

ISSN 1411-2027 e-ISSN 2541-5875

ANIMAL PRODUCTION

Accredited by

Dirjen Penguatan Risbang Kemenristek Dikti No 32a/E/KPT/2017



Sinta Score

Indexed by



Volume 22 (1), 2020

FACULTY OF ANIMAL SCIENCE JENDERAL SOEDIRMAN UNIVERSITY

in collaboration with

INDONESIAN SOCIETY OF ANIMAL SCIENCE

ANIMAL PRODUCTION

Scientific Journal of Farm Animals and Feed Resources in the Tropic

VOLUME 22, NUMBER 1, 2020

Accredited by Dirjen Penguatan Risbang Kemenristek Dikti No 32a/E/KPT/2017

ISSN 1411-2027

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BANK ACCOUNT

Bank Mandiri Purwokerto
Account name: Jurnal Animal Production
Account number: 139.001.227.2690

Indexed in: Google Scholar, ISJD, Crossref, BASE, Citeulike, Academic Research Index, Electronic Journal Library, Open Academic Journal Index, CiteFactor, Academic Journal Database, OCLC Worldcat, Index Copernicus

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***Indigofera zollingeriana* Leaf Extract Reduces Sheep Rumen Methane Production in Vitro**

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Abstract. An experimental study aimed at assessing the reduction of gas methane production of sheep using *Indigofera zollingeriana* leaf extract was conducted from May to September 2019 at the Nutrition and Animal Feed Laboratory, University of Jenderal Soedirman Purwokerto. A One Way Classification of Completely Randomized Design (CRD) experiment was employed. The treatment included *Indigofera zollingeriana* leaf extract with levels of 0%, 0.40%, and 0.80% of feed dry matter and each treatment was repeated six times, hence 18 experimental units. The feed provided consisted of 60% concentrate and 40% ammoniated rice straw. The concentrate consisted of coconut cake and rice bran with a ratio composition of 1:2. The material used was rumen fluid obtained from three sheep sampled from the Sokaraja slaughterhouse immediately after the sheep were slaughtered. In vitro incubation was carried out for four hours. The variables measured included protozoan population, methane gas production and bacterial populations. The data obtained were then subjected to analysis of variance and continued with the Orthogonal Polynomial test. Methane gas production observed in the present study ranges from 11.60 ± 0.76 to 14.37 ± 0.65 mM, thus the supplementation of *Indigofera zollingeriana* leaf extract decreases methane gas production by 19.28% compared to control. The use of 0.80% *Indigofera zollingeriana* leaf extract can reduce the protozoan populations, methane gas production and can increase the bacterial populations of sheep rumen fluid.

Keywords: *Indigofera zollingeriana*, leaf extract, protozoa, methane, bacteria

Abstrak. Penelitian eksperimental ini bertujuan untuk mengkaji penurunan produksi gas metana pada domba menggunakan ekstrak daun *Indigofera zollingeriana* dilakukan dari Mei hingga September 2019 di Laboratorium Nutrisi dan Makanan Ternak, Universitas Jenderal Soedirman Purwokerto. Penelitian menggunakan Rancangan Acak Lengkap (RAL) pola searah. Perlakuan berupa suplementasi ekstrak daun *Indigofera zollingeriana* dengan taraf 0%, 0,40%, dan 0,80% dari bahan kering pakan dan masing-masing perlakuan diulang enam kali, sehingga terdapat 18 unit percobaan. Pakan yang diberikan terdiri dari 60% konsentrat dan 40% jerami padi amoniasi. Konsentrat terdiri dari bungkil kelapa dan dedak dengan komposisi perbandingan 1: 2. Materi yang digunakan adalah cairan rumen yang diperoleh dari tiga ekor domba yang diambil dari rumah pemotongan hewan Sokaraja segera setelah domba dipotong. Inkubasi in vitro dilakukan selama empat jam. Variabel yang diukur yaitu populasi protozoa, produksi gas metana dan populasi bakteri. Data yang diperoleh kemudian dianalisis variansi dan dilanjutkan dengan uji Orthogonal Polinomial. Produksi gas metana yang diamati dalam penelitian ini berkisar antara $11,60 \pm 0,76$ dan $14,37 \pm 0,65$ mM, sehingga suplementasi ekstrak daun *Indigofera zollingeriana* mengurangi produksi gas metana sebesar 19,28% dibandingkan dengan kontrol. Penggunaan ekstrak daun *Indigofera zollingeriana* 0,80% dapat mengurangi populasi protozoa, produksi gas metana dan dapat meningkatkan populasi bakteri cairan rumen domba.

