Before ensiling, the whole forages were wilted for 4 h before chopped. Then, 250 g of elephant grass and 250 g of each legume (50:50 ratio) were weighted and mixed homogeneously (Dewhurst et al. 2003). In the T0 treatment, plant materials were stored in a plastic bag and allocated in the fridge (-20°C), while the plant materials of T1 treatment underwent the ensiling process for 36 d. These forages were placed in plastic bags and vacuum-sealed, then were stored in a container box lined with aluminum foil and anaerobically ensilaged under controlled conditions. At the end of ensiling day, the forages were then collected and were dried in an oven at 60°C for one day, and finally ground using a grinder with a 1 mm sieve size.

Determination of chemical composition, in vitro rumen fermentation, and methane production

The chemical composition of the samples, including Dry Matter (DM), Organic Matter (OM), and Crude Protein (CP), was conducted following the methodology outlined by AOAC (2005). The dry matter content was determined by drying about 1 g of the sample at 105°C to constant weight; then, the sample was burned at 600°C to obtain the ash content. Crude protein was analyzed using the Kjeldahl method with a process of destruction, distillation, and titration, then the protein was calculated by multiplying the nitrogen content by a factor of 6.25. Neutral Detergent Fiber (NDF) and Acid Detergent Fiber (ADF) adhered to the protocol described by Van Soest et al. (1991). The sample was weighed as much as 0.5 g and then put into a beaker glass. Next, 50 mL of Neutral Detergent Solution (NDS) was added and heated for 1 h and 15 min. Subsequently, the sample was filtered with a previously weighed glass. After being filtered, it was opened at 130°C for 1 h, and then was put into a desiccator and weighed. The procedure for Neutral Detergent Fiber (NDF) and Acid Detergent Fiber (ADF) was similar, but the ADF determination used an Acid Detergent Solution (ADS).

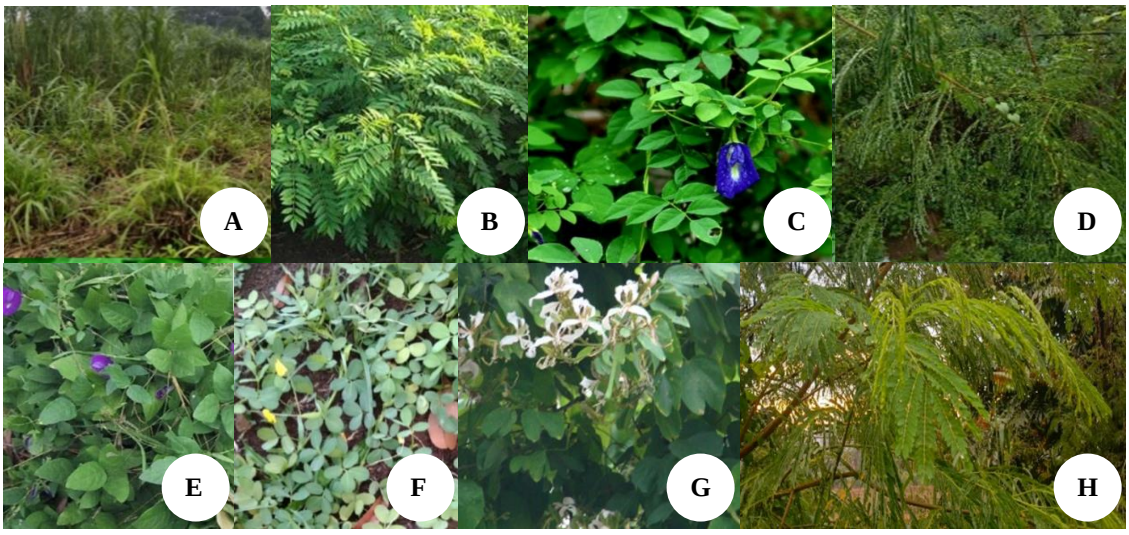

Figure 1. Grass and legume species used in the present study A. *Pennisetum purpureum*; B. *Indigofera zollingeriana*; C. *Calliandra callothyrsus*; D. *Clitoria ternatea*; E. *Centrosema pubescens*; F. *Arachis pintoi*; G. *Bauhinia purpurea*; H. *Leucaena leucocephala*

The in vitro rumen fermentation procedure was performed by following the method of Theodorou et al. (1994). Approximately 0.5 g of each sample was weighed and placed into a 100 mL serum bottle. Rumen fluid was obtained from three fistulated Ongole cattle, filtered, homogenized, and subsequently maintained in a water bath (Memmert WNB 45) at 39°C. Ongole cattle was chosen since it is a common breed of beef cattle in Indonesia and typically consumes such kind of grass and legume species used in this experiment. The use of three fistulated Ongole cattle was conducted according to the recommendation from Yáñez-Ruiz et al. (2016) in order to obtain a stable rumen microbial composition and activity. After measuring the pH of the rumen fluid, it was mixed with McDougall's buffer at a 1:2 ratio and continuously flushed with CO_2. The buffer solution was prepared by mixing 9.8 g $NaHCO_3$, 4.63 g $Na_2HPO_4.2H_2O$, 0.57 g KCl, 0.47 g NaCl, 0.12 g $MgSO_4.7H_2O$, and 0.053 g $CaCl_2.2H_2O$ with 1000 mL of distilled water. An amount of 50 mL buffered rumen fluid was then added to each CO_2-flushed bottle. The bottles were sealed with rubber stoppers, metal caps, and stainless steel clamps, and the samples were incubated in a water bath at 39°C for 48 h.

After 48 h of incubation, each liquor sample from the bottle was centrifuged (Hareus-Multifuge X3R, Thermo Scientific) at 6,000 rpm for 10 min using 50 mL falcon tube. The supernatant from each sample was subsequently separated from the sedimented substrate, and 50 mL of 0.2% pepsin-HCl solution was added before going through another 48 h incubation at 39°C. All of the fermented sediment samples then was filtered through Whatman No. 41 filter paper, then placed in pre-weighed porcelain dishes and oven-dried (Thermo Vacutherm 6025 Vacuum oven, Thermo Scientific) at 130°C for 8 h, then were transferred to a desiccator for 30 min and subsequently weighed to determine their in vitro DM digestibility (IVDMD). Furthermore, the oven-dried samples were then placed in a 600°C furnace (Heraeus M104 Muffle) for 3 h, transferred to a desiccator, then all samples were weighed to determine their in vitro OM digestibility (IVOMD). The in vitro rumen fermentation procedure was conducted in five replicates.

