

Effectivity of Probiotic, Micromineral Enriched Yeast and Their Combination with *Azadirachta indica* Leaves Containing Tannin on Fermentability and Digestibility of *Pennisetum hybrid*

Sofyan A, Sakti AA, Karimy MF, Julendra H, Istiqomah L, Herdian H, Damayanti E, Suryani AE

Division of Animal Feed and Nutrition, Research Unit for Processes Development and Chemical Engineering (BPPTK), Indonesian Institute of Sciences (LIPI), Jl. Jogja-Wonosari Km. 31.5, Gading, Playen, Gunungkidul, DI Yogyakarta 55861
Telephone/Faximile: 0274-392570/391168
E-mail: sofyan_lipi@yahoo.co.id/ahmad.sofyan@lipi.go.id

(received 16-02 2015; revised 13-03-2015; accepted 17-03-2015)

ABSTRAK

Sofyan A, Sakti AA, Karimy MF, Julendra H, Istiqomah L, Herdian H, Damayanti E, Suryani AE. 2014. Efektivitas probiotik, mikromineral terkorporasi khamir yang dikombinasikan dengan daun *Azadirachta indica* mengandung tanin terhadap fermentabilitas dan pencernaan *Pennisetum hybrid*. JITV 20(2): 95-104. DOI: <http://dx.doi.org/10.14334/jitv.v20i2.1164>

Eksplorasi imbuhan pakan organik untuk ternak saat ini terus dilakukan untuk menggantikan antibiotik sebagai pemacu pertumbuhan. Probiotik dari bakteri asam laktat telah digunakan secara luas untuk mendukung keseimbangan mikroba saluran pencernaan. Selain itu, mineral organik ditambahkan pada pakan untuk meningkatkan ketersediaan dalam mencegah kelainan metabolisme akibat defisiensi mineral. Penelitian ini bertujuan untuk mengevaluasi penambahan probiotik (*Pediococcus acidilactici* RS2) dan mikromineral terkorporasi khamir/*micromineral enriched yeast* (MEY) yang dikombinasikan dengan tanin dari daun mimba (*Azadirachta indica*) terhadap fermentabilitas rumput Raja (*P. hybrid*) dengan menggunakan teknik produksi gas *in vitro*. Perlakuan terdiri dari P0 (kontrol/hijauan tanpa imbuhan), P1 (P0+MEY); P2 (P0+MEY+tanin); P3 (P0+Probiotik); P4 (P0+Probiotik+MEY), dan P5 (P0+Probiotik+MEY+tanin) yang disusun dalam rancangan acak lengkap dengan 3 ulangan setiap perlakuan. Suplementasi MEY (P1), probiotik+MEY (P4) dan probiotik+MEY+tanin (P5) nyata ($P < 0,05$) meningkatkan produksi gas hijauan tanpa berpengaruh pada asam lemak volatil (VFA), jumlah protozoa, produksi metana dan pencernaan *in vitro*. Produksi gas tertinggi tercatat pada hijauan yang diberi perlakuan P4 diikuti P5, P1, P2, P3 dan kontrol. Pengaruh perlakuan terhadap kinetika produksi gas menunjukkan perbedaan yang nyata pada inkubasi setelah 8 jam. Walaupun perlakuan hanya berpengaruh terhadap kinetika produksi gas (b, c dan total gas), berdasarkan analisis kluster-hirarki menunjukkan beberapa parameter yang terdiri dari asetat, propionat, pencernaan *in vitro*, jumlah protozoa dan produksi gas metana sangat berkorelasi dengan parameter kinetika produksi gas. Dapat disimpulkan bahwa fermentabilitas hijauan dapat ditingkatkan dengan suplementasi mineral organik maupun kombinasinya dengan probiotik maupun probiotik+tanin tanpa berpengaruh negatif terhadap pencernaan *in vitro*.