Kata kunci: *Indigofera zollingeriana*, ekstrak daun, protozoa, metana, bakteri

Introduction

Smallholder farms in Indonesia are still dominated by traditional keeping systems, characterized by providing low-quality feed originated from agricultural wastes, including rice straw, which has several disadvantages, including low nutrient content, especially fermentable carbohydrate content. Its crude protein content is also low i.e. around 5.06%, its

lignin content is high and it binds cellulose to form lignocellulosic bonds making it difficult to digest by rumen microbes (Prasetyono et al., 2007; Herawati and Adang, 2010). Thus, rice straw-based animal feed will disturb the fulfillment of energy and protein needed by both the host animal and the microbes present in the rumen. In conditions of low feed quality, protozoa will ingest bacteria and convert

bacterial proteins into protozoan proteins. It causes the bacterial populations to decline and vice versa protozoan populations increase. It further causes protein supply to host animals to decrease because bacteria are the main supplier of protein for ruminants. The increase in protozoan populations in addition to bacterial populations' reduction, it also increases methane gas production because 9-25% of methanogenic bacteria are symbiotic by sticking to the protozoan surface (Santoso and Hariadi, 2007).

Ruminants produce methane gas (CH_4) which contributes to 18% of the total accumulated greenhouse gases in the atmosphere (Kreuzer and Soliva 2008). These methane emissions are not only related to environmental problems but also reflect the loss of some energy from livestock that cannot be utilized for the subsequent production process. Between 6-10% of the gross energy of ruminant animal feed is lost as methane (Jayanegara and Sofyan, 2008). Therefore it is necessary to develop a feeding strategy that is able to reduce ruminant livestock methane emissions that are beneficial both in the long term for reducing the rate of accumulation of greenhouse gases, and in the short term for reducing livestock energy loss.

Various techniques have been carried out to reduce the production of methane gas (CH_4) by livestock, including the use of chemical monensin, α -bromoethanesulfonic acid and nitrates/nitrites. However, the use of chemicals with high concentrations in the long term can cause residues in livestock products and toxic effects on livestock, thus these additives are not recommended for use in controlling CH_4 production (McAllister et al., 1996). Nowadays the use of natural food additives as a substitute for chemical additives including antibiotics and ionophores as fermentation manipulators in the rumen is increasingly popular. Susanti and Marhaeniyanto (2014) prove that saponins from plant leaves, including *Indigofera*

zollingeriana leaves can reduce methane gas production. This decrease can occur because saponins are able to kill or lyse protozoa by forming complex bonds with sterols found on the surface of the protozoan membrane so it interferes with the development of protozoa which causes membrane rupture, lysis cells and causes protozoa to die (Cheeke, 2000; Wahyuni et al. 2014). As many as 70% of the total methanogenic bacteria are symbiotic with protozoa, thus a decrease in the number of protozoa will be followed by a decrease in methanogenic bacteria (Jouany, 1991).

Saponins from plant leaves were reported to be able to increase the efficiency of the fermentation process through a mechanism of decreasing the protozoa population in the rumen by reducing the predatory nature of protozoa against bacteria (Wang et al., 2000). Suryapratama et al. (2018) prove that the use of Moringa leaf extract of up to 0.50% based on dry matter of feed can reduce the protozoa population, acetate concentration, methane gas production and increase the bacterial population and synthesis of rumen microbial protein. *Moringa leaf* as a defaunation agent has been widely studied. Therefore it is necessary to look for other defaunation agents which are easy to find and widely available but they have not been much explored, including *Indigofera zollingeriana*.