Gas production was recorded at 2, 4, 6, 8, 12, 24, and 48 h using 10 mL syringes, with a distinct syringe employed for each treatment and replication. Data were recorded manually from the syringe in mL, and gas readings were obtained throughout the in vitro incubation period. Methane gas measurements were conducted at 6, 12, 24 and 48 h. The collected gas was transferred to 10 mL vacutainer tubes using a plastic syringe, and methane gas was analyzed using gas chromatography (GC-MS Thermo Scientific TSQ 9610). The data for gas and methane production was fitted using the exponential equation of Ørskov and McDonald (1979) as follows:

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

Where:

p : cumulative gas production at time t (h)

a : gas production from the immediate substrate soluble fraction

b : gas production from the insoluble fraction of the substrate

c : gas production rate constant for the insoluble fraction (b)

t : incubation time (h) , (a + b) is the potential extent of gas production

Statistical analysis

All data were analyzed using a factorial Analysis of Variance (ANOVA) according to the following statistical model:

$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk}$

Where:

Y_{ijk} : observed value of each individual
μ : overall mean
α_i : effect of the forage type factor
β_j : effect of the ensiling process factor
$(\alpha\beta)_{ij}$: interaction between these two factors
ε_{ijk} : residual error

Tukey's test was employed to determine significant differences between treatments of the various types of forages and fermentation included in the silage. Statistical significance between groups was marked at $P<0.05$ and tended to be significant at $P<0.10$. All data were analyzed using SAS statistical software version 9.1.

RESULTS AND DISCUSSION

The results demonstrated that combining elephant grass with legumes and subjecting the mixtures to a fermentation process significantly influenced the nutritional composition of silage (Table 1). Nutrient composition analysis revealed highly significant effects ($P<0.001$) of both fermentation and forage types on DM. The inclusion of *I. zollingeriana* and *C. calothyrsus* resulted in the highest DM values. Fermentation significantly affected ($P<0.001$) OM, with the highest OM content observed in non-fermented treatments. A significant interaction ($P<0.001$) was observed between forage type and fermentation for crude protein, NDF and ADF. Treatments incorporating *C. ternatea* and *L. leucocephala* without fermentation exhibited the highest CP content. Following fermentation, the lowest NDF and ADF levels were observed in treatments with *A. pintoi*.

The inclusion of legumes and the duration of fermentation did not significantly affect ruminal pH (Table 2). However, IVDMD exhibited significant differences ($P = 0.001$), while IVOMD showed a tendency toward significance ($P = 0.086$) among the mixtures. The ensiling process did not significantly affect pH, IVDMD, or IVOMD. Among the legumes, *I. zollingeriana* demonstrated the highest IVDMD. Different legume species significantly reduced ($P<0.001$) ruminal gas production at 24 and 48 h, whereas the effects of ensiling and its interactions were not significant. *C. callothyrsus* exhibited the lowest total gas production, while ***B. purpurea*** produced the highest.

Table 1. Nutrient contents of different grass-legume mixtures

Variable	Ensilage	Treatments								Average	SEM	Ensiled	Legume	E*L
		EG	IZ	CC	CT	CP	LL	BP	AP					
Dry matter (%)	T0	94.58	96.71	96.45	94.86	95.9	94.47	95.78	92.82	95.21^{A}	0.24	<0.001	<0.001	0.123
	T1	94.95	95.4	94.8	92.43	93.66	94.25	94.13	92.11	94.00^{B}	0.26			
	Average	94.76^{bc}	96.05^{a}	95.62^{a}	93.47^{cd}	94.78^{bc}	94.36^{bcd}	94.95^{bc}	92.47^{d}					
Organic matter (%)	T0	84.68	87.92	90.65	89.27	89.90	89.39	87.78	88.95	88.60^{A}	0.29	0.300	<0.001	0.133
	T1	84.50	88.85	90.87	87.06	89.50	89.23	87.65	88.19	88.36^{B}	0.34			
	Average	84.59	88.39	90.76	88.00	89.68	89.31	87.71	88.57					
Crude protein (%)	T0	14.03^{h}	18.77^{defg}	18.75^{defg}	23.42^{a}	19.23^{cd}	23.35^{a}	18.58^{fg}	19.43^{c}	19.23	0.44	<0.001	<0.001	<0.001
	T1	10.81^{i}	20.46^{b}	18.71^{efg}	19.11^{cde}	18.61^{fg}	18.90^{def}	18.99^{cdef}	18.39^{g}	17.97	0.46			
	Average	12.42	19.61	18.73	20.96	18.92	21.13	18.78	18.91					
NDF (%)	T0	64.60^{fg}	64.44^{fg}	68.03^{ij}	66.47^{hi}	71.42^{k}	62.52^{cde}	67.78^{ij}	58.62^{b}	65.43	0.62	<0.001	<0.001	<0.001
	T1	69.00^{j}	61.16^{c}	61.29^{cd}	63.07^{def}	63.85^{efg}	65.37^{gh}	63.61^{efg}	54.54^{a}	62.73	0.65			
	Average	66.80	62.80	64.66	64.52	67.63	63.94	65.69	56.58					
ADF (%)	T0	38.98^{fg}	31.82^{b}	39.57^{g}	36.76^{e}	41.66^{h}	33.64^{c}	33.45^{c}	30.06^{a}	35.84	0.65	<0.001	<0.001	<0.001
	T1	35.85^{e}	36.26^{e}	34.14^{cd}	39.45^{g}	41.20^{h}	36.71^{e}	38.19^{f}	34.72^{d}	37.00	0.37			
	Average	37.42	34.04	36.86	38.30	41.43	35.17	35.82	32.65					

Notes: EG: 100% elephant grass; IZ: 50% elephant grass + 50% *Indigofera zollingeriana*; CC: 50% elephant grass + 50% *Calliandra callothyrsus*; CT: 50% elephant grass + 50% *Clittoria ternatea*; CP: 50% elephant grass + 50% *Centrosema pubescens*; LL: 50% elephant grass + 50% *Leuchaena leucocephala*; BP: 50% elephant grass + 50% *Bauhinia purpurea*; AP: 50% elephant grass + 50% *Arachis pintoi*. T0: without fermentation; T1:36 days fermentation; ^{abc} superscript with different letters indicates significant difference at P<0.05; NDF: neutral detergent fiber; ADF: acid detergent fiber; SEM: Standard error means; E: ensiled; L: Legume

Table 2. Ruminal pH, digestibility rates, total gas production and enteric methane production of non-ensiled and ensiled grass-legume mixtures

