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ORIGINAL PAPER

Evaluation of feed supplementation using legume leaf meal and spent substrate fermented by *Eupenicillium javanicum* as a potential feed for ruminants: an *in vitro* study

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ABSTRACT. Legume forage contains anti-nutrients that mitigate enteric methane production by reducing microbial activity in the rumen. Therefore, this study aimed to observe the inclusion of 49.6% legumes in concentrate, with or without *Eupennicillum javanicum* fermented spent substrate supplementation on *in vitro* feed fermentation and methane emission. The experimental diet consisted of a legume containing (L) or non-legume (NL) concentrate with (+S) or without (−S) spent substrate at 10% dry matter concentrate. This study used a complete randomised design in a 2 × 2 factorial arrangement with five replicates per treatment. The ratio of concentrate to forage was 1:3, with the legume component containing a mixture of Indigofera sp. and *Gliricidia* sp. at a ratio of 3:1. The results showed that *Indigofera* sp. and *Gliricidia* sp. could substitute 49.6% of the concentrate without affecting feed digestibility. Furthermore, the addition of *Indigofera* sp., *Gliricidia* sp., and spent substrate effectively reduced methane production in ruminants.

Introduction

Sufficient nutrient supply, particularly energy and protein, determines optimal livestock performance. Potential feed resources, such as crop residues, by-products, and legumes are available in Indonesia. Legume forage contains more protein than grass (Fadiyimu et al., 2016) and agricultural by-products (Fadiyimu et al., 2016; Idan et al., 2020), making it a cost-effective protein source (Idan et al., 2020).

Gliricidia sepium is a common legume species used as a protein supplement in Indonesia (Marhaeniyanto et al., 2020). It contains 17.85–25.08% protein on a dry matter (DM) basis (Aye and Adegun, 2013; Fadiyimu et al., 2016; Gunasekaran et al., 2017; Idan et al., 2020), but also several anti-nutrition factors: 3.51 mg/100 g tannin, 15.24 mg/100 g phytic acid, 4.3 mg/100 g phytic P, 4.82 mg/100 g oxalate, 7.51% saponin, 6.33% alkaloid, 5.25% flavonoid (Aye and Adegun, 2013), and 25.42 mg/100 g cyanide (Paranamana et al., 2015). According to a study by Paranamana et al. (2015), *Gliricidia* sp. has a higher content of tannins (9.04 g/100 g) and phytic acid (0.34 g/100 g), but lower saponin levels (3.94 g/100 g) compared to other forage plants. Additionally, this legume contains 5.67% acid detergent fibre (ADF), 4.72% neutral detergent fibre (NDF), 5.25% acid detergent lignin (ADL), 4.35% cellulose, and 2.66% haemicellulose (Aye and Adegun, 2013). The inclusion of *Gliricidia* sp. and other legume fodder in the diet can improve *in vivo* digestibility of DM, crude protein (CP), and crude

fibre (CF) in sheep (Adegun, 2014). Furthermore, the NH_3 content, *in vitro* dry matter digestibility (IVDMD), and *in vitro* organic matter digestibility (IVOMD) of *Gliricidia* was reported by Jayanegara et al. (2016) to amount to 5.68 mM, 43.5%, and 35.0%, respectively.

Indigofera zellingofera is another legume found in Indonesia. Feeding Boerka goat a diet consisting of 90% pellet of *Indigofera zellingofera* did not affect dry matter digestibility (DMD), organic matter digestibility (OMD), crude protein digestibility (CPD), pH, NH_3 (mM), and total volatile fatty acid (VFA) (mM) (Tarigan et al., 2018). An *in vitro* study using goat rumen liquid showed that the inclusion of 20% *Indigofera zellingofera* improved IVDMD, IVDOM, and *in vitro* CPD (Suharlina et al., 2016). The NH_3 content, IVDMD, and IVOMD of Indogofera obtained after fermentation were 12.28 mM, 70.3%, and 65.7%, respectively (Jayanegara et al., 2016). Additionally, extracts of this legume have also been reported to contain antioxidants (Singh et al., 2015).