Kata Kunci: Fermentabilitas, Kecernaan *In Vitro*, Mineral Organik, Probiotik, Tanin

ABSTRACT

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Organic additive for animal had been explored to replace antibiotic growth promoter. Probiotic from lactic acid bacteria was widely used to support the microbial balances in digestive tract, while organic mineral was added into diets to improve bioavailability for preventing mineral deficiency disorders. This experiment was aimed to assess probiotic (*Pediococcus acidilactici* RS2) and micromineral enriched yeast (MEY) combined with tannin from neem (*Azadirachta indica*) leaves containing tannin on king grass (*P. hybrid*) fermentability using *in vitro* gas production technique. Treatments consisted of P0 (control/forage without additive), P1 (P0+MEY); P2 (P0+MEY+crude tannin); P3 (P0+Probiotic); P4 (P0+Probiotic+MEY), and P5 (P0+Probiotic+MEY+crude tannin). The study was arranged in a completely randomized design (CRD) with three replications in each treatment. Probiotic, MEY or tannin supplementation significantly increased ($P < 0.05$) gas production without affecting volatile fatty acid, protozoa numbers, methane production and *in vitro* digestibility of forage. The highest cumulative gas production was found in forage treated by P4 followed by P5, P1, P5, P2, P3 and control. Kinetic of gas production was significantly affected by treatments after 8 h incubation. Although the treatments were only significantly affected gas production kinetic (b, c and total gas), the hierarchical cluster analysis indicated that some parameters consisted of acetate, propionate, *in vitro* digestibility, protozoa numbers, and methane production were closely correlated to the gas production kinetic parameters. It was concluded that either organic mineral supplementation or its combination with probiotic, and probiotic+tannin improved fermentability of forage without negative effect on *in vitro* digestibility.

Key Words: Fermentability, *In Vitro* Digestibility, Organic Mineral, Probiotic, Tannin

INTRODUCTION

Ruminant contribute to meat and milk supply for human consumption. Low quality feedstuffs is the biggest constrain in ruminant productivity. Supplementation of feed additive such as antibiotic growth promoters (AGP) had been reported could improve the nutrient utilization and animal performance. However, feed additive containing AGP had been banned since January 2006 in accordance with pathogenic resistant risk and antibiotic residues in animal products and by-products (EC 2012).

Feed additive based on organic material had been explored to replace antibiotic in order to support the ruminant performance. Probiotic from lactic acid bacteria are widely used to support the microbial balances in digestive tract. Probiotic is microorganism that supports animal health through maintaining and improving the intestinal microbial balance and its immune response. It is necessary to consider this probiotic, because rumen constitutes an effective animal-microbe interdependency system from that each partner derives benefit to the host animal (Arokiyaraj et al. 2014). The role interdependency system was played by complex microbial community in which the domains bacteria, archaea, and eukarya that responsible for degrading fiber particle from forage as major energy sources (Fraga et al. 2014).

Many species of lactic acid bacteria were used as probiotic cultures for ruminant such as *Lactobacillus animalis*, *L. paracasei*, *Bacillus coagulans* (Agazzi et al. 2014), *Pseudobutyrvibrio ruminis* (Fraga et al. 2014) and *Pediococcus acidilactici* (Cobos et al. 2011; Arokiyaraj et al. 2014). Moreover, Cobos et al. (2011) revealed that *P. acidilactici* isolated from rumen lamb could survive in low pH condition (4.71-4.98) without causing negative effect on dry matter digestibility. It implies that *P. acidilactici* can be used as probiotic.

On the other side, productivity of ruminant was influenced by mineral deficiencies. Although diets was formulated based on the nutrient requirement, in some cases, ruminant metabolic disorder caused by mineral deficiency. Haenlein & Anke (2011) reviewed that mineral deficiency is not only affect productivity parameters but also causes serious disease in ruminant. Organic mineral was added into diets to improve bioavailability for preventing mineral deficiency disorders. Artificial organic mineral (Selenium) involved yeast fermentation had been reported by Gresakova et al. (2013) that Se-yeast had higher absorption in digestive tract and body cell than Se-inorganic form. In addition, Rabiee et al. (2010) reviewed that complex trace mineral in organic form increased ruminant productivity and health.

The other strategy to optimize feed digestibility is through methane reduction by tannin. Neem tree

(*Azadirachta indica* A. Juss) is tropical plant, its leaves contain secondary compound such as flavanoid and tannin (Pandey et al. 2014; Bhatta et al. 2015). Many researchers reported that plant secondary metabolites such as tannin could be used to modify ruminal fermentation in which improving feed utilization through reducing methane emission and therefore tannin is considered as a natural compound possessing methane mitigating effect (Bodas et al. 2012; Jayanegara et al. 2012).

Nutrition management strategy for enhancing ruminant production by supplementation of organic feed additive consisting of probiotic and organic mineral was necessary done. The availability of mineral in digestive tract affects the rumen microbe activity. This research was conducted to evaluate addition of probiotic (*Pediococcus acidilactici* RS2) and micromineral enriched yeast (MEY) combined with leaves containing tannin (*A. indica*) on king grass (*P. hybrid*) fermentability using *in vitro* gas production technique.

