Condition of Rumen Fermentation as Impacted by Supplementation of Fermented Rice Brand Using *In Vitro* Gas Production Technique

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ABSTRAK

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Gas metana merupakan salah satu gas yang dihasilkan oleh ternak ruminansia dari fermentasi di dalam rumen. Adapun tujuan dari penelitian ini adalah untuk mengetahui produksi monakolin K pada fermentasi bekatul menggunakan kapang *Monascus purpureus* dan pengaruh suplementasi bekatul yang difermentasi dengan kapang *Monascus purpureus* pada pakan rumput gajah terhadap produk fermentasi dan produksi gas metana cairan rumen secara *in vitro*. Penelitian dilakukan dalam 2 tahap, yang pertama analisis kandungan monakolin K yang terkandung dalam bekatul yang difermentasi dengan kapang *Monascus purpureus* sebanyak 0, 4, 8, dan 12% (v/w) dari substrat (bekatul). Analisis kandungan monakolin K pada substrat dilakukan dengan menggunakan HPLC. Penelitian tahap II yaitu analisis *in vitro* produksi gas dari 3 perlakuan pakan yaitu rumput gajah (kontrol), rumput gajah:bekatul (1:1), dan rumput gajah: bekatul fermentasi (kandungan monakolin K tertinggi (12% dari substrat)) (1:1). Hasil penelitian tahap I menunjukkan bahwa fermentasi bekatul dengan dosis inokulum sebanyak 12% dari substrat memiliki kandungan monakolin K tertinggi (1,39 µg/ml). Hasil fermentasi *in vitro* pakan menunjukkan bahwa perlakuan pakan tidak berpengaruh (P>0,05) terhadap kandungan ammonia, VFA, pH, aktivitas CMC-ase, protein mikroba, KCBK dan KCBO. Namun penambahan bekatul yang difermentasi pada pakan rumput gajah dapat menurunkan (P <0,05) produksi gas metana (CH_4) sebesar 50% dan populasi protozoa sebesar 80%. Berdasarkan hasil tersebut dapat disimpulkan bahwa penambahan bekatul fermentasi yang mengandung monakolin K ke dalam pakan dapat menurunkan produksi gas metana secara *in vitro* tanpa mempengaruhi karakteristik fermentasi rumen.

Kata Kunci: Fermentasi, *In Vitro*, Metana, Monakolin K, *Monascus purpureus*

ABSTRACT

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Methane is one of the gases produced by ruminants during feed fermentation in the rumen. This experiment was aimed to investigate the production of monacolin K in rice bran fermented by *Monascus purpureus* mold and the influence of the supplementation of fermented rice bran using *Monascus purpureus* mold on elephant grass basal diet on fermentation products and methane production in an in vitro gas production method. The study consisted of two experiments. The first experiment analysis of monacolin K production in fermented rice bran using *Monascus purpureus*. Fermentation is done by the addition of *Monascus purpureus* at levels 0, 4, 8, and 12% (v/w) of substrate (rice bran) with 3 replications. Monacolin K in the substrate was analyzed using HPLC. The second experiment was the evaluation of supplementation of fermented rice bran to elephant grass basal diet using in vitro gas production. The treatment diet evaluated were *Pennisetum purpureum* (control), *Pennisetum purpureum*:rice bran (1:1 ratio), and *Pennisetum purpureum*:rice bran fermented. Each treatment was replicated 3 times. Results from the first experiment shows that rice bran with the highest monacolin K content was in rice bran fermented at 12% by *Monascus purpureus*. Result from the second experiment showed that supplementation of fermented rice bran to *Pennisetum purpureum* basal diet did not affect rumen ammonia concentration, VFA, protein microbial production, and dry matter and organic matter digestibility. However, methane production (CH_4) was reduced (P<0.05) by 50%, and the protozoal population was decreased (P<0.05) by 80%. It is concluded that supplementation of fermented rice brands containing monacolin K was able to reduce methane production and the protozoa population without affecting feed fermentation.

Key Words: Fermentation, *In Vitro*, Methane, Monacolin K, *Monascus purpureus*

INTRODUCTION

Global warming has been widely discussed in Indonesia and even in the world. Livestock is one of the contributors to greenhouse gas emissions (GHG). One source of GHG emissions from the livestock subsector is methane gas (CH4) from the enteric fermentation and manure removed by ruminants. Beef cattle are the

largest contributor to CH4 emissions (69.41%) compared to other ruminants (Widiawati et al. 2016).