The aforementioned legume forages contain high amounts of protein and secondary plant metabolites, and their addition to feeds can reduce enteric methane emissions. *Indigofera* sp. contains phenol as an antimicrobial agent (Gerometta et al., 2020), whereas *Gliricidia* sp. has 1% tannin, 0.04% condensed tannins (Santos et al., 2017), and 17 mg/g saponins (Molina-Botero et al., 2019).

On the other hand, ruminants produce the highest enteric methane emissions, which can be reduced through feeding management, rumen modifiers, and genetic improvements (Knapp et al., 2014). The first two are short-term emission mitigation strategies and involve providing high-quality nutrients and microbial inclusion (Knapp et al., 2014).

Common feed additives used for reducing methane emissions include saponin and tannins found in legumes. Saponin can decrease enteric methane production by reducing the abundance of total protozoa (Patra and Yu, 2013), which serve as methanogen hosts. Condensed tannins also diminish enteric methane production (Tan et al., 2011; Focant et al., 2019) by decreasing the total counts of ciliate protozoa and methanogens (Tan et al., 2011). Therefore, supplementation with *Indigofera* and *Gliricidia*, which contain phenolic compounds, is expected to effectively reduce methane emissions.

Eupenicillium javanicum produces the BS4 mannanase enzyme through the fermentation of copra meal substrate. These fungi also synthesise other enzymes, such as cellulase, xylanase, CMCase, β-glucosidase, pectinase, and mannosidase (Purwadaria et.al., 2003; Tao et al., 2011; Evelyn et al., 2020). The extraction residue generated during BS4 enzyme production contains a high protein content, making it a potential protein source for ruminants. Additionally, residual fungi may be present in the substrate, which may enhance rumen fermentation. Therefore, the objective of this study was to evaluate the potential inclusion of legumes in the concentrate and supplementation of spent substrate from BS4 enzyme production to improve feed efficiency and decrease methane production. The most significant finding was the possibility of utilising spent substrate from *E. javanicum* fermentation as feed for ruminants.

Material and methods

This research was conducted with the approval of the Animal Ethics Committee (Balitbangtan/Balitnak/Rm/02/2021). Rumen fluid was collected in a slaughterhouse in Bogor, West Java, and strained using cheesecloth.

Forage and feed preparation

Napier grass (*Pennisetum purpureum*), *Gliricidia sepium*, and *Indigofera* sp. were harvested from the research station farm of the Indonesian Research Institute for Animal Production (IRIAP), Ciawi, Bogor. After harvest, the forage was chopped and dried at 60 °C in a Memmert Seri UN-oven for 48 h until constant weight (AOAC International, 1995), then ground using a GRT-400A(K) Grinder (Yongkang Tiange Electric Co., Ltd., Jinhua, ZJ, China) and sieved through a 2 mm mesh size. Legume leaf meal and other feed ingredients were used to formulate the concentrate feed as a dietary treatment.

The non-legume (NL) and legume (L) diets were formulated using a mixture of NL concentrate and Napier grass, and L concentrate and Napier grass, respectively. The NL concentrate contained 14.8% rice bran, 11.1% cassava waste, 9.6% copra meal, 30% pollard, 10% molasses, 9.8% palm kernel meal, 12.2% distillers dried grains with solubles (DDGS), 0.4% urea, 0.4% $CaCO_3$, 0.4% dicalcium phosphate (DCP), 0.8% salt, and 0.5% crude palm oil (CPO). Meanwhile, the L concentrate included 10.4% cassava waste, 9.6% copra meal, 4% pollard, 10% molasses, 13.6% DDGS, 0.4% $CaCO_3$, 0.4% DCP, 0.8% salt, 1.2% CPO, and 49.6% mixed legume meal (*Indigofera* sp. to *Gliricidia* sp. ratio – 3:1).