MATERIALS AND METHODS

Sample preparation

King grass (*Pennisetum hybrid*) was harvested on 70 days after previous cutting (harvest) and used as forage sample, then chopped and dried in oven at 60°C up to reach 12-14% of moisture content. The sample was ground and sieved into two mm particle size. Similarly, this procedure was applied in preparing leaf of Neem (*Azadirachta indica* A. Juss) which was used as tannin source.

Probiotic lactic acid bacteria (*P. acidilactici* RS2) was isolated from cattle rumen and prepared by spray-drying method. Isolate was facultative anaerobically cultivated in deMann Rogosa Sharpe (MRS) Broth media for 18 h at 37°C. The culture was centrifuged at 4500 rpm for 10 minutes, then the pellet/biomass was mixed with the sterilized skim solution (20% w/v) and gum arabic (1% w/v). The solution was homogenized using the digital homogenizer at 8000 rpm for 5 minutes. The culture solution was dried into powder form using the spray dryer (Lab Plant SD-Basic). Spray dryer operating conditions as follows: inlet air temperature was 110°C, outlet air temperature was 55-60°C, and the speed of pump was '3' speed unit. The dried culture viability was evaluated by spread plate method and adjusted to 10⁹ cfu/g of bacterial cell density.

Micromineral enriched yeast (MEY) was produced by inoculating *S. cerevisiae* ATCC 9763 into media fortified by micro minerals consisted of Fe, Mn, Cu, Co, Zn, and I. Cassava (*Manihot* sp.) flour as a substrate for fermentation, and the formulation per kg substrate

consisted of $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (0.177 g), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (7.129 g), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (9.810 g), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (12.646 g), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.192 g), and KI (0.217 g). Fermentation was conducted for 7 days in facultative condition then dried in oven at 55°C (up to 24-48 h, DM 10%), followed by ground and sieved into one mm particle size.

Fermentability and *in vitro* digestibility assessment

The sample and rumen liquid were prepared prior to *in vitro* assessment. Two ruminally fistulated Ongole crossbreed cattle adapted by feeding forage (*P. hybrid*) and concentrate (80:20 in dry matter basis) were used as rumen liquor donor. Rumen fluid was taken using aspirator, and immediately transported in pre-warmed vacuum flask (39°C water temperature) and filtered.

In vitro fermentability was evaluated using *in vitro* gas production technique according to Menke & Steingass (1988). Gas production kinetics was calculated based on the exponential equation according to Ørskov & McDonald (1979). The exponential equation is $P = a + b(1 - e^{-ct})$ with describing P is total gas production, a is the gas production from soluble fraction, b is the gas production from insoluble fraction,

c is the rate of gas production, t is the time of incubation and e is Euler's constant (2.7183...). The estimated value of a , b and c were calculated by a fitting curve method using Neway Software developed by Chen (1997). This study was conducted based on Completely Randomized Design (CRD) with six treatments and three replications. Nutrient composition from each treatment was showed in Table 1. The treatments were described as follows:

- P0 = control/forage 380 mg (dry matter=92.5%)
- P1 = P0+3% micromineral enriched yeast (MEY)
- P2 = P0+3% MEY+2% crude tannin
- P3 = P0+0.1% probiotic (10^9 cfu/g)
- P4 = P0+3% MEY+0.1% probiotic
- P5 = P0+3% MEY+ 2% crude tannin+0.1% probiotic

Fermentation was conducted in 100 mL syringe glass (Fortuna model, Poulten and Graft GmbH Germany). Two syringes containing rumen-buffer without sample (blank) was used in the experiment. All of syringes consisted of samples and blank were randomly incubated for 48 hours in an incubator at 39°C .

Table 1. Nutrient composition of diets consisting of king grass supplemented by either MEY, probiotic or crude tannin

Nutrients	Treatment diets					
	P0	P1	P2	P3	P4	P5
DM	92.5	92.6	92.5	92.5	92.6	92.5
CP (%DM)	11.7	11.4	11.7	11.7	11.4	11.7
CF (%DM)	25.5	25.2	24.8	25.5	25.2	24.8
EE (%DM)	2.1	2.0	2.1	2.1	2.0	2.1
NFE (%DM)	45.0	46.0	46.1	45.0	46.0	46.1
CT (%DM)	-	-	0.28	-	-	0.28

P0 = *P. hybrid*;

P1 = P0+Micromineral Enriched Yeast/MEY;