The efforts to reduce CH4 emissions from ruminants have been carried out in Indonesia, one of them is the use of secondary metabolites of plants such as essential oils, saponins, and tannins (Benchaar & Greathead 2011; Bodas et al. 2012). However, the use of the compounds produced by bacteria and molds is still rarely done, such as the use of secondary metabolites from *Monascus purpureus* which has the potential to reduce about 30% methane production (Morgavi et al. 2013).

Monascus purpureus is one of the molds used in the production of food and medicine. Besides, this mold was known to produce the compounds of biologically active such as inhibitors 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR) (Shi & Pan 2011). These compounds inhibit methanogenic growth in the rumen by *in vitro* (Beltowski et al. 2009).

Based on this fact, this research was aimed to determine the effect of monacolin K as a secondary metabolite of *Monascus purpureus* on methane gas production in rumen.

MATERIALS AND METHODS

Materials used were mold of Monascus from the Central Microbiology Inter-University Laboratory (PAU), rice bran, elephant grass, and rumen fluid of Filial Ongole Cattle (PO) owned by Faculty of Animal Husbandry, Gadjah Mada University.

This research was conduct in 2 phases. The first phase about the fermentation of rice bran with the addition of 0, 4, 8, and 12% (v/w) inoculum of Monascus from the total substrate (rice bran), and the second phase was the evaluation of the effect of using fermented rice bran as an additional feed on the base feed of elephant grass against methane production by *In vitro* gas test according to the Menke & Steingass 1988 method.

Fermentation of rice bran by *Monascus purpureus*

A total of 50 g substrates was transferred to 250 ml Erlenmeyer flasks, added distilled water (40% DM) then autoclaved at 121^0C for 30 min, left it until the temperature becomes 25-30^0C. After that, the *Monascus purpureus* was inoculated into the substrate as much as 0, 4, 8, and 12% (v/w) from the dry weight of the substrate, then incubated for 9 days. Each level of inoculation was repeated 3 times. After the fermentation finished, weighed the product and dried in an oven at 100^0C for 30 minutes to inactivate the mold, then continue baking at 50^0C for 24 hours. After that, stir evenly, ground and samples were taken to measure the pH and levels of monacolin K.

PH measurement and analysis of monacolin K levels of fermented rice bran using HPLC

pH measurement of the substrate was carried out by a digital pH meter. A total of 1-gram substrate was dissolved with 10 ml of distilled water and then the pH was measured using a digital pH meter that had previously calibrated using a pH buffer of 4 and 7. Measurement of monacolin K content was performed by using HPLC at the Toxicology and Pharmacology Laboratory of the Faculty of Pharmacy UGM. A total of 1 mg the fermented product was milled and dissolved with 9 ml of ethanol 67% (v/v) then stirred at 50^0C for 2 hours. After that, precipitated and then the supernatant was taken to be analyzed for monacolin K levels using HPLC according Zhang et al. 2013.

In vitro gas production (Menke & Steingass 1988)

The feeds were tested using *in vitro* gas production from 3 treatments, namely elephant grass (control), elephant grass: rice bran (1:1), and elephant grass: fermented rice bran (1:1); each treatment consisted of 3 replications. The fermented rice brand used was the best result of the first phase, based on AOAC method (2006).

The fermentation medium was prepared by mixing 474 ml of McDougall's buffer, 0.12 ml of mineral B solution, 237 ml of buffered solution, 237 ml of mineral A solution, 1.22 ml of resazurin solution and 49.5 ml of reduced solution (Na_2S) put into the Erlenmeyer 2 L. then mixed with 2,000 ml of rumen fluid while continuously flushed with CO_2 in anaerobic conditions before being put into a syringe glass. The ratio of rumen fluid and the medium is 1:2 (v/v) (Karlsson et al. 2009).