Variable	Ensilage	Treatments								Average	SEM	Ensiled	Legume	E*L
		EG	IZ	CC	CT	CP	LL	BP	AP					
pH	T0	6.78	6.80	6.83	6.80	6.80	6.80	6.79	6.83	6.80	0.01	0.190	0.122	0.397
	T1	6.84	6.82	6.83	6.82	6.78	6.84	6.77	6.91	6.82	0.01			
Average		6.80	6.81	6.83	6.81	6.79	6.82	6.78	6.86					
IVDMD	T0	57.44	63.20	43.71	59.73	52.11	56.06	64.09	57.99	56.57	1.47	0.227	0.001	0.083
(%)	T1	50.11	68.15	51.52	51.49	52.29	56.64	49.61	51.32	54.26	1.70			
Average		54.18^{ab}	65.68^{a}	47.18^{b}	56.06^{ab}	52.20^{b}	56.32^{ab}	56.85^{ab}	64.66^{ab}					
IVOMD	T0	61.35	65.73	46.34	63.12	59.47	51.53	50.13	54.99	56.87	2.03	0.767	0.086	0.409
(%)	T1	53.57	64.69	53.12	56.71	53.22	53.78	64.14	55.98	57.65	1.62			
Average		58.44	65.20	48.28	60.27	56.34	52.65	57.92	55.36					
TGP 24 h	T0	48.60	46.26	26.78	41.58	38.66	37.32	48.72	40.54	40.86	1.51	0.457	<0.001	0.645
(mL)	T1	44.50	48.16	26.80	37.85	32.82	29.37	53.42	42.40	39.51	1.98			
Average		46.78^{cde}	47.21^{de}	26.79^{a}	39.92^{bcd}	35.74^{abc}	33.79^{ab}	51.33^{e}	41.37^{bcde}					
TGP 48 h	T0	65.68	59.84	36.58	54.92	50.60	48.32	60.35	52.58	53.43	1.69	0.241	<0.001	0.326
(mL)	T1	59.77	59.94	38.44	50.00	43.40	39.02	66.14	53.12	51.42	2.04			
Average		63.05^{cd}	59.89^{cd}	37.51^{a}	52.73^{bc}	47.00^{ab}	44.19^{ab}	63.57^{d}	53.27^{bcd}					
CH_4 24 h	T0	2.19^{de}	2.34^{e}	1.37^{a}	1.97^{bcde}	1.90^{bcde}	1.74^{abcd}	2.08^{cde}	1.95^{bcde}	1.94	0.05	0.325	<0.001	0.017
(mL)	T1	2.00^{bcde}	2.08^{cde}	1.59^{abc}	1.77^{abcd}	1.65^{abc}	1.55^{ab}	2.40^{e}	2.06^{cde}	1.89	0.06			
Average		2.10	2.21	1.48	1.87	1.77	1.65	2.24	2.00					
CH_4 48 h	T0	2.35^{efg}	2.62^{g}	1.86^{bcd}	2.04^{cdef}	2.22^{defg}	2.03^{cdef}	2.39^{efg}	2.32^{defg}	2.23	0.04	<0.001	<0.001	0.003
(mL)	T1	2.33^{defg}	2.43^{fg}	1.31^{a}	1.66^{abc}	1.37^{a}	1.46^{ab}	1.97^{cdef}	1.92^{bcd}	1.85	0.08			
Average		2.34	2.53	1.62	1.87	1.84	1.82	2.18	2.14					
CH_4/TGP	T0	4.56	5.37	5.81	4.78	5.20	4.74	4.04	4.88	4.95	0.22	0.612	0.219	0.920
24 h (%)	T1	4.53	4.44	6.52	5.05	5.30	5.30	4.74	4.87	5.11	0.23			
Average		4.55	4.90	6.16	4.90	5.25	4.99	4.43	4.88					
CH_4/TGP	T0	3.59	4.58	5.24	3.73	4.50	4.22	3.83	4.43	4.28^{B}	0.14	<0.001	0.094	0.096
48 h (%)	T1	3.95	4.16	3.34	3.32	3.09	3.40	3.03	3.55	3.47^{A}	0.13			
Average		3.75	4.37	4.29	3.55	3.80	3.86	3.38	4.04					

Notes: EG: 100% elephant grass; IZ: 50% elephant grass + 50% *Indigofera zollingeriana*; CC: 50% elephant grass + 50% *Calliandra callothyrsus*; CT: 50% elephant grass + 50% *Clittoria ternatea*; CP: 50% elephant grass + 50% *Centrosema pubescens*; LL: 50% elephant grass + 50% *Leuchaena leucocephala*; BP: 50% elephant grass + 50% *Bauhinia purpurea*; AP: 50% elephant grass + 50% *Arachis pintoi*. T0: without fermentation; T1:36 days fermentation; ^{abc}superscript with different letters indicates significant difference at P<0.05. IVDMD: in vitro DM digestability; IVOMD: in vitro OM digestability; SEM: standard error means; E: ensiled; L: Legume

Regarding ruminal methane production at 24 and 48 h, significant differences (P<0.001) were induced by different legume types, while the ensiling factor significantly influenced methane production at 48 h (P<0.001). Interactions between legumes and the ensiling factor were significant at both 24 (P = 0.017) and 48 h (P = 0.003). Non-ensiled *C. callothyrsus* produced the lowest methane levels at 24 h, while the ensiled form produced the lowest levels at 48 h. The methane-to-total gas production ratio (% CH_4/TGP) did not differ significantly during the 24 h incubation period but demonstrated a significant effect of ensiling (P<0.001) at 48 h. There was also a tendency toward significance among legume species (P = 0.094) and their interactions (P = 0.096). The lowest CH_4/TGP ratios were observed in ensiled *B. purpurea* and *C. pubescens*.

Significant differences were observed among legumes in the soluble fraction (a) of gas kinetics (P<0.001), with a tendency toward significance for the ensiling factor and its interaction (P<0.10). *C. pubescens* exhibited the lowest rates in this parameter (Table 3). The potential degradable fraction (b) and total gas production kinetics (a + b) also varied significantly among legumes (P<0.001), with no significant effects observed for the ensiling factor or its interaction. *L. leucocephala* showed the lowest b kinetics, while *C. callothyrsus* exhibited the lowest estimated total gas production kinetics (a + b). The soluble fraction (a) of methane kinetics was significantly affected by the ensiling factor (P = 0.049), though no significant differences were noted among legumes or their interactions (Table 4). The potential degradable fraction (b) of methane was unaffected by legumes or ensiling, but an interaction effect on methane degradation rates (c) approached significance (P = 0.089). The total enteric methane kinetics (a + b) varied significantly among legume species (P < 0.001) and were influenced by both the ensiling factor (P < 0.001) and its interaction (P = 0.005). Non-ensiled *L. leucocephala* recorded the lowest methane kinetics (a + b), while ensiled *C. callothyrsus*, *L. leucocephala*, and *C. pubescens* exhibited lower estimated total methane kinetics compared to other legumes.

