Influence of feed supplement based on *Andrographis paniculata* and irradiated chitosan on *in vitro* fermentation and methane production of selected forage

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Abstract. The Wallacean region of Indonesia, specifically the Nusa Tenggara and South Sulawesi regions in Indonesia, carry a large population of cattle. Therefore, it is necessary to evaluate methane production from forage-based feed in this region. The objective of this study was to determine the effect of feed supplement containing *Andrographis paniculata* and irradiated chitosan on the *in vitro* fermentation and methane production, of forage-based rations in this region. This study evaluates five forages that are mainly used as feed ingredients by smallholder farmers nnamely, (Sorghum forage, *Pennisetum purpureum cv. Mott*, *Pennisetum purpupoides*, *Indigofera zollingeriana* and cocoa pod husk. That five forages were supplemented with *Andrographis paniculata* and irradiated chitosan. There were a total of 10 treatments with four replications. The samples were subjected to *in vitro* analysis using the gas production technique. There was no differences between the treatment with and without feed supplements on *in vitro* gas production. There was no interaction between feed supplement addition and forage type. Feed supplement addition influence pH ($P<0.05$) and single chain fatty acids (SCFAs) value ($P<0.01$). The addition of feed supplements with added *Andrographis paniculata* and irradiated chitosan in the *Pennisetum purpupoides* forage can reduce the production of enteric methane ($P<0.05$). The results suggest that, except for sorghum forages, the addition of feed supplements tended to reduce methane gas emissions from forage samples. Moreover, except for pH and SCFAs, *In vitro* gas production and digestibility of forage from eastern Indonesia were not affected by the addition of feed supplement.

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1 Introduction

Minimising methane emissions is one of the most important ruminant nutrition objectives. Because ruminants generate greenhouse gas (GHG) emissions that are associated with climate change, the environmental sustainability of ruminant farming practices has been heavily criticised [1]. The data on livestock sector methane emissions in Indonesia are continually updated to ensure their accuracy. According to these data, methane gas emissions have increased each year [2]. Therefore, Indonesian livestock stakeholders must take a role in reducing methane emissions. The Wallacean part of Indonesia, specifically the Nusa Tenggara and South Sulawesi regions, is a major resource of large ruminants [3]. Therefore, it is necessary to evaluate methane production from forage-based feed in this region. Various farming methods, modifications to diets, feed additives, chemical methanogenesis modulators, probiotics, immunisation against the rumen microbiome, selective breeding, and genetic techniques are now being used to reduce methane emissions from ruminants [4]. The most recent advances in the understanding of methane production have led to a development of feed additives that may reduce methane emissions. Among these additives are tannin and saponin [1, 5, 6], 3-Nitroxypropanol [7], seaweed [8], chitosan [9], and fatty acids [10].

Indonesia is rich in fishery byproducts and herbal production, and has the potential to produce additives that reduce methane emissions. Chitosan can be used as methane-reducing compounds because it is antimicrobial and affects the fermentation process [11]. Chitosan may be utilised as a natural rumen modifier because it modifies rumen fermentation in desirable methods, such as increasing propionate and decreasing acetate, which is desirable for higher energy synthesis with possibly minimised methane emission [9]. As herbs, due to the availability of active compounds in *Andrographis paniculata*, it can be used as a supplement's ingredient [12, 13]. As ruminant feed supplements, it is necessary to evaluate the effectiveness of these two component combinations. Previous study reported that feed supplements based on herbs and chitosan could reduce methane emission without reducing forage digestibility [14]. However, to our knowledge, there are no studies that have evaluated the use of this feed supplement on some common forages from Wallacea region in Indonesia. Therefore, the aim of the experiment was to determine the effect of feed supplement with addition of *Andrographis paniculata* and irradiated chitosan on *in vitro* fermentation and methane production in forage-based rations.