Experimental design and data analysis

This study evaluated two types of diets, i.e., without legumes and with legume leaf meal inclusion, along with the addition or lack thereof of spent substrate for BS4 enzyme production. According to Haryati et al. (2019), BS4 is produced by fermentation of coconut cake by *Eupennicillium javanicum*. The study was conducted in a completely randomised design using a 2 × 2 factorial arrangement with five replicates per treatment. Two diet types (legume and non-legume), addition or lack of addition of BS4 enzyme spent substrate, and their interaction were used as fixed effects and repeated (n = 5) as random factors using the following model:

$$Y_{ijk} = \mu + L_i + P_j + (L \times P)_{ij} + e_{ij},$$

where: Y_{ijk} – is the dependent variable, μ is the overall mean, L_i – effect of diet type (i = 2), and P_j – effect of BS4 enzyme spent substrate addition (j = 2) (with and without the addition), $(L \times P)_{ij}$ – interaction between diet type and BS4 enzyme spent substrate addition, and e_{ij} – the error term.

The collected data were analysed using analysis of variance (ANOVA) in SPSS. Additionally, Duncan's test was applied to analyse the interaction of treatments, while the T-test was used to evaluate the effect of diet type or the addition of BS4 enzyme spent substrate. Differences between treatments were considered significant at $P < 0.05$.

The diets evaluated were as follows: (1) 75% non-legume concentrate + 25% Napier grass, (2) 75% concentrate containing 49.6% legume leaf meal (3:1 ratio of *Indigofera* sp. to *Gliricidia* sp.) + 25% Napier grass, (3) 75% non-legume concentrate supplemented with 10% BS4 spent substrate + 25% Napier grass, and (4) 75% concentrate containing 49.6% legume leaf meal (3:1 ratio of *Indigofera* sp. to *Gliricidia* sp.) supplemented with 10% BS4 spent substrate + 25% Napier grass. The nutrient contents of the concentrates and Napier grass were analysed for organic matter (OM), CP, and ether extract (EE), according to AOAC International (1995), as well as NDF, and ADF using the procedure of Van Soest et al. (1991) (Table 1).

Table 1. Chemical composition of diets supplemented with legume leaf meal and BS4 spent substrate formulated in 75% concentrate + 25% Napier grass

Item, %	NL		L	
	0% S	10% S	0% S	10% S
Dry matter	88.79	88.56	91.01	88.28
Ash	12.25	11.11	12.39	11.08
Organic matter	76.54	76.31	78.62	77.20
Crude protein	17.32	22.14	18.34	20.14
Ether extract	4.21	4.19	4.33	4.44
GE, kcal/kg DM	4265	4318	4305	4450
NDF	59.25	55.71	56.98	58.44
ADF	29.42	29.30	39.98	39.97

NL – diet with non-legume concentrate and Napier grass; L – diet with concentrate containing 49.6% legume leaf meal (*Indigofera* sp. to *Gliricidia* sp. ratio – 3:1) and Napier grass, S – BS4 spent substrate, GE – gross energy, NDF – neutral detergent fibre, ADF – acid detergent fibre

Fermentation

Fermentation was conducted for 48 h under anaerobic conditions at 39 °C, and the incubation medium was prepared as described by Theodorou et al. (1994). Diet samples weighing approximately 1 g were transferred to fermentation tubes. The incubation medium consisted of a mixture of 10 ml of rumen fluid and 90 ml of buffer added to the bottle. In addition, CO_2 was continuously injected into the tubes during preparation to ensure anaerobic conditions. The tubes were subsequently sealed and placed in a Memmert shaking water bath (Memmert, Germany) at 39 °C, and incubated for 48 h. *In vitro* digestibility of neutral detergent fiber and protein of *Pennisetum purpureum* incubated for 48 h did not differ from those incubated for 72 h (Ortiz and Vega, 2020). In line with these reports, gas production in the present study was measured at 0, 3, 24, 36, and 48 h of incubation.

Subsequently, the supernatant was filtered, and the substrate was dried in an oven at 60 °C until stable weight. The mass of the dry residue was used to calculate DMD, and the dried sample was ashed at 550 °C to calculate OMD. IVDMD and IVOMD were calculated using the following formula (Larsen and Jones, 1973):

$$\text{IVDMD} = \frac{\text{sample DM} - (\text{residual DM} - \text{DM residue of incubated blank})}{\text{sample DM}} \times 100$$

$$\text{IVOMD} = \frac{\text{sample OM} - (\text{residual OM - OM residue of incubated blank})}{\text{sample OM}} \times 100$$