Discussion

Fermentation can lead to a reduction in DM content, primarily due to microbial activity that consumes various available nutrients and produces water. This process causes a decrease in DM as volatile compounds, including carbon dioxide (CO_2) and water, are released (Xia et al. 2023). The combination of elephant grass with legumes such as *I. zollingeriana* and *C. calothyrsus* yields higher DM content compared to a mixture of elephant grass with *A. pintoi*. This observation aligns with the findings of Zain et al. (2021), who reported that the DM content of *Arachis* sp. was lower than that of other legumes. The reduction in OM during fermentation is attributed to microbial activity as well. During ensiling, microbes produce organic acids that lower the pH, which consequently leads to losses in both DM and OM (Auerbach and Nadeau 2020). According to Silva et al. (2010) and Schnaider et al. (2014), ADF content in *A. pintoi* was relatively low, ranging between 44% and 57%. Additionally, *A. pintoi* exhibited NDF content ranging from 28% to 35%, as noted by Gomes et al. (2021) and Silva et al. (2010). Fluctuations in NDF and ADF content are influenced by soluble carbohydrates, which are associated with DM levels and fermentation microbial activity during ensiling (Dos Santos et al. 2015; Shoe et al. 2021). These findings highlight the importance of choosing legume species that optimize nutrient composition and DM retention when combined with elephant grass in silage production.

With regard to the in vitro rumen fermentation profiles, the fermentation process of all grass-legume mixtures resulted in a normal pH range (from 6.77 to 6.91), indicating that none of the observed treatments adversely affected fermentation activity in the rumen (Santoso et al. 2020). However, among other legumes, either conditioned in a non-ensiled or ensiled treatment, *I. zollingeriana* achieved satisfactory digestibility rates, up to 44 % higher than the lowest digestibility treatments, i.e., non-ensiled and ensiled *C. callothyrsus*. It is widely known that leguminous forages are rich in protein contents but possibly also contain secondary metabolites that may play a role as anti-nutrient factors (Parastiwi et al. 2023; Samal et al. 2023). It had been reported that *L. leucocephala* and *C. callothyrsus* contained considerable levels of tannin, i.e., 6.21 and 8.70% from DM contents, respectively (Rimbawanto et al. 2015). *C. calothyrsus* contains tannins that reduce protein degradation in the rumen, leading to a lower dry matter digestibility. The digestibility of dry matter is influenced by the composition of organic matter and the presence of secondary metabolites, such as the tannins found in *C. calothyrsus* (Atmojo et al. 2020).

The high tannin content in *C. calothyrsus* can also inhibit rumen microbial enzyme activity and reduce the population of fiber-degrading microbes, leading to decreased fermentation efficiency (Jayanegara et al. 2020). Furthermore, tannin can alter the fermentation end-products by reducing volatile fatty acid (VFA) production, which is a key energy source for ruminants, thereby impairing overall nutrient utilization. On the other hand, *I. zollingeriana* is known for its high protein content and ability to improve the digestibility rates of ruminants, owing to its low tannin content (Abdullah 2010). Another study also reported higher digestibility rates in *Indigofera* and *Sesbania* than the other legumes (Rahmat and Permana 2021).

Regarding the Total Gas Production (TGP) and methane (CH_4) levels, the control treatment, namely grass silage, exhibited higher values than treatments with legume mixtures. This phenomenon can be attributed to the effects of *Rhizobium* symbiosis on plant nutrient metabolism, which synthesizes atmospheric nitrogen and subsequently influences the chemical reactions and formation of secondary metabolites (Darma et al. 2023). Consistent with the present study, *C. callothyrsus* demonstrated the lowest total gas production compared to other legume treatments. Such lower gas production is likely due to its high tannin content, which protects proteins from degradation, reduces feed degradation, and consequently results in lower gas production (Bueno et al. 2015). Reduction of gas production is possible through inhibition mechanisms with the formation of CO_2 and H_2 during fermentation process occurring in the rumen (Bodas et al. 2012).

Table 3. Dynamics of ruminal gas kinetics of non-ensiled and ensiled grass-legume mixtures

Variable	Ensilage	Treatments								Average	SEM	Ensiled	Legume	E*L
		EG	IZ	CC	CT	CP	LL	BP	AP					
a (mL)	T0	-3.19	-0.91	-0.66	-0.89	-0.84	-1.52	-2.28	-1.34	-1.43^{A}	0.21	<0.001	<0.001	0.056
	T1	-2.38	-2.80	-1.98	-1.69	-1.09	-2.00	-3.82	-1.93	-2.24^{B}	0.16			
Average		-2.84^{bc}	-1.86^{abc}	-1.32^{a}	-1.24^{a}	-0.96^{a}	-1.74^{ab}	-3.13^{c}	-1.60^{ab}					
b (mL)	T0	85.93	65.87	61.92	58.91	54.68	53.60	64.18	55.21	62.50	2.29	0.361	<0.001	0.731
	T1	77.05	73.59	74.00	56.53	53.46	51.33	74.49	60.10	65.49	3.01			
Average		81.98^{c}	69.73^{ab}	67.96^{ab}	57.85^{a}	54.07^{a}	52.59^{a}	69.90^{ab}	57.38^{a}					
c (mL/h)	T0	0.039	0.054	0.034	0.060	0.057	0.060	0.072	0.069	0.055	0.00	0.999	0.738	0.492
	T1	0.036	0.058	0.081	0.052	0.047	0.036	0.062	0.058	0.055	0.01			
Average		0.036	0.056	0.059	0.056	0.054	0.049	0.068	0.066					
a + b (mL)	T0	66.34	59.59	39.10	54.25	50.15	48.13	59.88	51.66	53.48	1.65	0.498	<0.001	0.321
	T1	60.82	60.35	41.98	50.04	43.57	39.83	66.68	54.25	52.30	1.98			
Average		63.89^{e}	59.97^{cde}	40.54^{a}	52.38^{bc}	46.48^{ab}	44.45^{ab}	63.66^{de}	52.81^{bcd}					