2 Experimental Procedure

2.1 Sample preparation

Sorghum forage, *Pennisetum purpureum cv. Mott*, and *Pennisetum purpureum* were collected from the field laboratory, Siwabessy Science and Technology Area, National Research and Innovation Agency, Jakarta, Indonesia. *Indigofera zollingeriana* was collected from the field laboratory, Ciawi, Ministry of Agriculture Republic Indonesia. Cocoa pod husk was obtained from the field laboratory, Faculty of Animal Science, Haluoleo University, Kendari, Southeast Sulawesi. Samples of forage were dried at 60oC for 72 h and then ground into 1 mm particles using a hammer mill. In preparation for nutrient and *in vitro* analysis, dry samples were kept in plastic containers at 4^{o}C. Feed supplement containing Andrographis

paniculata and irradiated chitosan was obtained from the Research Center for Animal Husbandry, BRIN.

2.2 Nutrient and fibre analysis

The content of organic matter (OM), ash, crude protein (CP), and ether extract (EE) were analysed according to Association of Official Analytical Chemist method [15]. Neutral detergent fibre (NDF), and acid detergent fibre (ADF) were analysed according to Ankom procedure protocol methods 6 [16] and 5 [17]. Hemicellulose was estimated using equations, as follows:

$$\text{Hemicellulose (\%)} = \text{NDF (\%)} - \text{ADF (\%)}$$

2.3 Experimental design

This study investigates five forages that are primarily utilized as feed ingredients by smallholder in Wallacea area, Eastern Indonesia Region (Sorghum forage, *Pennisetum purpureum cv. Mott*, *Pennisetum purpureum*, *Indigofera zollingeriana*, and cocoa pod husk). That five forages were supplemented with feed supplement with added Andrographis paniculata and irradiated chitosan (10 g/kg DM). There were in total ten treatments with four replications.

2.4 *In vitro* analysis

In vitro gas production was determined according to the Hohenheim gas test procedure [18] with slight modifications. Rumen fluids were collected from two fistulised ongole crossbreeds' bulls (approximate average 350 kg live weight) cared at Animal Nutrition field laboratory, Faculty of Animal Science, Gadjah Mada University, Yogyakarta, Indonesia. A 300 mg (DM basis) of each sample was weighed into a 100 ml glass syringe (Fortuna, Germany). Media (rumen fluid + buffer) consisting of 33% rumen fluid was prepared and put into a syringe (40 ml of each sample). The glass syringe was incubated at 38.5-39.5oC for 48 h. *In vitro* gas production was observed after 3, 6, 9, 12, 24, 36 and 48 h of incubation. Total gas production values were adjusted by following Ørskov and McDonald's equation [19] as follows: P=a+b (1-e^(-ct)), where P is the gas production at time t (ml), a is the *in vitro* gas production from the soluble fraction (ml), b is the *in vitro* gas production from the insoluble fraction (ml), c is the gas production rate constant, (a+b) is the potential gas production (ml), and t is the incubation time (h). After 48 h incubation, samples-rumen-buffer fluids were collected for pH, NH3 and single chain fatty acids (SCFAs) observation. Metabolizable energy (ME), *in vitro* organic matter digestibility (IVOMD) and microbial protein (MP) were calculated as follows [18, 20, 21]:

$$\text{ME (MJ/kg DM)} = 2.2 + (0.136 \times \text{GP}) + (0.057 \times \text{CP}) + (0.0029 \times \text{EE})$$
$$\text{IVOMD (\%)} = 14.88 + (0.889 \times \text{GP}) + (0.45 \times \text{CP}) + (0.0651 \times \text{ash})$$
$$\text{MP (g/kg IVOMD)} = \text{IVOMD} \times 19.3 \times 6.25$$

Where: GP is gas production at 12 h (ml/300 mg DM); CP is crude protein (% DM); and EE is ether extract (% DM).