Based on the description that has been elaborated, there is a need to research the reduction of thin tail rumen methane gas production through the use of *Indigofera zollingeriana* leaf extract.

Materials and Methods

The method used in this study was experimental in vitro. The design used was Completely Randomized Design (CRD), One Way Classification (Steel and Torrie, 1993). The treatments tested consisted of:

T1 = feed consisting of 60% concentrate and 40% ammoniated rice straw

T2 = T1 + 0.40% *Indigofera zollingeriana* leaf extract

T3 = T1 + 0.80% *Indigofera zollingeriana* leaf extract

Indigofera zollingeriana obtained from CV. Nuansa Baru Lampung was given based on the feed dry matter. Each treatment was repeated six times so that there were 18 experimental units. The concentrate consists of coconut cake and rice bran with a ratio proportion of 1:2. The results of lab analysis of saponins was presented with +++ symbols. The results of the proximate analysis of the nutrient content of the tested feed ingredients are listed in Table 1.

Indigofera zollingeriana leaf extraction was using ethanol with a ratio between ethanol and *Indigofera zollingeriana* leaves of 3:1 resulting in $8.6 \pm 0.52\%$ extract. Calculated based on the nutrient content listed in Table 1, the nutrient content of basal ration is listed in Table 2. The leaves of *Indigofera zollingeriana* were dried in an oven at 60°C for 2 x 24 hours, then made into flour and extracted using ethanol.

The material used was rumen fluid obtained from three sheep taken from the Sokaraja

slaughterhouse immediately after the sheep had been slaughtered. The feed consists of concentrate and ammoniated rice straw with a 60:40 ratio based on the dry matter of the feed. In vitro incubation was carried out for four hours.

The measured variables were (1) the number of bacteria computed according to Cappucino and Sherman (2014), (2) the number of protozoa following Suryahadi (1990) method and (3) methane gas production measured according to Tamminga (1982) method. As much as 1 ml of the sample was aseptically dissolved into 9 ml of sterile distilled water (10^{-3} dilution), then followed by a serial dilution up to 10^{-6} . The last two dilutions (10^{-5} and 10^{-6}) were planted in a Duplo pour plate in a nutrient agar medium and subsequently incubated at 37°C for 2x24 hours. Colonies growing on medium were counted and reported in cfu/ml units.

The living protozoa population was counted by making a solution of Methylgreen Formalin Saline (MSF) consisting of 20 ml formaldehyde (35%), 180 ml aquadest, 0.12 g methylgreen 1.6 g NaCl (pa). As much as 0.1 ml of the prepared sample rumen fluid was taken and added with

Table 1. Nutrient Content of Feed Ingredients

Nutrient	Feed ingredients		
	<i>Indigofera zollingeriana</i>	Concentrate	Ammoniated rice straw
Dry Matter (%)	86.65	89.08	90.12
Protein (% BK)	24.00	13.73	5.30
Crude Fibre (% DM)	38.79	12.48	33.50
Fat (% DM)	13.52	11.49	7.56
Ash (% DM)	5.29	10.04	18.64

Source: Proximate analysis was performed at Nutrition and Animal Feed Laboratory of Faculty of Animal Science, University of Jenderal Soedirman (UNSOED) in 2019

Table 2. Nutrient content of treatment rations

Nutrient	Treatment rations		
	P1	P2	P3
Dry Matter (%)	89.50	89.53	89.56
Protein (% BK)	10.36	10.37	10.38
Crude Fibre (% DM)	20.89	20.90	20.92
Fat (% DM)	9.92	9.93	9.94
Ash (% DM)	13.48	13.48	13.48

0.1 ml of then mixed using a vortex until homogeneous. Counter deck glass of protozoa cover glass was placed on the surface, then 0.1 ml suspension was taken using a Pasteur pipette. The pipette tip was affixed to a v-shaped indentation on the edge of the counter deck glass of protozoa cover glass and then was viewed under a microscope with a magnification of 40X. Protozoa counting was done in five areas, for example N samples were obtained. The total protozoa per ml of rumen fluid is $\frac{N}{5} \times 10^4 \times 5$. Methane gas was computed using the formula $CH_4 = (0.5 \text{ Acetate} - 0.25 \text{ Propionate} + 0.5 \text{ Butyrate}) \text{ mM}$.