Notes: EG: 100% elephant grass; IZ: 50% elephant grass + 50% *Indigofera zollingeriana*; CC: 50% elephant grass + 50% *Calliandra callothyrsus*; CT: 50% elephant grass + 50% *Clittoria ternatea*; CP: 50% elephant grass + 50% *Centrosema pubescens*; LL: 50% elephant grass + 50% *Leuchaena leucocephala*; BP: 50% elephant grass + 50% *Bauhinia purpurea*; AP: 50% elephant grass + 50% *Arachis pintoi*. T0: without fermentation; T1:36 days fermentation; ^{abc} superscript with different letters indicates significant difference at P<0.05; a: soluble fraction; b: potentially degradable fraction; c: rate of degradation; a + b: summary of a and b values; SEM: standard error means; E: ensiled; L: Legume

Table 4. Dynamics of ruminal enteric methane kinetics of non-ensiled and ensiled grass-legume mixtures

Variable	Ensilage	Treatments								Average	SEM	Ensiled	Legume	E*L
		EG	IZ	CC	CT	CP	LL	BP	AP					
a (mL)	T0	-1.07	-0.77	-0.65	-0.70	-0.27	-0.18	0.07	0.04	-0.45^{A}	0.08	0.049	0.107	0.149
	T1	-0.59	-0.20	-1.35	-2.54	-0.23	-1.82	-0.71	-0.42	-0.94^{B}	0.26			
Average		-0.85	-0.48	-1.00	-1.52	-0.25	-0.91	-0.36	-0.16					
b (mL)	T0	3.60	3.70	3.14	2.97	2.92	2.76	2.72	3.10	3.13	0.08	0.462	0.178	0.248
	T1	3.10	3.25	2.79	4.38	1.75	3.32	2.76	2.53	2.95	0.25			
Average		3.38	3.47	2.97	3.59	2.34	3.01	2.74	2.85					
c (mL/h)	T0	0.082	0.186	0.038	0.064	0.112	0.036	0.042	0.034	0.751	0.02	0.116	0.872	0.089
	T1	0.075	0.058	0.130	0.165	0.088	0.145	0.154	0.082	0.111	0.01			
Average		0.078	0.122	0.084	0.109	0.100	0.084	0.104	0.057					
a + b (mL)	T0	2.43^{cde}	2.74^{e}	1.91^{abc}	2.10^{bcd}	2.38^{cde}	2.10^{bcd}	2.36^{cde}	2.40^{cde}	2.30	0.05	<0.001	<0.001	0.005
	T1	2.38^{cde}	2.48^{de}	1.42^{a}	1.78^{ab}	1.47^{a}	1.47^{a}	2.03^{bcd}	2.05^{bcd}	1.88	0.08			
Average		2.40	2.60	1.67	1.96	1.93	1.82	2.18	2.24					

Notes: EG: 100% elephant grass; IZ: 50% elephant grass + 50% *Indigofera zollingeriana*; CC: 50% elephant grass + 50% *Calliandra callothyrsus*; CT: 50% elephant grass + 50% *Clittoria ternatea*; CP: 50% elephant grass + 50% *Centrosema pubescens*; LL: 50% elephant grass + 50% *Leuchaena leucocephala*; BP: 50% elephant grass + 50% *Bauhinia purpurea*; AP: 50% elephant grass + 50% *Arachis pintoi*. T0: without fermentation; T1:36 days fermentation; ^{abc}superscript with different letters indicates significant difference at P<0.05; a: soluble fraction; b: potentially degradable fraction; c: rate of degradation; a + b: summary of a and b values; SEM: standard error means; E: ensiled; L: Legume

In the present study, the lowest CH_4/TGP ratio at 48 h was observed in *B. purpurea*, with a value of 3.03% after 36 d of fermentation. This low CH_4/TGP ratio is attributed to the presence of bioactive compounds in *B. purpurea*, including tannins, alkaloids, flavonoids, saponins, polyphenols, and phenolics (Zakaria et al. 2011; Htay et al. 2023). Tannins have been demonstrated to affect methanogenic activity by reducing hydrogen availability and modifying the rumen microbiome, thereby decreasing degradation and methanogenic activity (Hassan et al. 2020; Alayón et al. 2023). Other bioactive compounds, such as saponins and polyphenols, also influence methane production. Polyphenols have been shown to reduce methane emissions, whereas tannins and saponins can lower methane emissions by inhibiting methanogen populations and reducing protozoan populations (Jayanegara et al. 2020).

In the present study, the dynamics of ruminal gas production kinetics between ensiled and non-ensiled legumes after 48 h in vitro batch culture incubation were significantly different among different legume species and treatments, which were neither preserved as non-ensiled nor ensiled manners. For instance, the lowest gas production from the immediately soluble fraction (a) was observed in ensiled *C. callothyrsus* (-1.98 mL), with a potential extent of gas production (a + b) of 40.54 mL. Moreover, the lowest (b) gas fraction was observed in *L. leucocephala* (52.59 mL), likely due to its high tannin content, which reduces the gas production rate constant for the legume insoluble fraction.

The lower gas production of the soluble and insoluble substrate fractions seems are likely attributable to the high tannin content in the *C. callothyrsus*. However, the tannin content of the observed legumes was not quantified in this study. Rimbawanto et al. (2015) confirmed that *C. callothyrsus* contains tannin (approximately 8.70%). Moreover, the *Leucaena leucocephala* is also known for its high tannin content (6.21-6.57%; Yusiati et al. 2018). Both tropical legumes are naturally enriched with polyphenols such as tannins that are able to bind proteins, and consequently protecting and reducing feed degradation during fermentation (Rimbawanto et al. 2015).

Furthermore, it is commonly known that rumen bacterial activities directly correlate with gas production, which consists of CO_2, free H_2, and CH_4 gases, as by-products of the substrate degradation process of the resulting Volatile Fatty Acids (VFA) during ruminal fermentation. Thus, the presence of polyphenols such as tannins in tropical legumes inhibits bacterial activity in degrading feed nutrients, consequently lowering the total gas production (Danielsson et al. 2017; Li et al. 2019). Additionally, less degradable feeds have also shown lower CH_4 production owing to less microbial breakdown of plant materials that are bound to tannins (Pal et al. 2015; Yanza et al. 2021).

There is limited literature on the dynamics of ruminal in vitro methane kinetics. Most of the studies measuring methane production in vitro employed a single point measurement at a certain incubation period, mainly either at 24 or 48 h. In recent years, the model only interprets the dynamics of total gas production during fermentation but does not appreciate the potential of the CH_4 kinetics pattern, which is also a part of ruminal gas production. Hence, this study attempted to assess the kinetics of CH_4 gas production during 48 h of fermentation. Although there were no differences in the methane production of potential degraded fraction of substrate (a + b), the ensiling process significantly influenced in vitro methane production. However, a high Standard Error of Means (SEM) was observed, indicating that the measured methane production kinetics increased with bias.

