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by Erwin Sondakh 5

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The use of coconut pulp as a feed substrate to methanogenesis inhibitor in *in vitro* rumen fluid fermentation

H.B. Sondakh, J.A.D. Kalele and F.S. Ratulangi

Faculty of Animal Science, Sam Ratulangi University,

Jl. Kampus-Bahu, Manado 95115 - Indonesia

Corresponding E-mail: erwin_sondakh@yahoo.com

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ABSTRAK

Penelitian ini bertujuan untuk mengetahui pengaruh penambahan ampas kelapa pada pakan ruminansia terhadap reduksi metan secara *in vitro*. Penelitian ini menggunakan cairan rumen dengan substrat hijauan dan konsentrat dengan perbandingan 60:40. Percobaan ini terdiri dari lima macam perlakuan ampas kelapa yakni: R1: tanpa penambahan ampas kelapa; R2: substrat pakan dengan penambahan ampas kelapa 5%; R3: substrat pakan dengan penambahan ampas kelapa 10%; R4: Substrat pakan dengan penambahan ampas kelapa 15% dan R5 substrat pakan dengan penambahan ampas kelapa 20%. Setiap perlakuan dilakukan empat kali ulangan. Fermentasi dilakukan menggunakan *Hohenheim Gas Test* (HGT) pada suhu 39°C selama 72 jam. Pada akhir fermentasi dilakukan pengukuran gas metan, parameter fermentasi, aktivitas mikrobia, pencernaan nutrient dan profil asam lemak cairan rumen. Data dianalisa menggunakan rancangan acak lengkap dan dilanjutkan dengan Duncan test (DMRT) untuk perbedaan rata-rata. Hasil penelitian menunjukkan bahwa produksi metan mengalami penurunan sekitar 13,74%-17,39 % ketika pakan diberi ampas kelapa sebanyak 15-20%. Dari hasil penelitian disimpulkan bahwa pemberian ampas kelapa 15- 20% dalam pakan ruminansia dapat mereduksi gas metan serta tidak mengganggu aktivitas mikrobia pada fermentasi cairan rumen secara *in vitro*. Supplementasi ampas kelapa tersebut dapat dijadikan acuan dalam mendesain pakan penghambat metanogenik.

Kata kunci : metan, ampas kelapa, fermentasi rumen, gas tes, parameter fermentasi

ABSTRACT

This experiment was conducted to determine the effect of the adding of coconut pulp in ruminant feed on methane reduction *in vitro*. Rumen fluid and substrate of forage and concentrate in ratio 60:40 were in this study. This experiment consisted of five treatments of coconut pulp as followed: R1: feed substrates without the coconut pulp; R2: substrate feed with coconut pulp 5%; R3: substrate feed with coconut pulp 10%; R4: Substrate feed with coconut pulp 15% and R5: substrate feed with coconut pulp 20%. Each treatment was conducted to four replications. Fermentation was done using the *Hohenheim Gas Test* (HGT) incubated at 39°C for 72 hours. At the end of fermentation was determined production of methane, parameters of fermentation, microbial activity, feed digestibility and fatty acids profile of the ruminal fluid. Data obtained were analyzed by using analysis of variance using completely randomized design. The differences of mean values were analyzed by Duncan multiple range test (DMRT). The results showed that methane production has decreased about 13.74%-17.39% when the feed was given coconut dregs 15-20%. Based on the results of the study was concluded that the supplementation of coconut pulp up to 15-20% of 100% dry matter can be used as a substrate of ruminant feed, because it can reduce methane and does not interfere the microbial activity in the fermentation of rumen fluid through *in vitro*. The supplementation of coconut pulp can be a reference in

designing the methanogenic inhibitor feed.