The data obtained were analyzed using analysis of variance followed by Honestly Significant Different and Orthogonal Polynomial Test (Steel and Torrie, 1993).

Results and Discussion

Protozoa

Protozoa are one of the microorganisms in the rumen, with a smaller amount compared to the number of bacteria, which is around 1 million/ml (McDonald et al., 2002). Its existence on one side is very necessary but on the other hand it can be detrimental. If the feed consumed by host animals is of low quality, protozoa will ingest bacteria. Therefore, the supply of bacteria to host animals will be reduced and the host animal productivity will decrease. The lowest average protozoan population in this study was achieved by rumen fluid which received a substrate by adding 0.8% *Indigofera zollingeriana* extract (Table 3).

Analysis of variance on the protozoan population showed that the addition of

Indigofera zollingeriana leaf extract affected the protozoan population ($P < 0.01$). The effect was linear with the equation $Y = 7.61 - 3.58 x$, the coefficient of determination (r^2) 0.89 (Figure 1).

The equation illustrates that the higher the level of *Indigofera zollingeriana* leaf extract, the more decrease the protozoan population. That is because the saponin compounds contained in the leaf extract of *Indigofera zollingeriana* act as defaunation agents. The research proves that the supplementation of *Indigofera zollingeriana* leaf extract up to 0.8% of the ration dry matter is able to reduce the protozoa count by 38.49% compared to ration without supplementation. According to Herdiawan et al (2014) the saponin content in *Indigofera* was 1.88% at 100% stress, whereas according to Abdulah (2010) 0.41-1.71% of the saponin content is dependent on the fertilizer dose.

Saponins are able to bind sterols contained in protozoan cell walls, thus affecting the surface tension of protozoan cell membranes. This results in increased cell wall permeability and eventually fluid from outside the cell will enter the protozoan cell. The entry of fluids from outside the cell causes rupture of the cell wall so that the protozoa experience death or lysis (Suharti et al., 2009). Yaghoubi et al. (2010) states that saponins function to interfere with the development of protozoan populations because saponins are able to make a bond with sterols on the surface of protozoan cell membranes. This causes the protozoan cell membrane to rupture, the cell undergoes lysis and ultimately causes the death of the protozoa (Thalib, 2008).

Table 3. Rumen Fermentation Products

Variables Measured	Treatments			P
	T1	T2	T3	
Protozoa ($\times 10^5/\text{ml}$)	7.43 ^a $\pm$ 0.54	6.52 ^b $\pm$ 0.25	4.57 ^c $\pm$ 0.19	<0.01
Methane Gas (mM)	14.37 ^a $\pm$ 0.65	18.88 ^b $\pm$ 1.31	11.60 ^c $\pm$ 0.76	<0.01
Bacteria (Cfu $\times 10^7/\text{ml}$)	2.66 ^a $\pm$ 0.27	11.31 ^b $\pm$ 0.81	12.85 ^{bc} $\pm$ 2.81	<0.01

Note: Different superscripts on the same rows indicate significant different ($P < 0.1$)

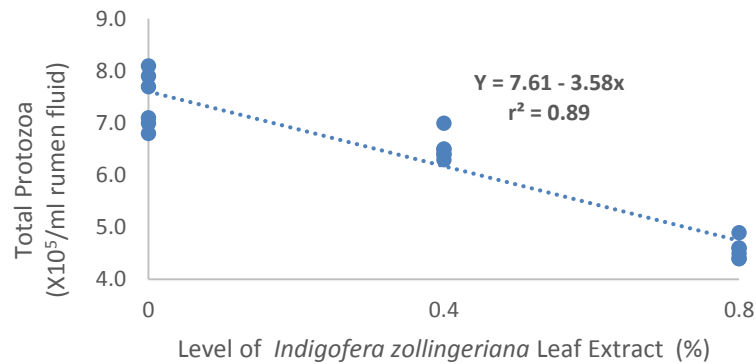


Figure 1. Effect of *Indigofera zollingeriana* leaf extract levels on number of Protozoa