Moreover, methane production from the soluble fraction (a) of non-ensiled samples showed lower ruminal methane levels production after the 48 h in vitro incubation, which also likely had a lower rate of nutrient degradation (c) to form CH_4. The fiber-degrading bacteria had an increasing proportion of easily fermentable carbohydrates, such as soluble sugars and organic acids from complex fibers of plant compounds during the ensiling process, further efficiently reducing the fermentation activity of ruminal bacteria in degrading the remaining nutrients and improving propionate production instead of acetate (Zhao et al. 2018). Such conditions can improve ruminal fermentation efficiency of feed nutrients and simultaneously reduce gas production, including methane production (Cui et al. 2020). Although no bacterial community was observed, the fermentation pattern was suspected to have changed during the ensiling process. The dominance of fiber-degrading bacteria was reduced over several days while propionate-producing microbes in the ensiling environment were favorably altered. Hence, ensiled forages resulted in higher lactic acid and propionate concentrations, and silage substrates were fed to ruminants with efficiently fermented, consequently reducing methane production (Ahmed et al. 2023; Pu et al. 2023).

The significant reduction in methane kinetics during in vitro ruminal fermentation has allegedly impacted how feed is metabolized and the production of methane by ruminal methanogens (Archaea). The formation of methane by Archaea depends on the available free hydrogen from the nutrient synthesis that was converted into acetate. However, instead of being attached by methanogens, several dominant ruminal bacteria shift to capture hydrogen to form propionates (Hill et al. 2016). Volatile fatty acids were not quantified in the present study, although Ku-Vera et al. (2020) confirmed that such conditions are strongly associated with reduced methane production during ruminal fermentation. Nonetheless, when the dynamics of methane production rates are expressed as (a + b), neither the legume type nor the interaction between legume ensiling factors showed significant results. *C. callothyrsus*, *L. leucocephala*, and *C. pubescens* were found to have a lower potential for ruminal methane production, especially in ensiled samples. Such conditions can be linked to the presence of polyphenols such as tannins, whereas some tropical legumes contain high tannins, inhibit the methanogenesis process of ruminal microbes (Soltan et al. 2012; Rira et al. 2022). The reduction in methane gas emissions can be influenced by phenolic compounds present in tropical legume plants. During the initial stages of wilting and chopping of legumes for silage, Polyphenol Oxidase (PPO) can be activated. The activation mechanism of PPO involves binding polyphenols, such as tannins, to

degradable nutrients like sugars, which are susceptible to degradation. This binding helps to protect nutrients within the rumen, thereby indirectly reducing rumen gas production, including CH_4 (Lee 2014; Lee et al. 2019; Li et al. 2019).

This study highlights the benefits of integrating non-ensiled and ensiled tropical legumes into ruminant diets to mitigate enteric methane production and improve in vitro feed digestibility (Aragadvay-Yungan et al. 2022). The addition of several tropical legumes to grass silage can reduce the total gas production by up to 40% and methane emissions by up to 30%. Among the experimented *I. zollingeriana* demonstrated the highest digestibility, with no significant influence on lowering the methane. Meanwhile, *C. callothyrsus*, *C. pubescens*, and *L. leucocephala*, resulted in reduced ruminal methane production.

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REFERENCES

Abdullah L. 2010. Herbage production and quality of shrub *Indigofera* treated by different concentration of foliar fertilizer. Media Peternakan 33 (3): 169-169. DOI: 10.5398/medpet.2010.33.3.169.

Ahmed M, Al-Sagheer A, El-Waziry A, El-Zarkouny S, Elwakeel E. 2023. Ensiling characteristics, in vitro rumen fermentation patterns, feed degradability, and methane and ammonia production of berseem (*Trifolium alexandrinum* L.) co-ensiled with artichoke bracts (*Cynara cardunculus* L.). Animals 13 (9): 1543. DOI: 10.3390/ani13091543.

Alayón JA, Albores-Moreno S, Jiménez-Ferrer G, Alarcón-Zúñiga B, Miranda-Romero LA, Pérez-Luna EJ, Canul-Solís J. 2023. Tropical tree foliage supplementation in ruminants improves rumen fermentation and the bacterial profile and decreases methane production. Anim Biotechnol 34: 4510-4522. DOI: 10.1080/10495398.2023.2165935.

AOAC [Association of Official Analytical Chemists]. 2005. Official Methods of Analysis (18th ed). Association of Official Analytical Chemists, Gaithersburg, MD, USA.

Aragadvay-Yungán RG, Barros-Rodríguez,M, Ortiz L, Carro MD, Navarro Marcos C, Elghandour MMMY, Salem AZM. 2022. Mitigation of ruminal methane production with enhancing the fermentation by supplementation of different tropical forage legumes. Environ Sci Pollut Res 29 (3): 3438-3445. DOI: 10.1007/s11356-021-15749-7.

Atmojo FA, Suhartanto B, Zulfa IH, Kustantinah K. 2020. Evaluation use of *Calliandra calothyrsus* substituted soybean meal supplement on feed nutrient intake and digestibility in the Kacang goat. Key Eng Mater 840: 107-112. DOI: 10.4028/www.scientific.net/KEM.840.107.

Auerbach H, Nadeau E. 2020. Effects of additive type on fermentation and aerobic stability and its interaction with air exposure on silage nutritive value. Agronomy 10: 1229. DOI: 10.3390/agronomy10091229.

Bodas R, Prieto N, García-González R, Andrés S, Giráldez FJ, López, S. 2012. Manipulation of rumen fermentation and methane production with plant secondary metabolites. Animl Feed Sci Technol 176 (1-4): 78-93. DOI: 10.1016/j.anifeedsci.2012.07.010.

Bueno IC, Brandi RA, Franzolin R, Benetel G, Fagundes GM, Abdalla AL, Louvandini H, Muir JP. 2015. In vitro methane production and tolerance to condensed tannins in five ruminant species. Anim Feed Sci Technol 205: 1-9. DOI: 10.1016/j.anifeedsci.2015.03.008.

Cieslak A, Szumacher-Strabel M, Stochmal A, Oleszek W. 2013. Plants components with specific activities against rumen methanogens. Animal 7: 253-265. DOI: 10.1017/S1751731113000852.

Cui X, Sun H, Wen X, Sobhi M, Guo J, Dong R. 2020. Urea-assisted ensiling process of wilted maize stover for profitable biomethane production. Sci Tot Environ 757: 143751. DOI: 10.1016/j.scitotenv.2020.143751.

Danielsson R, Dicksved J, Sun L, Gonda H, Müller B, Schnürer A, Bertilsson J. 2017. Methane production in dairy cows correlates with rumen methanogenic and bacterial community structure. Front Microbiol 8: 226. DOI: 10.3389/fmicb.2017.00226.