Keywords : coconut pulp, gas tes, methane, parameter of fermentation, ruminal fermentation

INTRODUCTION

Livestock is considered as the largest contributor of greenhouse gas emissions (methane) in the atmosphere. According to Steinfeld *et al.* (2006), livestock is the biggest methane contributor in the atmosphere for about 35%. The impact of accumulated methane gas in the atmosphere could cause global warming and the effects on climate change. According to Steinfeld *et al.* (2006), the calculation of average methane in the last 20 years has increased 72 times greater than CO₂. Ironically, today the world precisely focuses on the strategy to reduce CO₂ emissions, and only few are concentrating on the reduction of methane emissions.

In the livestock sector, methane is one of gases from fermentation products of feedstuffs by rumen microbes. The methane forming (methanogenic) occurring in the rumen is influenced by protozoa. Methanogen bacteria attach themselves to ciliate protozoa to get a constant supply of hydrogen (Kamra, 2005). Ciliate protozoa is known to have a specific role in the transfer of hydrogen interspecies (Machmuller, 2006; Thalib, 2004; Zhu *et al.*, 2008).

Animal nutritionists are challenged to seek ways in reducing methane production because they feel responsible for the contribution of livestock sector to atmospheric pollution by methane, as one of pollutants that is always associated with ozone depletion and global warming. Efforts to reduce methane gas emissions on ruminants, have been conducted by several researchers such as by the addition of various chemical compounds in livestock rations using fumaric acid, acrylate, 2-oxoglutarate, nitrate, unsaturated fatty acids and anthraquinone (Yusiati *et al.*, 2008). However, the addition of chemicals to the feedstuffs must be very risk, either for the animal itself or food safety. The decrease of methane can also occur when cattle are given feed containing medium chain fatty acids (MCFA) and there has been a study conducted by Machmuller (2006) using canola oil. Sondakh *et al.* (2015) conducted a research on reducing of methane using virgin coconut oil as a medium chain fatty acids source, in which the results of such research

could decrease methane and increase propionic acid in rumen fluid fermentation *in vitro*. Medium chain fatty acids are fatty acids that function as defaunation agents (Sondakh *et al.*, 2012). Research of Machmuller (2006) and Sondakh *et al.* (2015) using canola oil and VCO was very expensive and it will be difficult for further applications.

Based on some of the considerations about the role of medium chain fatty acids capable of reducing methane production in ruminal fermentation, so it had been conducted research on the effect of coconut pulp on methane reduction. The use of coconut pulp because coconut pulp contain lots of medium chain fatty acids, as waste and easy to obtain and not compete with human needs. The use of coconut pulp as lowering agents of methane gas production needs to be investigated. Coconut pulp is agricultural waste derived from coconut meat squeezed to be made coconut milk. Coconut meat from wet method processing will obtain byproduct, namely coconut pulp. Coconut pulp has been frequently used as cattle feed as a protein source, especially for non-ruminants. The use of coconut pulp for ruminant feed is rarely done, however the interesting side of coconut pulp is that it contains many MCFA. This study was conducted to obtain the best formula and concentration of coconut pulp as a defaunation agent to ciliate protozoa in reducing methane production with no negative impact on parameters of fermentation and other rumen microbial activities.

MATERIALS AND METHODS

Materials

The materials used in this research were ruminal fluid. It was taken from rumen of ruminant (Ongole Grade cattle). The coconut pulp used was taken from mechanical palm oil processing. Substrate used were elephant grass and concentrate (soybean cake, ricebran, coconut pulp). The solution for in vitro test, fermentor, syringe, and waterbath.

Methods

The supplementation coconut pulp was given

in this study with treatments as follows: R1: 0% coconut pulp, 40% concentrate, 60% forage); R2: 5% coconut pulp, 35% concentrate, 60% forage); R3 (10% coconut pulp, 30% concentrate, 60% forage); R4 (15% coconut pulp, 25% concentrate, 60% forage); R5 (20% coconut pulp, 20% concentrate, 60% forage). Every treatments was conducted four replications. The feed composition and nutrient ingredients are presented in Table 1.

Procedures

The medium for fermenting was made by mixing 474.0 mL of aquades, 0.12 mL of trace mineral, 237 mL of buffer, 1.22 mL of resazurin, and 49.5 mL of reducer solution. All the materials are homogenized and flowed CO₂ at anaerobic conditions. The ratio of ruminal fluid and the medium of fermentation was 1:2 (v/v).