P2 = P0+MEY+crude tannin;

P3 = P0+Probiotic;

P4 = P0+Probiotic+MEY;

P5 = P0+Probiotic+MEY+crude tannin;

DM = dry matter;

CP = crude protein;

CF = crude fiber;

EE = ether extract;

NFE= nitrogen free extract;

CT = condensed tannin

Nutrient composition was calculated by reference consisted of King grass (Rumiyati 2006); *A. indica* (Obun et al. 2013; Bhatta et al. 2015);

DM content was confirmed by re-analysing in our experiment

Cumulative gas production was recorded at 0, 1, 2, 4, 6, 8, 12, 18, 24, 36 and 48 hours. After 48 h incubation gas was released and the fluid contained in syringe was taken for analysis of protozoa population, VFA, and *in vitro* dry matter and organic matter digestibility. Dry matter (DM) and organic matter (OM) of substrate from each syringe was measured according to AOAC (2012) method. Percentage of DM or OM differences between initial and after incubation and corrected with blank were calculated as *in vitro* digestibility as followed the formula:

$$\text{IVDMD} = \frac{[\text{DM}_f - (\text{DM}_r - \text{DM}_b)]}{\text{DM}_f} \times 100 \%$$

and

$$\text{IVOMD} = \frac{[\text{OM}_f - (\text{OM}_r - \text{OM}_b)]}{\text{OM}_f} \times 100 \%$$

Where IVDMD: *in vitro* dry matter digestibility, IVOMD: *in vitro* organic matter digestibility, DM_f: dry matter of feed, DM_r: dry matter of residue, DM_b: dry matter of blank, OM_f: organic matter of feed, OM_r: organic matter of residue, and OM_b: organic matter of blank.

Analysis of volatile fatty acid, methane, and counting protozoa

Volatile fatty acid (VFA) product from fermentation was measured according to Sun et al. (2013). Sample was prepared by addition of meta-phosphoric acid and stored at -20°C before analysis. Analysis of VFA was performed by gas chromatography (Shimadzu type 8A) with *packed column* GP10% SP-1200/1% H₃PO₄ on 80/100 Chromosorb WAW (Supelco, Bellefonte, PA).

Analysis of methane (CH₄) from gas production was conducted after 18 h of incubation. Ten ml of gas from each sample was taken using vacuum syringe then injected into a vacuum tube. Gas samples were analyzed using gas chromatography (Shimadzu GC-14B) completed with ProparakQ Column (50°C) with helium (He) carrier gas at 60 ml/min flow rate and a flame ionization detector (150°C) as described by Duan et al. (2013). The number of protozoa was counted using hemocytometer and stained with methyl green formalin saline/MFS. MFS solution contained 100 mL of 35% formaldehyde solution, 900 mL of distilled water, 0.6 g of methyl green and 8.0 g of NaCl as described by Sun et al. (2013).

Data analysis

Variables measured were *in vitro* digestibility (IVDMD and IVOMD), fermentability (gas production kinetics, *a*, *b* and *c*), individual volatile fatty acids

(VFA), non-glucogenic ratio (NGR), protozoa numbers, methane production (CH₄) and CH₄/OMD ratio. The protozoa cell number was converted to logarithmic transformation. Data were evaluated by analysis of variance (ANOVA) and the differences among mean treatments were analyzed using post hoc test of Duncan's Multiple Range Test performed by the CoSTAT statistical software (Cohort 2008).