Approximately 300 mg of each test feed was put into the glass syringe which contains 30 ml of fermentation medium. All glasses were then incubated in a modified water bath at 39oC for 72 hours then its gas production was observed. At 0, 1, 2, 4, 6, 8, 12, 24, 36, 48, and 72 h measurement volumes were recorded; samples of gases produced were taken in Vacutainer® tubes for CH4 concentration analysis using Gas Chromatography (GC) and then released. At the end of this incubation (72 h), the liquid phase was centrifuged at a rate of 3,000 g. Its filtrate was used for testing rumen fermentation parameters (ammonia levels, VFAs, pH, and methane gas production) and microbial activity (CMCase, microbial proteins, and protozoal). The remaining material was filtered through sintered crucibles to determine *in vitro* apparent dry matter and

organic matter degradability. The residual dry matter and organic matter contents were determined to refer to the AOAC (2006). Dry matter (DM) and ash contents were determined by drying at 105 ^{0}C for 8 h and at 550 ^{0}C for 6 h, respectively.

Ammonia was determined according to Chaney & Marbach (1962). A total of 0.5 mL filtrate was centrifuged at 10,000 g for 10 minutes, then 20 µL of the sample supernatant was added with 2.5 mL LC (a mixture of phenol 50 mg Na nitroprusside and 10 gr crustal phenol which dissolved with aquadest to 1 L volume) and 2.5 mL LD (a mixture of hypochlorite and 5 gr NaOH, 21.31 gr Na_2HPO_4 anhydrous or 269.7125 gr Na_2HPO_4 1 $2H_2O$ which dissolved with 100 mL aquadest and 25 mL sodium hypochlorite 5%) then homogenized.

Measurement of VFA produced during *in vitro* fermentation was carried out according to Filípek & Dvořák (2009). A total of 0.2 mL of filtrate was added with 1 mL of metaphosphoric acid, then centrifuged at 10,000 g for 10 minutes. A total of 1µL of the supernatant sample was taken and injected into gas chromatography.

CMase activity was carried out using 0.1 acetate buffered solution; 1% CMC solution; cyanide carbonate solution; Na_2CO_3 solution; and 0.05% potassium ferricyanide solution (Halliwell & Lovelady 1981).

Protozoal populations were taken from the incubation medium at the end of fermented (72 h). The population was counted in the counting chamber thick as 0.2 mm using a microscope with a magnification of 40 times (Diaz et al. 1993).

The determination of microbial protein levels was measured according to the Lowry method (Plummer 1987). A total of 0.5 mL sample was put into a test tube then added with 2.5 mL of Lowry B solution then homogenized and allowed to stand for 30 minutes. After that, it was added 0.25 mL of Lowry A solution and allowed to stand for 10 minutes at room temperature then read using a spectrophotometer at a λ of 750 nm.

The data obtained were statistically analyzed using a completely randomized directional pattern design using the SPSS Program version 16.0. If there are differences, the analysis continued with the Duncan Test.

RESULTS AND DISCUSSION

Content of monacolin K of fermented rice bran

The effect of *Monascus purpureus* level on pH and monacolin K production of rice bran is presented in Table 1. Table 1 shows that the inoculum dose of *M. purpureus* had a significant effect (P <0.05) on the pH and production of monacolin K substrate. The pH of the substrate with the addition of 12% inoculum was still within the normal range for the growth of *M. purpureus* is 7-8. This result was also supported by an increase of monacolin K levels. The highest production of monacolin K in this study was in the addition of 12% (v/v) inoculum that is 1.39 µg/ml or equivalent to 154 mg/kg. This result was lower than that reported by Morgavi et al. 2013 which produced 570 mg/kg monacolin K with fermented rice used as substrate and ammonium sulfate addition to increasing monacolin K production. This is caused by the carbohydrate content of rice bran is lower than rice, while the effectiveness of the fermentation of Monascus in producing monacolin is influenced by carbohydrate content (Liu et al. 2020). Besides, the addition of ammonium sulfate can also increase the content of monacolin K (Su et al. 2003).

Characteristics of rumen fermentation

The effect of fermented rice bran addition to elephant grass basal diet on rumen fermentation characteristics of 72 h in vitro incubations is presented in Table 2.