Substrate 300 mg (forage and concentrate) were entered into the syringe (fermentation bottle) in accordance with the treatment tested. Insert ruminal fluid have mixed with element solution to 30 mL into the syringe to test in vitro. The fermentation of anaerob was done for 72 hours at 39°C. The fluid of fermentation was filtered to separate feedstuffs undegradated to measure methane production, pH and account of protozoa. After taking for sampling fluid to measure parameter, the filtrate was centrifuged at 3,000 rpm for 15 minutes to determine VFA and NH₃. And then, the filtrate was centrifuged again at 10,000 rpm for 15 minutes to measure microbe protein. The residue/sediment was taken to

determine biomass based on microbe protein.

Testing of Fermentation Parameters and Rumen Microbe Activity

After finishing of incubation for 72 hours, a total sample gas volume should be taken 10 mL using *sput* and injected to gas chromatography (GC). GC was used HITACHI 263-50 brand, the column of active carbon of 1 m of height, diameter of 0.5 m, the detector temperature of 190°C, injector temperature of 190°C, the column temperature of 150°C, and nitrogen gas of 50 mL/min. CH₄ can be measured by following (area of standard x area of sample)/area of standard (Lopez *et al.*, 1996). The fermented liquid was filtered using a gochroosh that given glass wool, until no feed sample was left in the syringe. The fermented liquid was used to measure pH. The measurement of pH used pH meter. Preparation of protozoa calculation was done by adding 0.8 mL of formaldehyde saline solution (37% v/v) + 0.9% (v/v) NaCl, with 1: 9 proportion) at 1 ml sample. And then the sample was calculated by microscopic technique with calculations using a microscope with magnification 40 times (Diaz *et al.*, 1993).

A total of 0.5 centrifugal filtrate of 3000 g was taken for NH₃ (Chaney and Marbach, 1962). Next the sample was centrifuged at 13000 g for 10 min, then 20 µL supernatant sample plus 2.5 mL LC and added with 2.5 mL LD, then mixed. Next it was incubated in a waterbath with a temperature of 40°C for 30 minutes. After the

Table 1. Composition of Feedstuff

Variables	Coconut Pulp Added (% of 100% Dry Matter)				
	0	5	10	15	20
Feedstuff (%)					
Forages	60	60	60	60	60
Concentrate	40	35	30	25	20
Coconut pulp	0	5	10	15	20
Nutrients ingredient (%)					
Crude Protein	17.08	17.65	17.46	17.28	17.01
Crude Fat	5.93	5.07	5.12	5.23	5.27
Crude fiber	23.27	23.34	23.36	24.39	24.46
Nitrogen Free Extract	42.34	43.21	43.05	42.35	43.28
Ash	10.46	10.04	10.24	9.84	9.96

blue is formed, the sample is cooled to room temperature. Furthermore, the reading was done by using λ 630 nm spectrophotometer. LC is a mixture of phenol 50 mg Na nitroprusside plus 10 gr of crystal phenol so the solution becomes 1 L with aquades (41) LD is a mixture of hypochloride, added with 5 g NaOH pellet, 26.7125 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ diluted first with 100 mL of aquades, and 25 mL of 5% sodium hypochloride then mixed and diluted into 1 liter of aquades. The centrifugal filtrate of 3000 g for 15 minutes was taken a total of 0.2 mL and added by 1 mL of metaphosphoric acid, then centrifuged 9700 g for 10 minutes, the centrifugal sample was used for the measurement of volatile fatty acids (VFA) by gas chromatography (Doreau *et al.*, 1993).

The sediment of centrifugal sample of 13.000 g was used to measure microbial protein level by using Lowry method according to Plummer (1987). A 0.5 mL sample was inserted into the test tube and then added 2.5 mL of Lowry I solution (2% Na_2CO_3 in 0.1 NaOH, 2% sodium tartrate, 1% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 48) in mixed with a ratio of 100: 1: 1) and ignored at room temperature for 10 minutes. Furthermore, 0.25 ml of Lowry II solution (1N 53) in, H_2O , mixed with a ratio of 1:1) was ignored at room temperature for 30 minutes. Then reading to spectrophotometer at λ 750 nm.