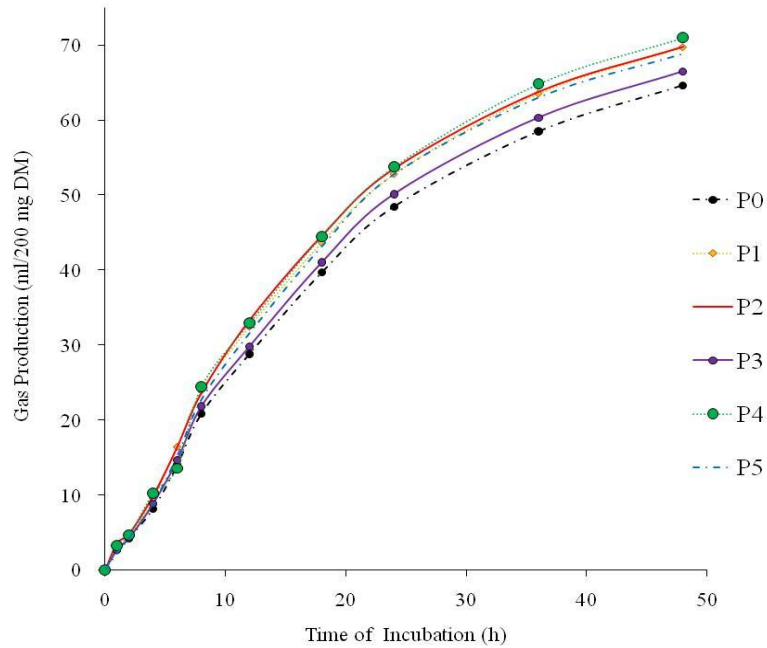
Analysis of interrelationship parameters was performed by hierarchical clustering analysis/HCA (Ametaj et al. 2010). Visualization of HCA was performed using dendrogram-heatmap that constructed using `heatmap.2` function from `gplots library` in the R-statistical software (R Core Team 2013). Pre-treatment data were calculated based on the relative differential data from treatments and control using formula $x_i/x_o - 1$ where x_i and x_o denote treatment and control data respectively.

RESULTS AND DISCUSSIONS

Ruminal fermentability characteristics evaluated by *in vitro* gas production were showed on Table 2. Total gas, gas production rate (*c*) and gas production from insoluble fraction (*b*) of forage was significantly affected by treatments (P<0.05). The other parameters consisted of gas production from soluble fraction (*a*) or total fraction (*a+b*) from forage had tendency (P~0.11) affected by treatments. Forage was supplemented by probiotic+MEY (P4) generated the highest gas production compared to the others.

Gas production generated from the soluble fraction (*a*) resulted negative value that indicated ruminal microbes need adaptation time (*lag* phase) before degrading the insoluble particle. The previous study reported by Arhab et al. (2010) that negative value could be interpreted as *lag* time of ruminal microbes to degrade soluble fraction and then to adhere to cellulosic fraction.

Production of total volatile acid, acetate (C2), propionate (C3), butyrate (C4) and non glucogenic ratio (NGR) indicated no significant difference (P>0.05) among treatments (Table 2). Total VFA was varied between 43 ~ 62 mM per mL of rumen fluid. Production of VFA from forage treated by P1, P3, P5, and P4 were 43.2%, 26.2%, 19.3% and 7.7% higher than P2 and control (P0). Non glucogenic ratio seemed constant in all treatments with average value 4.42 except for P5 treatment (13% higher than control), however proportion of C2:C3:C4 was constant about 70:20:10 for all treatments. Based on the ruminal fermentation stoichiometry, individual VFA proportion consisting of C2, C3 and C4 were 60-70%, 20-30% and 10-15% respectively (Wolin et al. 1997).



P0 (*P. hybrid*); P1 (P0+micromineral enriched yeast/MEY); P2 (P0+MEY+crude tannin); P3 (P0+Probiotic); P4 (P0+Probiotic+MEY); P5 (P0+Probiotic+MEY+crude tannin)

Figure 1. Cumulative gas production of forage supplemented by probiotic, micromineral enriched yeast (MEY) and crude tannin incubated during 48 h

In the present study, *in vitro* dry matter (IVDMD), organic matter (IVOMD) digestibility and production of volatile fatty acid (VFA) were not influenced by treatments either probiotic or combination with MEY and tannin. The similar result was also reported by Sun et al. (2013), that probiotic *Bacillus subtilis* supplementation on total mix ration (TMR) did not affect the dry matter digestibility during 24 h, moreover it decreased significantly ($P < 0.05$) neutral detergent fiber (NDF) digestibility. In contrast, Fraga et al. (2014) studied that addition of probiotic consisted of *Pseudobutyrvibrio ruminis* significantly increased total VFA concentration of wheat straw. It seems that probiotic supplementation affected *in vitro* digestibility might be influenced by culture adaptation associated with complexity in ruminal ecosystem.

Compared to other animal, supplementation of feed additive complex containing chelated/organic mineral, probiotic, yucca extract indicated that no significant improvement of feed digestibility in horse (Gordon et al. 2013). This varying result could be caused by many factors such as age of animal, viability of probiotic culture, and initial mineral status of the animals.

Fermentability evaluated by *in vitro* gas production was indicated by kinetic gas production parameters. Based on the kinetic curve of gas production, all treatments did not significantly influence gas production up to 8 hours of incubation. However, after 8 hours incubation, gas production from forage was significantly affected by treatment (Figure 1). The highest cumulative gas production found at forage treated by P4 followed by P5, P1, P5, P2, P3, and control. It was closely related to the kinetics parameter (*a*, *b*, and *c* values) which indicated that gas production in probiotic treatment and control was lower than others (Table 2).