Methane Gas Production

Ruminant animals produce methane gas (CH₄) which contributes to the accumulation of greenhouse gases (GHGs) in the atmosphere. Methane gas production from ruminants contributes 95% of total world methane emissions and about 18% of the total greenhouse gas in the atmosphere (Martin et al., 2008). Cattle emit nearly 73% of CH₄ gas during the fermentation process of feed in rumen. Buffaloes and sheep each produce 10% methane gas whereas goats produce 4% methane gas (US Environmental Protection Agency, 1994). The highest contributor to methane gas emissions in the livestock sector is beef cattle, which is about 65% of ruminant livestock emissions or 59% of total livestock emissions (DITNJENNAK, 2007)

The results obtained from this study showed that the lowest average methane gas production was achieved by rumen fluids receiving 0.8% *Indigofera zollingeriana* (Table 2). The addition of *Indigofera zollingeriana* leaf extract upto 0.8% of the feed dry matter is able to reduce the amount of methane gas production by 19.28% compared to that of control. Analysis of variance proved that the addition of *Indigofera zollingeriana* affected the production of methane gas in a quadratic response with the equation $Y = 14.365 + 26.044 X - 36.88 X^2$; the highest methane gas

production was achieved at P (0.35; 18.96). The coefficient of determination (R^2) was 0.92 (Figure 2), meaning that methane gas production is influenced by 92% *Indigofera zollingeriana* leaf extract, while the other % is influenced by other factors not examined in the study. At the addition of 0.8% *Indigofera zollingeriana* leaves, the lowest methane gas production was obtained because, as stated by Abdullah (2010), the leaves of *Indigofera zollingeriana* contain 0.41% saponins. The compound can act as a defaunation agent; therefore, the higher the level of the extract of the leaves of *Indigofera zollingeriana*, the higher the saponin which leads to a decrease in protozoan population and methane gas production. This is because protozoa are the host of methanogenic bacteria. Low methane gas production is expected whereas high methane gas production is not expected. Formation of methane gas in the rumen system can cause ruminants to lose some digestible chemical energy.

Bacteria Population (Total Plate Count)

Among the microorganisms in the rumen, bacteria have the largest population and are the largest source of protein for host animals. Therefore the presence of bacteria needs to be maintained, among others by reducing the protozoan population using defaunation agents.

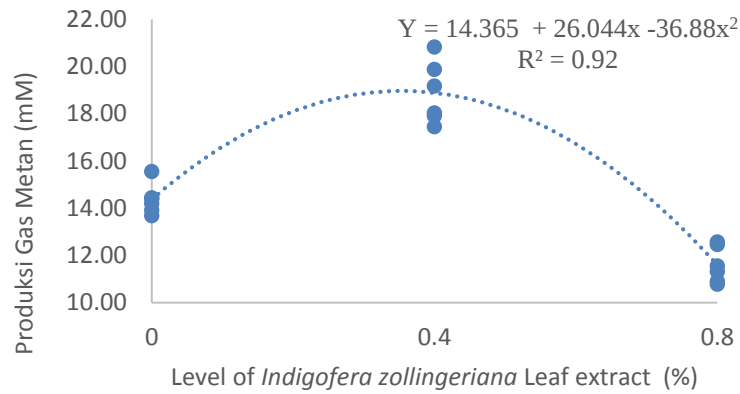


Figure 2. Effect of *Indigofera zollingeriana* leaf extract levels on methane gas production