Data analysis

The data obtained were statistically analyzed using analysis of variance with a completely randomized design in the direction pattern of each treatment with 29 4 replications. Differences between treatments were tested by using test Duncan multiple range test (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

The Production of Methane Gas

The results of this study showed that the addition of coconut pulp on in vitro fermentation is able to reduce the production of methane gas. The provision of coconut pulp in feed amounting to 15-20% has significant effects on the formation of methane gas ($P < 0.05$). The provision of coconut pulp 15% in 100% BK can reduce methane gas amounting to 13.74% and if the coconut pulp is raised up to 20% in 100% BK, there will a decrease of 17.38% when compared to substrates without coconut pulp. Similarly, there is a difference in the average methane gas production on the provision of 10% and 15%

coconut pulp. The provision of 15% coconut pulp shows that methane gas production 3.6% is lower than the provision of 10% coconut pulp. In this study, the highest decrease is in the provision of 20% coconut pulp. When compared with the provision of 20% and 15% coconut pulp, then the methane gas production has decreased by 4.22%.

It is clearly seen that the influence of coconut pulp levels can reduce methane gas production on in vitro fermentation. The decrease in methane gas production is suspected because of content contained in coconut pulp which contains medium chain fatty acids. Coconut pulp contains about 56% medium chain fatty acids (Table 2). This fatty acid inhibits the activities of protozoa.

Lauric acid (C12:0) is found to be more effective than myristic acid (C14:0) in suppressing the formation of methane. Myristic acid (C:14) can support the intermediate inhibition effect of lauric acid against methanogenic bacteria and methane gas emission (Soliva *et al.*, 2003). Lauric acid is the most powerful antiprotozoal that inhibits the growth and activity of ciliated protozoa (Hristov *et al.*, 2004). According to Sondakh *et al.* (2012), the ciliated protozoa is a good place for methanogen bacteria to get nutrients for life sustainability. The decrease of methane gas is closely associated with the presence of protozoa in rumen. Hegarty (1999) states that protozoa is the agent which serves as a unifier between methanogen and its ciliate in a symbiosis manner, so that 57) causes the formation of methane. Sondakh *et al.* (2012) reported a study that the provision of MCFA substrate 1.0-1.5% on in vitro fermentation can decrease the population number of protozoa and can reduce methane gas production.

Activity of Ruminal Microbe

The Number of Protozoa. The results of this study show that the addition of coconut pulp on in vitro fermentation of rumen fluid can decrease the number of protozoa. There is difference in the number of protozoa ($P < 0.05$) on the feed added with coconut pulp compared with feed without coconut pulp on in vitro rumen fermentation. The provision of coconut pulp up to 10% do not lead to differences in the number of protozoa. The decline in the number of protozoa later on occurs in the provision of 15% - 20% coconut pulp. 47) provision of 15% - 20% coconut pulp can reduce the number of protozoa by 31% - 35% compared with feed substrate that is not given coconut pulp.

Table 2. Production of Methane Gas at Ruminal Fermentation in vitro with the Addition of Different Level of Coconut Pulp

Replication	Added Coconut Pulp (% of 100% Dry Matter)				
	0	5	10	15	20
1	14.23	12.16	13.84	12.03	12.02
2	15.37	13.21	12.03	13.8	11.1
3	13.64	14.25	13.17	12.17	11.87
4	15.02	13.86	13.03	12.21	13.08
Average	14.55±0.77 ^a	13.37±0.91 ^a	13.02±0.74 ^a	12.55±0.83 ^b	12.02±0.81 ^c

Different superscripts in the same row indicate significantly different (P <0.05)