The pH of the supernatant was measured after 48 h of incubation in the medium before filtration, and the NH_3 concentration was measured according to the method of Conway and Malley (1942). Boric acid mixed with an indicator, was placed in the central chamber unit, while the sample and half-saturated potassium carbonate were placed in the outer chamber unit. After overnight incubation, NH_3 was absorbed by boric acid and titrated back with HCl. Rumen microbial populations were counted using

the method of Ogimoto and Imai (1981). A 0.5 ml sample of rumen fluid was mixed with 0.5 ml of trypan blue formalin saline. The protozoa populations were directly counted under a microscope (40×) on 5 squares of a counting chamber and calculated using the following formula:

$$P = \left(\frac{n}{5}\right) \times 10^4 \times d,$$

where: P – number of ciliates/1 ml rumen contents, n – number of squares counted in the counting chamber, d – dilution factor of the sample.

The population of total bacteria was also counted according to Ogimoto and Imai (1981) using the roller tube method and rumen fluid glucose cellobiose agar modification. In this method, 45 ml of anaerobic dilution solution and 0.5 ml of rumen sample were transferred to a Hungate tube. The samples were diluted serially 10 times. Samples (0.5 ml) from dilutions 6 to 10 were placed into petri dishes containing rumen fluid glucose cellobiose agar (RGCA) (HiMedia Laboratories Pvt. Ltd., Mumbai, India) and rotated to form a lemniscate for homogeneous mixing of the sample. The samples were incubated for 48 h at a temperature of 37–40 °C. Calculation of bacteria populations were conducted using the following formula:

$$BP = C \times 10^n \times 2,$$

where: BP – total bacteria population, C – number of colony forming units, n – dilution factor.

Methane measurement

Gas production during fermentation was recorded and collected at 0, 3, 24, 36, and 48 h during the 48 h of incubation. The gas collection process involved capturing gas from the fermentation tube using a glass vessel connected by a pipe. The gas was then injected into 100 ml of 1 N sodium hydroxide (NaOH) in a bottle connected to other glass vessels, to measure methane production. Total methane production was estimated based on the volume gas collected in a glass vessel connected to a bottle (T system connector). This technique, based on the protocol described by Maarif and Januar (2009), utilises NaOH to react with CO_2 forming Na_2CO_3. Since methane does not react with NaOH, the CO_2 in the fermentation gas, which contains both CH_4 and CO_2, reacts with NaOH, allowing CH_4 to flow through the pipe into the glass vessel. The prediction of methane production using NaOH at different concentrations and volumes (4 ml of 10 M NaOH) was also conducted as described by Fievez et al. (2005).

Results

Fermentation

The outcomes of *in vitro* fermentability of the diet during 48 h of incubation for IVDMD, IVOMD, and fermentation products are presented in Table 2. They showed that IVDMD, NH_3, and bacteria population were affected by the interaction between legume and spent substrate supplementation. On the other hand, these factors did not affect IVOMD and pH ($P > 0.05$). The addition of spent substrate to the NL diet reduced IVDMD, and resulted in the most abundant bacteria population ($P > 0.05$). The main effects of legume inclusion and spent substrate supplementation were record-

Table 2. *In vitro* fermentability of diets supplemented with legume leaf meal and BS4 spent substrate during 48 h of incubation

Protein source	Diet				*P*-value		
	NL		L				
Spent substrate	0% S	10% S	0% S	10% S	PS*	SS*	PS × SS
Digestibility, %							
IVDMD	77.98 ± 2.25^{a}	72.52 ± 3.70^{b}	74.29 ± 2.70^{ab}	75.85 ± 3.98^{ab}	0.900	0.200	0.028
IVOMD	74.08 ± 2.85	71.50 ± 6.94	69.35 ± 4.55	71.48 ± 4.82	0.305	0.923	0.308
Fermentability characteristic							
pH	6.5	6.8	6.7	6.9	NA	NA	NA
NH_3, mM	21.80 ± 2.08^{b}	23.90 ± 2.07^{b}	23.80 ± 3.11^{b}	28.20 ± 1.92^{a}	0.024	0.019	0.004
Rumen microbial counts							
bacteria, 10^9 CFU/ml	4.18 ± 0.31^{c}	6.14 ± 0.22^{a}	2.81 ± 0.24^{d}	5.37 ± 0.21^{b}	<0.001	<0.001	<0.001
protozoa, 10^5 CFU/ml	27.20 ± 1.68	19.30 ± 2.02	57.80 ± 4.83	45.80 ± 1.64	<0.001	<0.001	0.148