Table 2 shows that the addition of fermented rice bran using *M. purpureus* did not affect rumen ammonia levels. Ammonia levels in this study were 25 to 28 mg/100 mL, which was still in the normal range to support the growth of rumen microbes that is 10.21 to 35.76 mg/100 mL (Olijhoek et al. 2016). Ammonia levels in the rumen are indicative of protein degradation. Protein will be degraded to oligopeptides, then to peptides and amino acids, then the process of amino acid deamination will produce ammonia (Goldberg 2013). Besides, ammonia levels in the rumen also describe degradation and protein synthesis process by rumen microbes. If the feed is protein-deficient, the ammonia concentration in the rumen will be decreased, and the growth of rumen microbes will be slow, that causes decreased digestibility of feed (Suharti et al. 2019). As in this study, with the same ammonia levels in each treatment caused no difference in the digestibility of dry matter and organic matter (Table 4).

The addition of the fermented rice bran did not affect average VFA levels (acetate, propionate, and butyrate) and the acetate: propionate ratio. In this study, the proportion of acetate was higher than propionate. This caused the feed in the fermented liquid to contain a lot of fiber. Glucose-rich food increased propionate production while fiber-rich feed increased acetate production (Suryani et al. 2014). The addition of rice bran without or with fermentation using *M. purpureus* did not affect (P <0.05) on pH (Table 2). The addition of fermented rice bran in this study resulted in a range of pH values that were still within the normal pH range for the rumen fermentation process, which is 6-7. These results were in line with that reported by Candyrine et al. (2018), that the addition of 2 mg/kg body weight/day lovastatin to goat feed resulted in rumen pH of 6.59.

Table 1. Average pH and monacolin K production of rice bran after fermentation with *Monascus pupureus* at various levels

Parameters	*Monascus purpureus* % DM			
	0	4	8	12
pH	6.53[a] ± 0.06	8.50[b] ± 0.30	9.00[c] ± 0.21	8.20[b] ± 0.06
Monacolin K (µg/ml)	0.00[a] ± 0.00	0.01[a] ± 0.01	1.07[b] ± 0.14	1.39[c] ± 0.21

[a,b,c] Means within rows and subtitles followed by distinct superscripts differ (Duncan test at 5%)

Table 2. Ammonia levels, VFA, and pH of fermented rice bran with *M. purpureus* used as inoculum of 72 hours *in vitro* incubations

Parameters	Feed		
	Elephant grass	Elephant grass:rice bran (1:1)	Elephant grass:fermented rice bran (1:1)
Ammonia (mg/100 mL)	25.62 ± 0.34	28.05 ± 1.60	25.80 ± 1.95
VFA (%)			
Acetate	78.00 ± 1.83	77.06 ± 0.51	76.39 ± 0.84
Propionate	15.77 ± 0.53	14.28 ± 0.40	15.99 ± 1.48
Butyrate	6.23 ± 2.08	8.66 ± 0.44	7.63 ± 0.67
Acetate:propionate	4.95 ± 0.17	5.40 ± 0.18	4.81 ± 0.50
pH	6.25 ± 0.04	6.26 ± 0.08	6.30 ± 0.07
Methane (mL/100 mg DM)	5.52 ± 0.71[b]	5.53 ± 1.29[b]	2.75 ± 1.14[a]

[a,b] Means within rows and subtitles followed by distinct superscripts differ (Duncan Test at 5%)
VFA: Volatile Fatty Acid

The addition of fermented rice bran reduced methane production (P <0.01) to 50.2% (Table 2). These results were in line with that reported by Morgavi et al. (2013) that the use of rice bran fermented with *Monascus sp*. and hay with a ratio of 1: 1 in sheep reduced 30% methane production *in vitro*. This result caused by the *Monascus sp*. that produces secondary metabolites such as monacolin K. Monacolin K is an HMG-CoA reductase inhibitor, which is an enzyme that plays a role in cholesterol formation (Sharpe & Brown 2013a). With monacolin K, the formation of cholesterol will be disrupted so that the development of protozoal inside will also be disrupted because cholesterol is one of the constituent components of the protozoal cell membrane. Protozoal live in symbiosis with methanogenic bacteria (methane-producing bacteria) in the rumen. Methanogenic bacteria get a constant supply of hydrogen from protozoal, so a decrease in the protozoal population in the rumen will indirectly reduce methane production (Martin et al. 2010).

Microbial activity

Effect of fermented rice bran addition to elephant grass basal diet on rumen microbial activity of 72 h *in vitro* incubations is presented in Table 3.