Darma ING, Jayanegara A, Sofyan A, Budiartilaconi E, Ridla M, Herdian H. 2023. Evaluation of nutritional values of tree-forage legume leaves from Gunungkidul District, Indonesia. Biodiversitas 24 (5): 2733-2745. DOI: 10.13057/biodiv/d240527.

Dewhurst RJ, Evans RT, Scollan ND, Moorby JM, Merry R J, Wilkins RJ. 2003. Comparison of grass and legume silages for milk production. 2. In vivo and in sacco evaluations of rumen function. J Dairy Sci 86 (8): 2612-2621. DOI: 10.3168/jds.S0022-0302(03)73856-9.

Dos Santos WCC, do Nascimento WG, Magalhães ALR, Silva DKA, Silva WJCS, Santana AVS, Soares GSC. 2015. Nutritive value, total losses of dry matter and aerobic stability of the silage from three varieties of sugarcane treated with commercial microbial additives. Anim Feed Sci Technol 204: 1-8. DOI: 10.1016/j.anifeedsci.2015.03.004.

Franco M, Rinne M. 2023. Dry matter content and additives with different modes of action modify the preservation characteristics of grass silage. Fermentation 9 (7): 640. DOI: 10.3390/fermentation9070640.

Gomes FM, Ribeiro KG, De Souza IA, de Lima Silva J, Agarussi MCN, Da Silva VP, Da Silva TC. Pereira OG. 2021. Chemical composition, fermentation profile, microbial population and dry matter recovery of silages from mixtures of palisade grass and forage peanut. Trop Grassland-Forrajes 9 (1): 34-42. DOI: 10.17138/tgft(9)34-42.

Hassan FU, Arshad MA, Ebeid HM, Rehman MSU, Khan MS, Shahid S, Yang C. 2020. Phytogenic additives can modulate rumen microbiome to mediate fermentation kinetics and methanogenesis through exploiting diet-microbe interaction. Front Vet Sci 7: 575801. DOI: 10.3389/fvets.2020.575801.

Hill J, McSweeney C, Wright A, Bishop-Hurley G, Kalantar-zadeh K. 2016. Measuring methane production from ruminants. Trends Biotech 34 (1): 26-35. DOI: 10.1016/j.tibtech.2015.10.004.

Htay TM, Sann KK, Haini H. 2023. Comparative study on phytochemical screening and antioxidant activity of aqueous extract from various parts of *Bauhinia purpurea*. Bioactivities 1 (1): 24-31. DOI: 10.47352/bioactivities.2963-654X.183.

Ineichen S, Seiler AB, Wyss U, Malisch CS, Reidy B. 2023. Ensilibility and protein degradation characteristics of forage from mountain grasslands containing tanniferous species. Grass Forage Sci 78 (4): 622-635. DOI: 10.1111/gfs.12620.

Jayanegara A, Goel G, Makkar HP, Becker K. 2015. Divergence between purified hydrolysable and condensed tannin effects on methane emission, rumen fermentation and microbial population in vitro. Anim Feed Sci Technol 209: 60-68. DOI: 10.1016/j.anifeedsci.2015.08.002.

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. DOI: 10.3390/ani10091531.

Kondo M, Hirano Y, Kita K, Jayanegara A, Yokota HO. 2014. Fermentation characteristics, tannin contents and in vitro ruminal degradation of green tea and black tea by-products ensiled at different temperatures. Asian-Australas J Anim Sci 27: 937-945. DOI: 10.5713/ajas.2013.13387.

Ku-Vera J, Jiménez-Ocampo R., Valencia-Salazar S, Montoya-Flores M, Molina-Botero I, Arango J, Gómez-Bravo C, Aguilar-Pérez C, Solorio-Sánchez F. 2020. Role of secondary plant metabolites on enteric methane mitigation in ruminants. Front Vet Sci 7: 584. DOI: 10.3389/fvets.2020.00584.

Lee MRF, Fychan R, Tweed JK, Gordon N, Theobald V, Yadav R, Marshall A. 2019. Nitrogen and fatty acid rumen metabolism in cattle offered high or low polyphenol oxidase red clover silage. Animal 13 (8): 1623-1634. DOI: 10.1017/S1751731118003294.

Lee MRF. 2014. Forage polyphenol oxidase and ruminant livestock nutrition. Front Plant Sci 5: 694. DOI: 10.3389/fpls.2014.00694.

Li R, Teng Z, Lang C, Zhou H, Zhong W, Ban Z, Yan X, Yang H, Farouk M, Lou Y. 2019. Effect of different forage-to-concentrate ratios on ruminal bacterial structure and real-time methane production in sheep. PLoS One 14 (5): e0214777. DOI: 10.1371/journal.pone.0214777.

Niderkorn V, Jayanegara A. 2021. Opportunities offered by plant bioactive compounds to improve silage quality, animal health and product

quality for sustainable ruminant production: A review. Agronomy 11 (1): 86. DOI: 10.3390/agronomy11010086.

Ørskov ER, McDonald I. 1979. The estimation of protein degradability in the rumen from incubation measurements weighted according to the rate of passage. J Agric Sci 92 (2): 499-503. DOI: 10.1017/S0021859600063048.

Pal K, Patra A, Sahoo A. 2015. Evaluation of feeds from tropical origin for in vitro methane production potential and rumen fermentation in vitro. Spanish J Agric Res 13: 0608. DOI: 10.5424/SJAR/2015133-7467.

Parastiwi HA, Lestari NSH, Yanza YR, Niderkorn V, Ridwan R, Jayanegara A. 2023. Estimating nutrient composition and polyphenol concentration using Near-Infrared Spectroscopy (NIRS) in tropical forages. Biodiversitas 24: 6652-6660. DOI: 10.13057/biodiv/d241227.

Pu X, Zhang X, Yi S, Wang R, Li Q, Zhang W, Qu J, Huo J, Lin B, Tan B, Tan Z, Wang M. 2023. Mixed ensiling plus nitrate destroy fiber structure of rape straw, increase degradation and reduce methanogenesis through in vitro ruminal fermentation. J Sci Food Agric 104 (6): 3428-3436. DOI: 10.1002/jsfa.13228.

Rahmat SFI, Permana IG. 2021. Rumen degradation properties of tropical legumes feed under in sacco studies. IOP Conf Ser: Earth Environ Sci 888: 012071. DOI: 10.1088/1755-1315/888/1/012071.

Ridla M, Asikin N, Jayanegara A, Samsudin AA. 2023. Mitigating methane emissions for rural tropical livestock through selecting appropriate grass types. Livest Res Rur Dev 35 (7): 1-6.