2.5 Methane production estimation

Methane (CH4) emission estimation was calculated based on SCFAs proportions by following equation [22]:

$$\text{Methane emission (mM)} = (\text{acetate proportion} \times 0.5) + (\text{butyrate proportion} \times 0.5) - (\text{propionate proportion} \times 0.25)$$

Methane production was converted to mM/100 mg IVOMD.

2.6 Statistical analysis

The data were analysed based on a completely randomised design with two factors. The first factor was forage type, followed by feed supplement addition as the second factor. SPSS version 25.0 was used for analysis of variance on the data. The Duncan's Multiple Range Test was used to evaluate all mean comparisons [23].

3 Result and discussion

Nutrient and fibre composition from the five samples of forages are presented in Table 1. All nutrient and fibre compositions differed significantly ($P<0.01$) between forage types. Pennisetum purpureum cv. Mott had the greatest ($P<0.05$) ash concentration (17.43%DM) and the lowest OM concentration. Cocoa pod husk had the lowest ($p<0.05$) concentration of EE (0.81 %). *Indigofera zollingeriana* demonstrated the highest level of CP (25.22 %), followed by Pennisetum purpureum cv. Mott (12.81%), and *Pennisetum purpupoides* (11.01%). In contrast, sorghum forage and cocoa pod husk had the lowest CP ($P<0.05$). In terms of fibre composition, the concentration of NDF and ADF in *Indigofera zollingeriana* was lowest ($p<0.05$), by 50.55 and 31.64%, respectively. Even though Sorghum forage had the highest NDF and ADF, there are no significant differences with *Pennisetum purpupoides*. Cocoa pod husk and *Indigofera zollingeriana* had lower hemicellulose content than other forages ($p<0.05$).

Table 1. Nutrient and fibre composition forage samples

	Nutrient and fibre composition (% DM)						
Forage	**Ash**	**OM**	**EE**	**CP**	**NDF**	**ADF**	**Hemi**
Sorghum forage	10.23^{a}	89.77^{c}	4.21^{b}	7.25^{b}	73.01^{c}	47.74^{c}	25.27^{b}
Pennisetum purpureum cv. Mott	17.43^{c}	82.57^{a}	3.46^{b}	12.81^{d}	71.77^{c}	43.37^{b}	28.40^{c}
Pennisetum purpupoides	15.31^{b}	84.69^{b}	3.79^{b}	11.01^{c}	72.51^{c}	45.44^{bc}	27.07^{bc}
Indigofera zollingeriana	10.51^{a}	89.49^{c}	3.86^{b}	25.22^{e}	50.55^{a}	31.64^{a}	18.90^{a}
Cocoa pod husk	10.56^{a}	89.44^{c}	0.81^{a}	6.45^{a}	58.78^{b}	42.21^{b}	16.57^{a}
SEM	0.812	0.812	0.309	1.550	2.042	1.257	1.118

DM (dry matter), OM (organic matter), EE (ether extract), CP (crude protein), NDF (neutral detergent fiber), ADF (acid detergent fiber), Hemi (hemicellulose).
Different superscripts in the same row indicate significant differences ($P < 0.05$), Standard Error of the Means (SEM).

Table 2. *In vitro* gas production and gas characteristics of forage and feed supplement treatment (ml/300 mg DM)