CMCase: Carboxymethyl cellulase

Carboxymethyl cellulose is a cellulose degradation enzyme which is a polysaccharide contained in the feed (Sitoresmi et al. 2009). Results showed that the addition of rice bran without or with fermentation using *M. purpureus* did not affect the activity of CMCase fermentation fluid. Results were in line with that reported by Candyrine et al. (2018), the use of HMG-CoA reductase inhibitors (mevastatin and lovastatin) did not affect the growth of a fiber-degrading bacteria in the rumen.

Table 3. CMCase activity and the number of protozoal of fermented rice bran with *M. purpureus* used as inoculum of 72 h *in vitro* incubations

Parameters	Feed		
	Elephant grass	Elephant grass:rice bran (1:1)	Elephant grass:fermented rice bran (1:1)
CMCase (U/g)	1.98 ± 0.67	2.21 ± 0.54	2.23 ± 0.15
Protozoal ($x10^3$)/mL	24.39^{ab} ± 13.81	30.67^{b} ± 6.29	6.31^{a} ± 4.83
Microbial protein (mg/mL)	0.20^{a} ± 0.06	0.36^{b} ± 0.04	0.18^{a} ± 0.63

[ab] Means within rows and subtitles followed by distinct superscripts differ (Duncan test at 5%)

Table 4. The digestibility of dry matter and organic matter of fermented rice bran with *M. purpureus* used as inoculum of 72 h *in vitro* incubations

Parameters	Feed		
	Elephant grass	Elephant grass:rice bran (1:1)	Elephant grass:fermented rice bran (1:1)
Dry matter digestibility	41.63 ± 8.53	43.68 ± 0.47	44.46 ± 8.79
Organic matter digestibility	41.06 ± 6.12	41.63 ± 1.96	43.74 ± 12.20

The addition of fermented rice bran using *M. purpureus* reduced the number of rumen fluid protozoal. As stated earlier, secondary metabolites (monacolin K) produced by *M. purpureus* are HMG-CoA reductase inhibitors (Sharpe & Brown 2013b). Monacolin K compounds competed with HMG-CoA reductase enzymes in binding HMG-CoA so that will be inhibited the formation of mevalonic acid, which is the stage of cholesterol formation. Cholesterol is one of the constituent components of the protozoal cell membrane so that with monacolin K the growth of protozoa in the rumen will also be disrupted, causing the protozoal population in the rumen to be reduced. In this study, the use of fermented rice bran reduced 74.13% the protozoa population. These results are in line with that reported by Dinesh et al. (2014), the addition of statin compounds (atorvastatin and simvastatin) can inhibit the growth of *Leishmania donovani* which is one type of protozoal.

The addition of fermented rice bran using *M. purpureus* did not affect the microbial protein. Microbial protein derived from bacteria, fungi, and protozoa in the rumen. The use of rice bran in the diet increased the population of protozoa and rumen microbes, which caused the addition of carbohydrates and fiber in the rice bran. As reported by Martínez et al. (2010), an increase in the ratio of carbohydrates in the feed will increase the protozoal population in the rumen. Whereas, the addition of fermented rice bran decreased the protozoal population due to the presence of Monacolin K. Therefore, the protozoal population decline causes a decrease in microbial protein production.

Effect of fermented rice bran on the digestibility of dry matter and organic matter

Effect of fermented rice bran addition to elephant grass basal diet on the digestibility of dry matter and organic matter of 72 h *in vitro* incubations are presented in Table 4.

The results show that the addition of rice bran without or without fermentation using *M. purpureus* did not affect (P> 0.05) digestibility of dry matter and organic matter diet *in vitro*. These results are in line with that reported by Candyrine et al. (2018), the addition of fermented oil palm cake using *Aspergillus terreus* (lovastatin 850 mg/kg DM) on goat diet, did not affect the total rumen microbial population and feed digestibility. This result also indicated that there is not significantly different on the VFA, NH3 (Table 2), and rumen microbial protein (Table 3)..

CONCLUSION

Fermentation of *Monascus purpureus* in rice bran produced Monacolin K with the best results at the level of 12% DM. The fermented rice bran reduced methane production by 50%, protozoal population, and microbial proteins without affecting ammonia production, pH, CMCase enzyme content, and nutrient rumen fluid in *vitro*. Monacolin K derived from *M. purpureus* has the potential to be used as an additive to animal feed for reducing methane production in the rumen. *In vivo* research needs to be done to see the benefits of using *M. purpureus* as a food additive in reducing emissions of enteric methane.

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