Methane emission from ruminant reflected the energy lost. The amount of methane production conversely indicated the nutrient utilization. Methane (CH_4) production and methane corrected by organic matter digestibility (CH_4/OMD) during incubation showed similar result in all treatments, around $14.05 \pm 0.35\%$ and $32.11 \pm 4.25\%$ respectively. CH_4 and CH_4/OMD clearly tended to decrease on treatments P2 and P3.

Table 2. Gas production kinetic, volatile fatty acid production and *in vitro* digestibility of forage supplemented by probiotic, micromineral enriched yeast (MEY) and crude tannin

Variables	Treatments						P-value
	P0	P1	P2	P3	P4	P5	
Fermentability/Gas production kinetic parameters							
<i>a</i> (mL)	-0.68±0.27	-0.81±0.14	-0.65±0.29	-0.76±0.15	-0.66±0.10	-1.09±0.06	0.097
<i>b</i> (mL)	47.52±0.61 ^{ab}	48.12±1.27 ^{ab}	46.92±0.80 ^b	47.72±0.16 ^{ab}	48.87±0.48 ^a	48.86±0.67 ^a	0.046*
<i>c</i> (mL/h)	0.042±0.002 ^b	0.046±0.001 ^a	0.047±0.002 ^a	0.043±0.001 ^b	0.045±0.001 ^{ab}	0.044±0.001 ^{ab}	0.036*
<i>a+b</i> (mL)	46.84±0.35	47.32±1.38	46.28±1.01	46.95±0.08	48.21±0.49	47.76±0.67	0.111
Gas (48h) (mL)	40.25±0.57 ^d	41.71±0.69 ^{ab}	40.92±0.56 ^{bcd}	40.39±0.16 ^{cd}	42.22±0.42 ^a	41.24±0.27 ^{bc}	0.002*
Volatile fatty acids							
Acetate (mM)	30.55±16.18	43.82±7.36	29.66±11.64	39.28±1.37	33.43±17.34	36.80±10.78	0.684
Propionate (mM)	8.64±4.32	12.10±1.69	8.96±2.82	10.80±0.23	9.12±4.03	9.40±2.93	0.691
Butyrate (mM)	3.97±1.54	5.88±1.27	4.56±1.59	4.39±1.58	3.94±2.04	5.30±2.73	0.587
Total VFA (mM)	43.16±22.03	61.80±10.27	43.19±15.80	54.48±1.39	46.49±23.40	51.50±16.37	0.703
NGR	4.44±0.14	4.58±0.18	4.27±0.28	4.45±0.15	4.38±0.52	5.01±0.16	0.308
In vitro digestibility							
IVDMD (%)	39.40±1.68	42.64±2.82	43.10±3.27	42.58±4.84	41.95±1.02	42.70±1.31	0.638
IVOMD (%)	41.11±1.61	43.46±2.95	45.11±4.33	49.50±12.33	42.80±0.86	50.39±12.09	0.586

P0 (*P. hybrid*); P1 (P0+micromineral enriched yeast/MEY); P2 (P0+MEY+crude tannin); P3 (P0+Probiotic); P4 (P0+Probiotic+MEY); P5 (P0+Probiotic+MEY+crude tannin). Gas production from soluble fraction (*a*); and insoluble fraction (*b*); rate of gas production (*c*); IVDMD: *In vitro* Dry Matter Digestibility; IVOMD: *In vitro* Organic Matter Digestibility; * Significant Difference