Forage	**FS**	**Incubation time (h)**							**gas characteristics**	
		3	**6**	**9**	**12**	**24**	**36**	**48**	**a+b**	**c**
Sorghum forage	-	12.08^{a}	18.03^{ab}	24.34^{a}	31.87^{a}	53.40^{a}	64.26^{ab}	70.39^{ab}	85.21^{bc}	0.038^{a}
Sorghum forage	+	10.14^{a}	15.74^{a}	21.51^{a}	29.03^{a}	49.50^{a}	59.81^{a}	65.41^{a}	79.31^{ab}	0.038^{a}
Pennisetum purpureum cv. Mott	-	11.94^{a}	20.13^{bc}	30.11^{b}	43.22^{bc}	66.47^{b}	80.88^{d}	88.26^{fg}	102.91^{d}	0.042^{ab}
Pennisetum purpureum cv. Mott	+	13.75^{ab}	22.38^{c}	31.33^{b}	43.95^{bc}	65.63^{b}	81.90^{d}	89.36^{g}	103.69^{d}	0.042^{ab}
Pennisetum purpupoides	-	12.59^{a}	21.20^{bc}	29.82^{b}	41.09^{b}	62.30^{b}	73.57^{c}	79.53^{cde}	89.00^{c}	0.048^{b}
Pennisetum purpupoides	+	13.45^{a}	20.18^{bc}	31.12^{b}	41.72^{b}	63.09^{b}	74.19^{c}	80.08^{cde}	89.06^{c}	0.049^{b}
Indigofera zollingeriana	-	17.44^{bc}	27.64^{de}	36.52^{c}	46.71^{cd}	64.31^{b}	72.70^{c}	77.47^{bcd}	80.15^{ab}	0.067^{c}
Indigofera zollingeriana	+	17.27^{bc}	26.83^{d}	36.56^{c}	45.62^{bcd}	61.72^{b}	70.11^{bc}	74.80^{bc}	76.86^{a}	0.069^{c}
Cocoa pod husk	-	21.60^{d}	31.40^{ef}	39.54^{c}	47.84^{cd}	67.43^{b}	82.21^{d}	84.16^{defg}	90.37^{c}	0.061^{c}
Cocoa pod husk	+	20.50^{cd}	32.14^{f}	36.56^{c}	49.07^{d}	67.36^{b}	80.87^{d}	87.37^{efg}	90.77^{c}	0.060^{c}
SEM		0.655	0.884	0.961	1.054	0.983	1.228	1.311	1.535	0.002
Forage		**	**	**	**	**	**	**	**	**
Feed Supplement		ns	ns	ns	ns	ns	ns	ns	ns	ns
Forage x Feed Supplement		ns	ns	ns	ns	ns	ns	ns	ns	ns

FS (Feed supplement addition), a+b (optimum gas production), c (gas production rate constant.
Different superscripts in the same row indicate significant differences (P<0.05), Standard Error of the Means (SEM), ** (P<0.01), * (P<0.05)

It is necessary to examine nutrient profiles to identify nutritional characteristics that will influence feed fermentation. Due to its origin in the legume class, *Indigofera zollingeriana* has the greatest crude protein content. According to Kumalasari [24] experiment, *Indigofera zollingeriana* contains a protein content of between 25.17 and 26.44%. Sorghum forage, *Pennisetum purpupoides* and *Pennisetum purpureum cv.* Mott had higher fibre contents than other forages. According to Wahyono et al. [25], fiber components in forages have a negative correlation with digestibility and nutritive value. This complex combination may make foodstuff less digestible. Methane emissions are correlated with high hemicellulose and NDF concentration [26]. Low-nutrient forages can increase methane emissions since ruminants' digestive systems process them more slowly [14, 27].

In vitro gas production represents degradability characteristics from forage samples. *In vitro* gas production and gas characteristics of forage and feed supplement treatment are shown in Table 2. Significant differences were observed between forages for total gas production and gas characteristics (P<0.01). In contrast, there was no difference between the treatment with and without feed supplements. There was no interaction between feed supplement addition and forage type with regard to gas characteristics and total gas production. Cocoa pod husks (with and without feed supplement) had the highest *in vitro* gas production (3 – 36 h; P<0.05). Furthermore, after 36 h incubation, Pennisetum purpureum cv. Mott produced the highest gas production (P<0.05). Pennisetum purpureum cv. Mott (with and without feed supplement) also had the greatest optimum gas production (a+b). With

regard to gas production rate constant (c), *Indigofera zollingeriana* and cocoa pod husk had the highest rate constant (P<0.05).