The results of this study indicate that there is a tendency that the number of protozoa decreases along with the addition of coconut pulp. The decline in the number of protozoa is suspected to be caused by the content of medium chain fatty acids contained in coconut pulp. The analysis results of medium chain fatty acid profile consisting of lauric acid and myristic acid in coconut pulp by about 56%. This fatty acid contributes substantially in lowering the number of protozoa on the rumen fluid fermentation. This is in line with what was presented by Machmuller (2006) that medium chain fatty acids can be toxic to ciliated protozoa. The decline in the number of protozoa on the fermentation of rumen which is given substrate containing medium chain fatty acids has been reported by Sondakh *et al.* (2012) that medium chain fatty acids amounting to 1.0 - 1.5% in 100% dry matter can reduce the number of protozoa by 29,84%. Likewise, the results of a study reported by Sitoresmi *et al.* (2009) that the provision of coconut oil up to 5% can reduce the number of protozoa by 23.95%. Machmuller (2006) and Dohme *et al.* (1999) states that medium chain fatty acid is the most powerful antiprotozoal that inhibits the growth and activity of ciliate protozoa (*entodinium spp*). The content of lauric acid contained in the medium chain fatty acids can increase the microbial sensitivity on cell wall, which causes inhibition of ciliate protozoa and gram-positive of archaea.

Microbial protein. The effect of giving coconut pulp with different levels on the microbial protein synthesis in vitro can be seen in Table 3. The results show that the provision of coconut pulp up to 20% in 100% dry matter has

not led to changes in the microbial protein synthesis. The provision of 0-20% coconut pulp on the substrate of forage feed and concentrates with a ratio of 60:40 is in the range of 0.29-0.37 mg/mL. This study is in line with a research from Sitoresmi *et al.* (2009) stating that the addition of coconut oil up to 7.5% could not bring an effect on microbial protein synthesis, microbial protein synthesis is in the range of 0.38 - 0.39 mg/mL. The results of this study are still far below the results of research conducted by Yusiati *et al.* (2008), which states that the addition of lemuru fish oil up to level of 7.5% dry matter on the fermentation of king grass and fine bran with a ratio of 80:20 in vitro has microbial protein synthesis on the range of 0.51-0.56 mg/mL. Precursor for microbial protein synthesis depends on the availability of sufficient carbon chain, NH₃ and energy. Pak (2008) states that microbial protein is the main source of amino acid supply for ruminants. The contribution of microbial protein amounted to 30-50% of the total protein utilized by ruminants. Orskov (1992) stated that precursor for the synthesis microbial proteins is the availability of sufficient carbon, NH₃ and energy. The ideal conditions of microbial protein synthesis will be achieved if the fermentable carbohydrate source is available simultaneously with the protein source.

Parameter of Ruminal Fermentation

Ammonia (NH₃). The effect of giving coconut pulp up to 20% from 100% dry matter has not led to difference in NH₃ on the fermentation of rumen fluid in vitro using substrates of elephant grass and concentrates

(Table 4). The provision of coconut pulp up to 20%, NH₃ is in the range of 24.49-27.81 mg/100 mL. The results of this study are still far below the results of research conducted by Sitoresmi *et al.* (2009) stated that the level of ammonia (NH₃) on the addition of oil up to 7.5% ranges from 33.24- 34.53 mg/100m. Owen and Zinn (1988) stated that concentration of NH₃ varies greatly depending on the magnitude of feed degradation. The excessive production of NH₃ is not beneficial to the formation of microbial protein and will be discarded. Ammonia is the result of protein degradation by proteolytic bacteria which will then be used by the components to form microbial protein. The decrease in protein degradation of feed will be followed by a decrease in ammonia produced.