NL – diet with non-legume concentrate and Napier grass, L – diet with concentrate containing 49.6% legume leaf meal (*Indigofera* sp. to *Gliricidia* sp. ratio – 3:1) and Napier grass, PS – protein source, SS – spent substrate, S – BS4 spent substrate, IVDMD – *in vitro* dry matter digestibility, IVOMD – *in vitro* organic matter digestibility, CFU – colony forming unit; *P*-value column significant difference at α = 0.05, *analysed using T-test; interaction between PS and SS analysed using Duncan's test; $^{a–d}$ means within a row with different superscripts are significantly different

ed for the protozoa population. Legume inclusion increased the protozoa population, whereas spent substrate addition decreased it. Additionally, the *in vitro* assay showed that the NL diet without spent substrate resulted in higher IVDMD, while the NL diet with spent substrate showed lower IVDMD ($P < 0.01$).

The inclusion of the spent substrate tended to improve the rumen bacteria population and decrease the protozoa population ($P < 0.001$ for spent substrate (SS). In contrast, the inclusion of legumes resulted in an increasing trend of the abundance of the protozoa population and decreasing the bacteria population ($P < 0.001$ for (protein source PS).

Similar findings were reported by Yuliana et al. (2019), where the inclusion of *Gliricidia sepium* did not affect IVOMD of the diet (Yuliana et al., 2019).

The IVDMD of the legume leaf meal feed was influenced by the interaction between legume and spent substrate supplementation. Specifically, the spent substrate in the NL diet reduced IVDMD. A similar study by Lee et al. (2014) showed that the addition of β-mannanase decreased IVDMD. There was no significant interaction between the supplementation of BS4 spent substrate and legume on IVOMD, as shown in Table 2. The similarity of IVOMD in all diets could be attributed to the nutrient content, particularly the availability of NDF

Table 3. *In vitro* gas production of diet supplemented with legume leaf meal and BS4 spent substrate during 48 h of incubation

Protein source	Diet				*P*-value		
	NL		L				
Spent substrate	0% S	10% S	0% S	10% S	PS*	SS*	PS × SS
Total gas							
ml	97.6 ± 1.14^{a}	83.4 ± 1.67^{b}	84.4 ± 1.67^{b}	82.6 ± 1.34^{b}	<0.001	<0.001	<0.001
ml/g DM of feed	219.86 ± 3.04^{a}	188.82 ± 4.79^{b}	183.75 ± 3.58^{c}	187.48 ± 2.52^{c}	<0.001	<0.001	<0.001
ml/g OM of feed	290.86 ± 4.03^{a}	246.74 ± 6.26^{b}	234.87 ± 4.58^{c}	245.40 ± 3.30^{b}	<0.001	<0.001	<0.001
Methane gas							
ml	30.10 ± 0.55^{a}	24.00 ± 0.00^{c}	25.60 ± 1.08^{b}	24.40 ± 0.55^{c}	<0.001	<0.001	<0.001
%	30.84 ± 0.70	28.79 ± 0.57	30.34 ± 1.43	29.54 ± 0.53	<0.001	<0.001	0.133
ml/g DM of feed	67.80 ± 1.27^{a}	54.33 ± 0.36^{b}	55.75 ± 2.65^{b}	55.38 ± 1.36^{b}	<0.001	<0.001	<0.001
ml/g OM of feed	89.70 ± 1.68^{a}	71.00 ± 0.47^{b}	71.25 ± 3.39^{b}	72.5 ± 1.77^{b}	<0.001	<0.001	<0.001

NL – diet with non-legume concentrate and Napier grass, L – diet with concentrate containing 49.6% legume leaf meal (*Indigofera* sp. to *Gliricidia* sp. ratio 3:1) and Napier grass, PS – protein source, SS – spent substrate, S – BS4 spent substrate, DM – dry matter, OM – organic matter; *P*-value column indicates significant difference at α = 0.05, *analysed using T-test; interaction between PS and SS analysed using Duncan's test; ^{abc} means within a row with different superscripts are significantly different

In vitro gas production

The total gas and methane production is presented in Table 3. The results indicated that treatments significantly affected both total gas and methane gas production ($P < 0.05$), while the proportion of methane generation to total gas production was not significantly altered ($P > 0.05$). The diet with the non-legume concentrate showed higher total gas and methane gas production compared the other treatments.