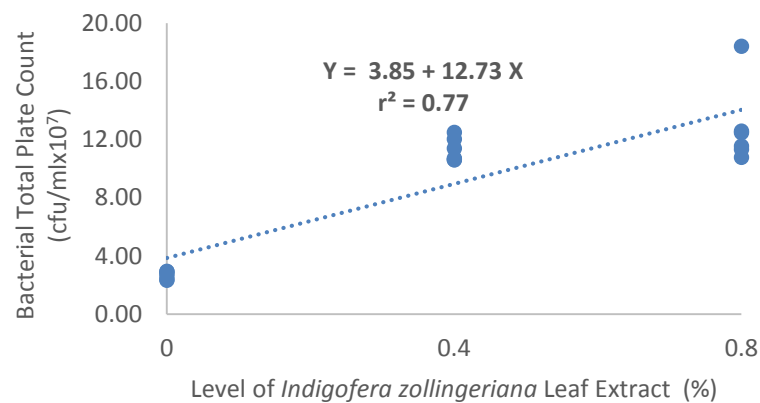


Figure 3. Effect of *Indigofera zollingeriana* leaf Extract on Total Bacteria (Total Plate Count)

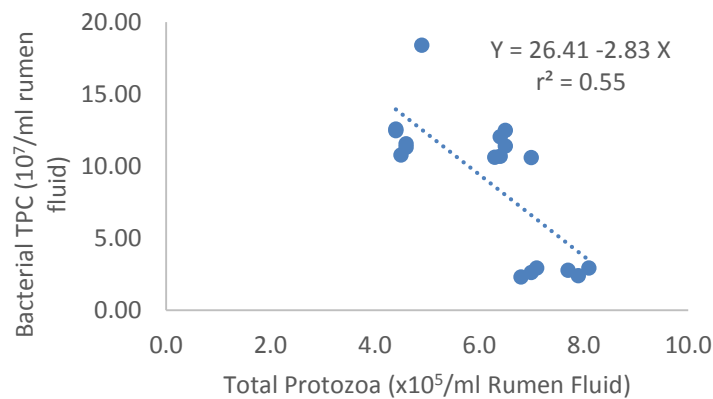


Figure 4. Relationship between the number of protozoa and the number of bacteria in rumen fluid

The highest number of bacteria produced by the rumen fluid obtaining the substrate with the addition of 0.8% *Indigofera zollingeriana* (Table 2).

The analysis of variance on the number of bacteria showed that the addition of *Indigofera*

zollingeriana leaf extract affects the total bacteria ($P < 0.01$). The effect is in linear fashion with the equation $Y = 3.85 + 12.73 X$ and the coefficient of determination (r^2) of 0.77 (Figure 3). This increase can occur because saponins from plant leaves are reported to be able to

increase the efficiency of the fermentation process through the mechanism of decreasing the protozoan population in the rumen by decreasing the protozoan predatory nature of bacteria (Wang et al., 2000). A decreased protozoan population in the rumen results in a higher bacterial population. The study proved that the supplementation of *Indigofera zollingeriana* leaf extract up to 0.8% of feed dry matter could increase the number of bacteria up to 383.08%; nearly four times of that without supplementation. The increase of bacteria count is very beneficial because bacteria are the main source of protein for host animals. According to Storm et al (1983) microbial proteins supply 60-85% amino acids to the host animals. The relationship between the two microorganisms is explained in Figure 4 where the higher the protozoa population, the lower the total bacteria, and vice versa, with the equation of $Y = 26.41 - 2.83 X$ and the coefficient of determination (r^2) of 0.55.

Saponins from plants can increase the efficiency of the fermentation process through a mechanism of decreasing the protozoan population in the rumen by reducing the predatory nature of protozoa to bacteria (Wang et al., 2000). The decrease in protozoan population in the rumen results in higher bacterial population (Hess et al., 2004).

Conclusion

The use of *Indigofera zollingeriana* leaf extract as much as 0.80% (based on dry matter) in feed consisting of 60% concentrate and 40% ammoniated rice straw can reduce the production of methane gas by 19.28% and the total number of protozoa count by 38.49% and can increase the total number of bacteria up to 383.08% in rumen fluid of sheep.

Acknowledgement

The author thanks the Rector and Head of the *Institute of Research and Community Service (LPPM)* of UNSOED for research

facilitation funds for the Special Task for Professors through the DIPA BLU LPPM UNSOED funds number: Kept. 182/UN23.14/PN.01.00/2019.

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