Volatile Fatty Acids. The results of this study show that the provision of coconut pulp has an effect on propionate acid. There is an increase

of propionic acid content in line with the higher level of coconut pulp provision. However, unlike acetic and butyric acids, the provision of coconut pulp up to 20% from 100% dry matter (DM) has not shown any difference. Likewise, the ratio of acetate propionate has not given any different effect. The improvement of propionic acid begins with the provision of 10% to 20% coconut pulp in 100% BK on the fermentation of rumen fluid. There is an increase in propionate acid by 33.8% - 36.0% in the provision of 10-20% coconut pulp in 100% BK. The provision of coconut pulp leads to a difference in the average of propionate profile. The difference in the average of propionate content when given coconut pulp is allegedly because of the coconut pulp that can lower methane content (Table 3). According to Sondakh *et al.* (2012), there was a correlation between the decrease in methane gas production and increase in propionic acid on the fermentation of rumen

Table 3. The Number of Protozoa and Microbe Protein Synthesis in *in vitro* Ruminal Fermentation with Addition of Different Level of Coconut Pulp

Variables	Added Coconut Pulp (% of 100% Dry Matter)				
	0	5	10	15	20
Number of protozoa (x10 ³)	8778±11 ^a	7543±1496 ^a	6621±881 ^a	6086±1395 ^b	5863±692 ^b
Microbe protein (mg/mL) ^{ns}	0.29±0.04	0.31±0.07	0.33±0.06	0.34±0.07	0.37±0.08

ns = non significant
Different superscripts in the same row indicate significantly different (P < 0.05)

Table 4. Parameter of *in vitro* Ruminal Fermentation with Addition of Different Level of Coconut Pulp

Parameter	Added Coconut Pulp (% of 100% Dry Matter)				
	0	5	10	15	20
NH ₃ (mg/100 mL) ^{ns}	37.75±0.45	36.61±1.34	36.51±6.14	35.59±0.49	41.42±1.76
VFA					
Asetic ^{ns}	14.30±1.60	13.74±0.80	14.12±0.90	14.25±1.00	13.85±0.90
Propionic	3.75±0.38 ^a	4.00±0.80 ^a	5.02±0.58 ^b	5.07±0.55 ^b	5.10±0.74 ^b
Butyric ^{ns}	1.53±0.23	1.51±0.10	1.49±0.75	1.55±0.90	1.71±0.27
Asetic:Propionic	3.83±0.45	3.57±0.89	2.85±0.52	2.85±0.52	2.77±0.59
pH ^{ns}	6.6±0.10	6.8±0.30	7.0±0.20	6.9±0.30	6.8±0.30

^{ns} = non significant
Different superscripts in the same row indicate significantly different (P < 0.05)

fluid. This is in line with what was stated by Wang *et al.* (2009) that VFA concentration has an influence on the methane production in rumen. Similarly, Hu *et al.* (2005) stated that defaunation process causes VFA concentration declining, but propionate molar increases.

Degree of Acidity (pH). The results of research show that provision of coconut pulp up to 20% in 100% dry matter (DM) on the fermentation of rumen *in vitro* has not led to changes in pH of rumen fluid. In this study, ruminal pH was stable on the range of 6.6-7.0. The pH value on the fermentation of rumen fluid which is *in vitro* associated with the methane production has been reported also by Sitoresmi *et al.* (2009); Harwanto *et al.* (2014). The researchers stated that the fermentation of rumen fluid *in vitro* uses feed substrate which allegedly can reduce methane production, has not led to changes in pH value. Krause *et al.* (2002) stated that pH value is influenced by the concentration of fiber in the feed. The pH value of rumen varies; feed containing grains can lower the pH value to below 5, while feed that contains a lot of fiber can cause an increase in pH to above 7. According to Owen and Zinn (1988), the range of normal pH for the activity of rumen microbes to degrade feed and carry out the process of fermentation is 5.5-7.6. This statement was also confirmed by researcher (Harwanto *et al.*, 2014; Keidane and Birgile, 2003) that ruminal fermentation survival at level pH 6.0-8.0.

CONCLUSION

Based on the results of the study, it can be concluded that the supplementation of coconut pulp up to 15-20% of 100% dry matter can be used as a substrate of ruminant feed, because it can reduce methane and does not interfere the microbial activity in the fermentation of rumen fluid through *in vitro*. The supplementation of coconut pulp can be a reference in designing the methanogenesis inhibitor feed.

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