Rimbawanto EA, Yusiati LM, Baliarti E, Utomo R. 2015. Effect of condensed tannin of *Leucaena* and *Calliandra* leaves in protein trash fish silage on in vitro ruminal fermentation, microbial protein synthesis and digestibility. Anim Prod 17: 83-91. DOI: 10.20884/1.anprod.2015.17.2.505.

Rira M, Morgavi DP, Popova M, Maxin G, Doreau M. 2022. Microbial colonisation of tannin-rich tropical plants: Interplay between degradability, methane production and tannin disappearance in the rumen. Animal 16 (8): 100589. DOI: 10.1016/j.animal.2022.100589.

Samal I, Bhoi TK, Raj MN, Majhi PK, Murmu S, Pradhan AK, Kumar D, Paschapur AU, Joshi DC, Guru, PN. 2023. Underutilized legumes: nutrient status and advanced breeding approaches for qualitative and quantitative enhancement. Front Nutr 10: 1110750. DOI: 10.3389/fnut.2023.1110750.

Santoso B, Widayati TW, Hariadi BT. 2020. Improvement of fermentation and the in vitro digestibility characteristics of agricultural waste-based complete feed silage with cellulase enzyme treatment. Adv Anim Vet Sci 8 (8): 873-881. DOI: 10.17582/journal.aavs/2020/8.8.873.881.

Schnaider MA, Ribeiro HMN, Kozloski GV, Reiter T, Orsoletta ACD, Dallabrida AL. 2014. Intake and digestion of wethers fed with dwarf elephant grass hay with or without the inclusion of peanut hay. Trop Anim Health Prod 46: 975-980. DOI: 10.1007/s11250-014-0594-5.

Schuba J, Südekum KH, Pfeffer E, Jayanegara A. 2017. Excretion of faecal, urinary urea and urinary non-urea nitrogen by four ruminant species as influenced by dietary nitrogen intake: A meta-analysis. Livest Sci 198: 82-88. DOI: 10.1016/j.livsci.2017.01.017.

Shoe HMN, Hai JB, Bo PT, Gong XW, Liu CJ, Dang K, Tian LX, Soomro RN, Aung KL, Feng BL. 2021. Evaluation of nutritive values through comparison of forage yield and silage quality of mono-cropped and intercropped maize-soybean harvested at two maturity stages. Agriculture 11 (5): 452. DOI: 10.3390/agriculture11050452.

Silva VP, Almeida FQD, Morgado EDS, Rodrigues LM, Santos TMD, Ventura HT. 2010. In situ caecal degradation of roughages in horses. Rev Bras Zootec 39: 349-355. DOI: 10.1590/S1516-35982010000200018.

Soltan YA, Morsy AS, Sallam SMA, Louvandini H, Abdalla AL. 2012. Comparative in vitro evaluation of forage legumes (*Prosopis*, *Acacia*, *Atriplex*, and *Leucaena*) on ruminal fermentation and methanogenesis. J Anim Feed Sci 21: 759-772. DOI: 10.22358/jafs/66148/2012.

Sońta M, Rekiel A, Sońta M. 2020. Legumes - use for nutritional and feeding purposes. J Elementol 25 (3): 835-849. DOI: 10.5601/jelem.2020.25.1.1953.

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: 185-197. DOI: 10.1016/0377-8401(94)90171-6.

Van Soest PJ, Robertson JB, Lewis BA. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J Dairy Sci 74 (10): 3583-3597. DOI: 10.3168/jds.S0022-0302(91)78551-2.

Voisin AS, Guéguen J, Huyghe C, Jeuffroy MH, Magrini MB, Meynard JM, Mougel C, Pellerin S, Pelzer E. 2014. Legumes for feed, food, biomaterials and bioenergy in Europe: a review. Agronom Sustain Develop 34: 361-380. DOI: 10.1007/s13593-013-0189-y.

Xia GH, Wu CR, Zhang MZ, Yang F, Chen C, Hao J. 2023. The metabolome and bacterial composition of high-moisture Italian ryegrass silage inoculated with lactic acid bacteria during ensiling. Biotech Biof Biop 16 (1): 91. DOI: 10.1186/s13068-023-02346-8.

Xue Z, Wang Y, Yang H, Li S, Zhang, Y. 2020. Silage fermentation and in vitro degradation characteristics of orchardgrass and alfalfa intercrop mixtures as influenced by forage ratios and nitrogen fertilizing levels. Sustainability 12: 871. DOI: 10.3390/su12030871.

Yáñez-Ruiz DR, Bannink A, Dijkstra J, Kebreab E, Morgavi DP, O'Kiely P, Reynolds CK, Schwarm A, Shingfield KJ, Yu Z, Hristov AN. 2016. Design, implementation and interpretation of in vitro batch culture experiments to assess enteric methane mitigation in ruminants - a review. Anim Feed Sci Technol 216: 1-18. DOI: 10.1016/j.anifeedsci.2016.03.016.

Yanza YR, Fitri A, Suwignyo B, Elfahmi, Hidayatik N, Kumalasari NR, Irawan A, Jayanegara A. 2021. The utilisation of tannin extract as a dietary additive in ruminant nutrition: A meta-analysis. Animals 11: 3317. DOI: 10.3390/ani11113317.

Yusiati LM, Kurniawati, A, Hanim C, Anas DM. 2018. Protein binding capacity of different forages tannin. IOP Conf Ser*:* Earth Environ Sci 11: 012007. DOI: 10.1088/1755-1315/119/1/012007.

Zain M, Despal, Tanuwiria UH, Pazla R, Putri EM and Amanah U. 2023. Evaluation of legumes, roughages, and concentrates based on chemical composition, rumen degradable and undegradable proteins by in vitro method. J Vet Sci 12: 528-538. DOI: 10.47278/journal.ijvs/2022.218.

Zakaria ZA, Abdul Hisam EE, Rofiee MS, Norhafizah M, Somchit MN, Teh LK, Salleh MZ. 2011. In vivo antiulcer activity of the aqueous extract of *Bauhinia purpurea* leaf. J Ethnopharmacol 137 (2): 1047-1054. DOI: 10.1016/j.jep.2011.07.038.

Zhao J, Dong Z, Li J, Chen L, Bai Y, Jia Y, Shao T. 2018. Effects of lactic acid bacteria and molasses on fermentation dynamics, structural and nonstructural carbohydrate composition and in vitro ruminal fermentation of rice straw silage. Asian-Australas J Anim Sci 32: 783-791. DOI: 10.5713/ajas.18.0543.