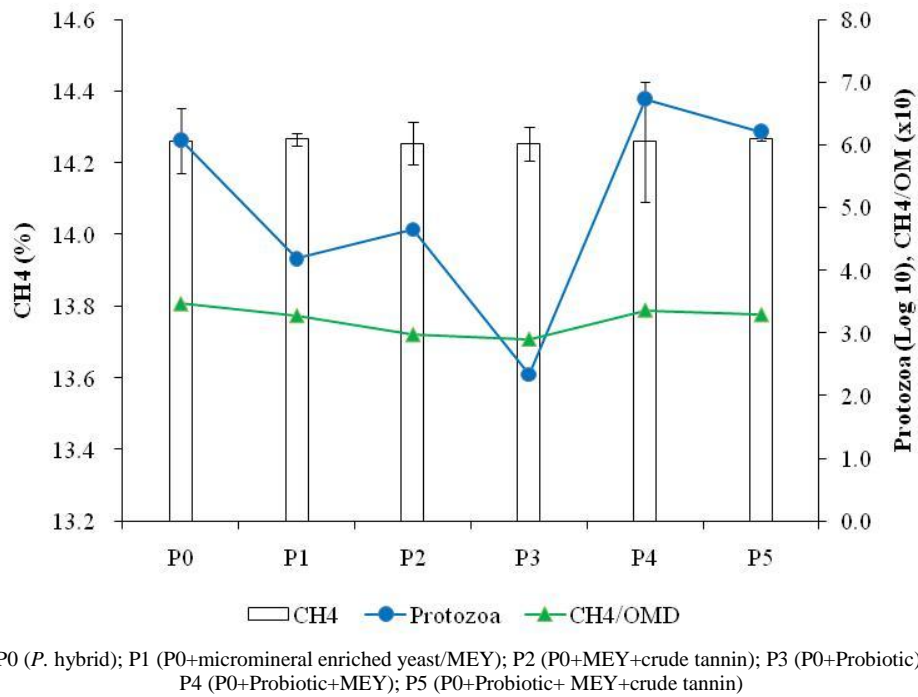
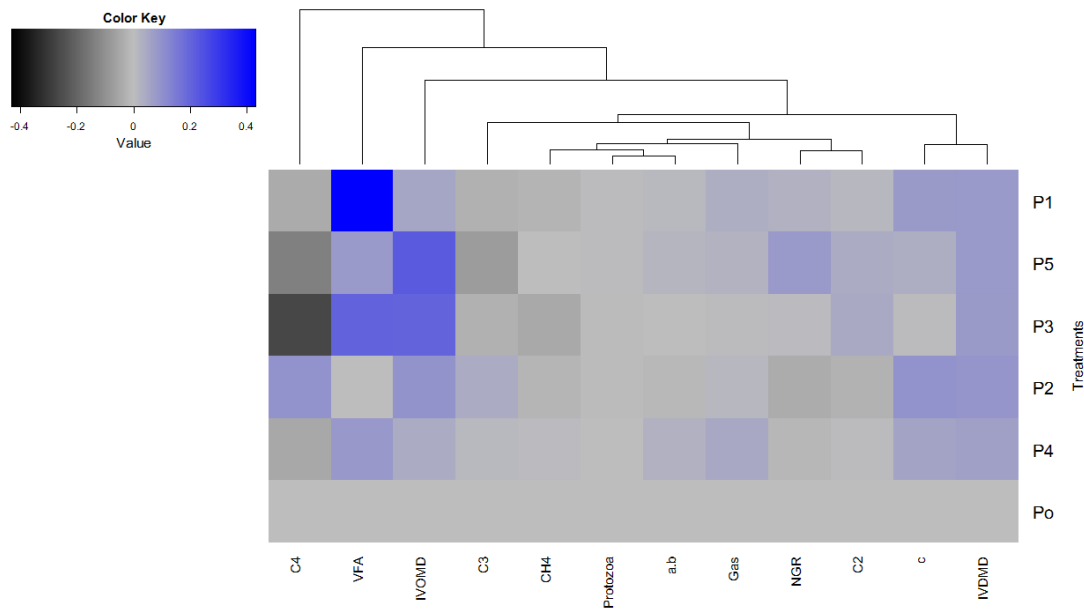


Figure 2. Protozoal cell number, methane production, and methane per organic mater digestibility (CH₄/OMD) of forage supplemented by probiotic, micromineral enriched yeast (MEY) and crude tannin



VFA: volatile fatty acids; C2: acetate; C3: propionate; C4: butyrate; CH₄:methane; NGR: non-glucogenic ratio; IVDMD: *in vitro* dry matter digestibility; IVDMD: *in vitro* organic matter digestibility; P0 (*P. hybrid*); P1 (P0+micromineral enriched yeast/MEY); P2 (P0+MEY+crude tannin); P3 (P0+Probiotic); P4 (P0+Probiotic+MEY); P5 (P0+Probiotic+MEY+crude tannin). Color key: (0) denotes no change; (+) denotes value of proportion increase; (-) denotes value of proportion decrease

Figure 3. Dendrogram-heatmap visualized hierarchical-clustering analysis of altering ruminal VFA, protozoa, methane production, fermentability, and *in vitro* digestibility

Moreover, protozoa number appeared similar in all treatments was around 6.05 Log₁₀ per mL rumen fluid (Figure 2). Supplementation of probiotic *Propionibacterium* could not alter methane production (Alazzeah et al. 2014). Indeed, Vyas et al. (2014) recommended to combine probiotic supplementation with concentrate to increase molar proportion of propionate and subsequently reduced the CH₄ emissions.