Previous research indicated that feed supplements containing irradiated chitosan and Andrographis paniculata influenced the characteristics of forage gas production [14]. Haryati et al. [11] also stated that the addition of feed additives chitin and chitosan has a significant impact on total gas production. However, these findings are not consistent with the findings presented by Yusuf et al. [12], who reported that the addition of *Andrographis paniculata* had no effect on the total gas production of basal feed. This difference may be related to variations in the nutrient composition of the forages. The findings of this study indicate that the addition of feed supplements has no negative effect on the digestibility of forage.

Table 3. *In vitro* fermentation characteristics of forage and feed supplement treatment

Forage	FS	ME (MJ/kg DM)	IVOMD (%)	MP (g/kg IVOMD)	pH	NH_3 (mg/100 ml)	SCFAs (mMol)		
							C_2	C_3	C_4
Sorghum forage	-	9.89^{a}	47.14^{a}	56.86^{a}	7.00^{c}	44.77^{b}	47.67^{a}	13.75^{a}	7.37^{a}
Sorghum forage	+	9.36^{a}	44.62^{a}	53.82^{a}	7.03^{c}	44.49^{b}	47.36^{a}	13.11^{a}	7.14^{a}
Pennisetum purpureum cv. Mott	-	11.98^{bc}	60.21^{bc}	72.63^{bc}	6.93^{ab}	46.71^{b}	64.88^{c}	17.93^{bc}	9.60^{cd}
Pennisetum purpureum cv. Mott	+	11.87^{bc}	60.86^{bc}	73.41^{bc}	6.91^{a}	45.69^{b}	61.46^{c}	17.20^{b}	8.80^{b}
Pennisetum purpupoides	-	11.31^{b}	57.36^{b}	69.19^{b}	7.07^{c}	48.57^{b}	62.51^{c}	16.65^{b}	8.94^{bc}
Pennisetum purpupoides	+	11.42^{b}	57.92^{bc}	69.86^{bc}	6.92^{a}	48.70^{b}	53.70^{b}	14.00^{a}	7.55^{a}
Indigofera zollingeriana	-	12.39^{c}	68.45^{d}	82.57^{d}	7.04^{c}	60.07^{c}	70.97^{d}	18.75^{cd}	10.17^{d}
Indigofera zollingeriana	+	12.04^{bc}	67.48^{d}	81.39^{d}	7.05^{c}	58.14^{c}	63.64^{c}	16.82^{b}	9.06^{bc}
Cocoa pod husk	-	11.74^{bc}	61.00^{bc}	73.58^{bc}	6.90^{a}	38.85^{a}	66.70^{cd}	20.46^{e}	8.95^{bc}
Cocoa pod husk	+	11.73^{bc}	62.09^{c}	74.89^{c}	6.88^{a}	38.28^{a}	64.56^{c}	19.60^{de}	8.67^{b}
SEM		0.186	1.398	1.686	0.014	1.514	1.358	0.413	0.175
Forage		**	**	**	**	**	**	**	**
Feed Supplement		ns	ns	ns	*	ns	**	**	**
Forage x Feed Supplement		ns	ns	ns	**	ns	ns	ns	ns

FS (feed supplement); ME (metabolizable energy); IVOMD (*in vitro* organic matter digestibility), MP (microbial protein); NH_3 (ammonia); SCFAs (short chain fatty acids); C_2 (acetate); C_3 (propionate); C_4 (butyrate)

Different superscripts in the same row indicate significant differences (P<0.05), Standard Error of the Means (SEM), ** (P<0.01), * (P<0.05)