Discussion

Fermentation

The average IVOMD, pH, and NH_3 values were 71.60%, 6.7, and 24.44 mM, respectively. The inclusion of legume leaf meal or BS4 spent substrate had no effect on IVOMD production. This suggests that legumes can be used as concentrate components without negatively affecting feed digestibility. and ADF in the feed (Table 1). This indicated that the inclusion of BS4 spent substrate did not affect NDF and ADF availability in the feed.

The feed in the current study contained 4.8% copra meal (CM) in the concentrate. Previous studies have reported that copra meal can substitute 50–75% of soybean meal (Paengkoum, 2011). Copra meal contains 68.99% carbohydrates, 79.77% of which is mannose (Khuwijitjaru et al., 2012), and it has been shown to decrease feed digestibility in Brahman crossbreeds (Chuntrakort et al., 2014). Cellulose and hemicellulose are other carbohydrate components of copra meal that reduce feed digestion, as reflected by the differences between NDF and ADF. Additionally, legume cell wall materials (CWM) were found to be similar between NL and NLS or L and LS.

The inclusion of *E. javanicum* and its products has been previously shown to potentially increase feed digestibility. According to Haryati et al. (2019), *E. javanicum* produces enzymes such as mannanase, which can improve the digestibility of palm kernel

meal, containing a similar carbohydrate. Fungi can produce several enzymes such as mannanase (Haryati et al., 2019) and proteases (Neto et al., 2014; 2019), and an *in vivo* study showed that the inclusion of β-mannanase increased the average daily gain and decreased the FCR of heifers (Seo et al., 2016).

However, the results in Table 2 indicate that the inclusion of spent substrate from BS4 production did not significantly improve IVDMD, possibly because CM was used only in a small proportion of the diet. The spent substrate exerted a slight effect on digestibility, as only low levels of cellulase (Evelyn et al., 2020) and mannanase activities were recorded.

Low mannanase activity could be due to either a limited mannanase concentration in the spent substrate or its reduced activity. The highest mannanase activity was recorded at pH 6 and a temperature of 60–70 °C. However, stable enzyme activity was observed at lower temperatures of 3 °C to 4 °C, while at 70 °C, this activity lasted for only 2 h (Ariandi et al., 2015). Feed incubation in the present experiment was carried out at 39–40 °C, and the pH range of 6.5–6.9, which could have led to reduced enzyme activity.

E. javanicum also produces a protease, which is expected to improve peptide availability, and an increase in its activity in the feed can be detected by higher rumen NH_3 levels. The results showed that the inclusion of this fungus and its product elevated rumen NH_3 concentrations, likely because the optimum proteolytic activity of this enzyme occurs at pH 6 and a temperature of 60 °C (Neto et al., 2017).

The optimal conditions for *E. javanicum* to produce both mannanase and protease are pH of 5.8 and temperatures of 50 °C and 30 °C, respectively (Haryati et al., 2019; Neto et al., 2013). Despite the suboptimal conditions in the present experiment, the inclusion of this fungus improved feed digestibility and NH_3 levels of rumen fluid.

The experiment demonstrated that IVDMD and IVOMD affected gas production (Table 3). Higher digestibility was predicted to increase gas production, as observed for the NL diet, which showed a trend of greater digestibility and correspondingly higher gas production.

The IVDMD of the samples exceeded that reported by Ding et al. (2015), who used barley grain. However, it was comparable to the *in vivo* and IVDMD results published by Sato et al. (2020).