Probiotic from lactic acid bacteria could reduce methane production by the mechanism of formate utilization in rumen, in turn formate would be used by methanogenic bacteria as methane precursor (Jeyanathan et al. 2014). In this study, inability probiotic to reduce methane might be correlated with constraint adaptability of culture in ruminal ecosystem. Similarly, Alazzeah et al. (2014) revealed that probiotic have no effect on methane reduction could be associated with adaptability with other ruminal microbes and including utilization of methane precursor. Moreover, probiotic also did not influence number of protozoa. Both of probiotic and protozoa indicated no antagonistic relationship as similar previously reported by Qadis et al. (2014).

Supplementation of tannin from *A. indica* leaves did not affect the methane production and protozoa number. It might be caused by low dosage of tannin (0.28%) used in the present study. Currently, Bhatta et al. (2015) reported that addition of 2.5% *A. indica* leaves (contained 13.8% of condensed tannin) did not reduced methane production and protozoa number, however the methane production was inhibited when the addition was increase to 25%.

Interrelationship parameters evaluated by hierarchical cluster analysis (HCA) indicated that proportion change of IVOMD, VFA and C4 were higher independently changed by treatment than other parameters (cluster I). Other parameters were categorized into cluster II (C3, CH₄, protozoa, *a+b*, gas), cluster III (IVDMD and *c*), cluster IV (C2 and NGR). Cumulative gas production and IVDMD had higher similarities related to the change of treatment (Figure 3.). The tree clusters and their shorter *Euclidean* distance indicated higher similarities. Similarity between two metabolites or parameter was represented by branch height (Ametaj et al. 2010).

Organic matter digestibility of forage treated by P5 (probiotic+MEY+tannin) and P3 (probiotic), then total VFA from P1 reflected the higher increased differences than others while forage treated P4 (MEY+tannin) was not affect VFA and gas production. This result revealed that forage treated by probiotic either combining with organic mineral or tannin affected ruminal fermentation. Improving ruminal fermentation by feed additive containing lactic acid bacteria was previously

reported Hillal et al. (2011). Otherwise, presence of tannin in P2 (MEY+tannin) resulted in VFA and fermentability lower than P1 might attribute to characteristic of condensed tannin in *A. indica* could inhibit ruminal microbes. Previously, Seresinhe et al. (2012) reported supplementation of condensed tannin could reduce forage fermentability by indicating gas production.

Methane production was closely related to the protozoa number. In the rumen, methane was generated by *Archaea bacteria* that consumed hydrogen. *Archaea* activity was closely symbiosis with protozoa (Bhatta et al. 2015) and endosymbiotic between protozoa and *Archaea* responsible to methane formation (Belanche et al. 2015). Change of NGR was closely related to C2, C3 and C4 in which C2 was dominantly affected due to 60% of VFA. Moreover, gas production and degradation rate of particle affected the IVDMD. Sandoval-Castro et al. (2002) studied the relationship between digestibility and production gas parameter. Significant correlation between gas production and digestibility were influenced by many factors consisted of nutrient composition in which associated microbial ability to adhere and degrade the fraction of feed materials.

Overall, *in vitro* digestibility indicated nutrient utilization in the rumen which was attributed by gas production kinetic parameters and others parameters consisting of VFA, protozoa and methane production. In this study, presence of probiotic complemented by mineral organic or crude tannin had a positive effect on ruminal fermentation of forage.

CONCLUSION

Supplementation of feed additive contained micromineral enriched yeast (MEY) in combination with probiotic *P. acidilactici* RS2 or probiotic+*A. indica* leaves improved fermentability of king grass without affected volatile fatty acid, protozoa numbers, methane production, and *in vitro* digestibility.

ACKNOWLEDGEMENT

The authors would like to gratefully appreciate to the Indonesian Ministry of Research, Technology and Higher Education (Kemenristekdikti) and the Indonesian Institute of Sciences (LIPI) for supporting the research. We thank to the technician staff of Animal Feed and Nutrition Laboratory, the Research Unit for Processes Development and Chemical Engineering (UPT. BPPTK LIPI) for assistance during the experiment.

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