In vitro fermentation characteristics from forage samples and feed supplement treatment are shown in Table 3. Except pH, there was no significant interaction between forage type and feed supplement treatment. Significant differences were found between forages (pP<0.01). Feed supplement addition influences pH (P<0.05) and SCFAs value (P<0.01). Sorghum forage (with and without feed supplement) had the lowest ME, IVOMD and MP (P<0.05). *Indigofera zollingeriana* (with and without feed supplement) produced the highest IVOMD, MP and NH3 (p<0.05). The pH value ranges of ten treatments were from 6.88-7.05. Feed supplement addition in *Indigofera zollingeriana* and *Pennisetum purpupoides* could reduce C_2, C_3, and C_4 production (P<0.05). However, for other forages, feed supplement addiction treatment obtained numerically lower SCFAs concentration than without supplement treatment. Enteric methane estimation from forage-based ruminant rations, with and without supplementation are presented in Figure 1. Differences in forage samples and the addition of feed supplements influence methane production (P<0.05). Regarding methane production, there was no interaction between forage type and feed supplement treatment. The addition of feed supplements containing *Andrographis paniculata* and irradiated chitosan in the *Pennisetum purpupoides* sample reduced the production of enteric methane (P<0.05).

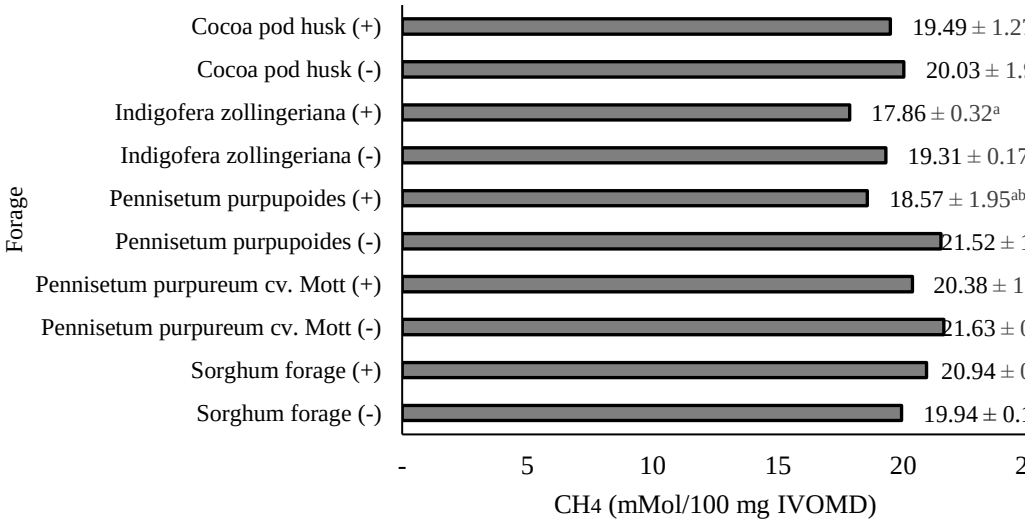

Fig. 1. Enteric methane estimation from forage-based ruminant rations, before (-) and after (+) supplementation. *In vitro* organic matter digestibility (IVOMD)

As a feed additive, chitosan may reduce methane production, enhance propionate production, decrease the acetate/propionate ratio, and contribute to improved animal performance [28]. Shah et al. [29] reported that chitosan has antibacterial properties that change the structural bacterial community, continuing the rumen fermentation pattern to propionate acid productions, which explains the reduced methane emission. This mechanism can be seen from changes in the SCFAs composition, particularly the propionate concentration shown in Table 3. However, chitosan's ability to reduce methane production is also influenced by the extraction method. Strongly hydrophobic chitosan had the best methane hydrate inhibition effectiveness and might extend the hydrate induction period [30]. It has been suggested that the bioactive compounds found in *Andrographis paniculata* play

a role in methane reduction. Tannins present in this herb can be used as methane inhibitors [13].

4 Conclusion

In vitro gas production and digestibility of forages in this study were not affected by the addition of *Andrographis paniculata* and irradiated chitosan-based feed supplements. However, the addition of feed supplements changed the pH value and characteristics of SCFAs. Except for sorghum forages, the addition of feed supplements tended to reduce methane gas emissions from some tested forage samples.

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