The IVOMD in this study was comparable to the OMD values in previous studies by Roca-Fernández (2020), Rufino-Moya et al. (2019), and Sato et al. (2020), while it was higher than that obtained by Akakpo et al. (2020) for groundnut and soybean fodder. This difference could be attributed to the specific concentrates utilised in the present study. The addition of BS4 spent substrate in the current experiment neither affected rumen fermentation in the NL nor L diets. Therefore, BS4 spent substrate can be used as a component in rations rather than as a source of enzymes or fungi.

The inclusion of legumes increased the protozoa population and tended to decrease the abundance of bacteria. Rira et al. (2015) reported that the supplementation of tannin-rich plants (*Gliricidia sepium*, *Leucaena leucocephala*, and *Manihot esculenta*) reduced the total counts of bacteria but did not affect the protozoa population.

This study demonstrated that the inclusion of legumes increased the protozoa population, while showing a trend towards decreased methane gas production. This finding was consistent with studies of Aziz et al. (2018) and Ramos et al. (2021), who observed a decrease in ruminal pH and total protozoa with increasing concentrate levels in feed. The present study replaced some concentrate using legumes, which increased the protozoa population. This increase did not relate to methanogenesis (Bhatta et al., 2013) as a methane gas producer..

The physical form of feed have also been shown to affect the total counts of protozoa, with block-form feeds showing more positive effects on their numbers compared to mashed and pelleted forms (Karimizadeh et al., 2017). Feed in the form of blocks may increase chewing activity, leading to increased saliva flow to the rumen. The more intensive chewing with respect to highfiber feeds also affects the buffer capacity in the rumen due to elevated saliva production, creating optimal conditions for protozoa. Moreover, Bohatier (1991) claimed that protozoa often attach to plant substrates, which means that feeds with higher fiber content offer more attachment sites for these organisms.

In vitro gas production

The concentrate diet supplied in this study caused higher *in vitro* gas production (ml/g OM) compared to the findings of Guzatti et al. (2017), which used only mixed forages, and obtained similar *in vitro* results (ml/g) to Sato et al. (2020), who administered a mix of concentrate and forage. The interaction between legume and spent substrate supplementation affected total gas and methane production. The result showed that the inclusion of

BS4 spent substrate in the NL and L diets tended to decrease gas production (ml). Moreover, total gas production in the case of the NL diet without BS4 spent substrate was the highest and closely related to feed digestibility. Although OM digestibility was similar between diets, gas production was higher in the NL sample without spent substrate.

The lower gas production in the L diet sample could be due to the lower digestibility of non-starch polysaccharides in legumes, which typically produce less gas compared to starch-rich diets. Molina-Botero et al. (2020) also reported that replacing *Brachiaria brizantha* with 15% *Gliricidia sepium* decreased gas production over a 48-h period.

In contrast, Rufino-Moya et al. (2019) observed higher methane/total gas production, likely due to the utilisation of legumes containing more condensed tannins. Methane production (ml/g DM and ml/g OM) decreased in the NL diet supplemented with BS4 spent substrate, as well as in both L diets, regardless of spent substrate supplementation. However, the proportion of methane in total gas production (%) was not affected by legume leaf meal and BS4 spent substrate supplementation. Comparatively, the L diet exhibited a higher methane-to-total gas ratio (%) than the NL diet, while supplementation with BS4 spent substrate resulted in a lower ratio. Additionally, both the inclusion of legumes (*Indigofera* sp. + *Gliricidia* sp.) and BS4 spent substrate demonstrated potential in reducing methane production, which could be attributed to the activity of phenolic compounds, such as tannins and saponins, present in both legumes (Gerometta et al., 2020).

Conclusions

The present study found that *Indigofera* sp. and *Gliricidia* sp. could effectively replace 49.6% of the concentrate without significantly affecting feed digestibility. Furthermore, their inclusion in the concentrate diet significantly reduced methane production. Moreover, the BS4 spent substrate can be used as a feed source in the L diet with concentrate containing 49.6% legume leaf meal (*Indigofera* sp. to *Gliricidia* sp. ratio – 3:1) and Napier grass without adversely affecting feed digestibility, total gas, and methane production.

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Conflict of interest

The Authors declare that there is no conflict of interest.

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