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THE EFFECT OF CONCENTRATE CONTAINING PROBIOTICS ON FERMENTATION CHARACTERISTICS, METHANOGENESIS AND *In vitro* NUTRIENT DIGESTILITY

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ABSTRAK

Tujuan penelitian ini adalah untuk mengevaluasi pengaruh penambahan probiotik dalam konsentrat terhadap karakteristik fermentasi, produksi metana dan kecernaan nutrien secara *in vitro*. Penelitian ini disusun dalam rancangan acak lengkap yang terdiri atas 4 perlakuan yaitu silase rumput (G); silase rumput + konsentrat tanpa probiotik (G+A); silase rumput + konsentrat mengandung *L. plantarum* dan *S. cerevisiae* (G+B); silase rumput + konsentrat mengandung *L. acidophilus* dan *S. cerevisiae* (G+C); silase rumput + konsentrat mengandung *L. plantarum* dan *L. acidophilus* (G+D). Data dianalisis menurut rancangan acak lengkap dan dilanjutkan uji wilayah berganda Duncan. Hasil penelitian menunjukkan bahwa konsentrat mengandung bakteri asam laktat (BAL) bervariasi 1.5×10^6 dan 3×10^7 cfu/g, dan *S. cerevisiae* 3×10^3 cfu/g. Kombinasi *L. plantarum* dan *S. cerevisiae* (G+C) dan *L. acidophilus* dan *S. cerevisiae* (G+D) meningkatkan (P<0,01) konsentrasi asam propionat. Rata-rata produksi metana pada konsentrat mengandung probiotik (G+B, G+C, G+D) menurun (P<0,01) sebesar 6,9% dibandingkan konsentrat tanpa probiotik (G+B). Kecernaan bahan kering dan neutral detergent fiber (NDF) meningkat (P<0,01) berturut-turut sebesar 25,7% and 6,3% pada konsentrat mengandung probiotik (G+B, G+C, G+D) dibandingkan konsentrat tanpa probiotik (G+A). Disimpulkan bahwa penambahan probiotik pada konsentrat meningkatkan proporsi asam propionat, kecernaan nutrien dan menurunkan produksi metana (*in vitro*).

Kata kunci: konsentrat, kecernaan, metana, probiotik, ruminansia

ABSTRACT

The aim of the experiment was to evaluate the effect of probiotic addition in concentrate on fermentation characteristics, methane (CH_4) production and *in vitro* nutrient digestibility. Two strains lactic acid bacteria (LAB) i.e. *Lactobacillus plantarum* and *Lactobacillus acidhophilus*, and one strain yeast of *Saccharomyces cerevisiae* was used as probiotic.This experiment was arranged in a completely randomized design consisted of 4 treatments as follows grass silage (G); grass silage + concentrate without probiotic (G+A); grass silage + concentrate containing *L. plantarum* and. *S. cerevisiae* (G+B); grass silage + concentrate containing *L. acidophilus* and. *S. cerevisiae* (G+C); grass silage + concentrate containing *L. plantarum* and *L. acidophilus* (G+D). Data were analyzed as completely randomized design and followed by Duncan's multiple range test. The results showed that the concentrate containing LAB varied 1.5×10^6 and 3×10^7 cfu/g, and 3×10^3 cfu/g of S. cerevisiae. Combination between *L. plantarum* and *S. cerevisiae* (G+B), and *L. acidophilus* and *S. cerevisiae* (G+C) in concentrate increased (P<0.01) propionic acid proportion. The average CH_4 production in concentrate containing probiotic (G+C, G+D and D+E) was lower by 6.9% (P<0.01) compared to concentrate without probiotic (G+B).

The *in vitro* dry matter (DM) and neutral detergent fiber (NDF) digestibility were higher (P<0.01) by 25.7% and 6.3% respectively, in grass silage substrate with concentrate containing probiotic (G+C, G+D and G+E) than in grass silage with concentrate without probiotic (G+B). In conclusion, addition of probiotic in concentrate could increase *in vitro* propionic acid proportion, DM and NDF digestibility and reduce CH_4 production.

Keywords: concentrate, digestibility, methane, probiotics, ruminant

INTRODUCTION

There is an increasing interest in research activities to evaluate the potential of secondary plant compound as feed additives instead of chemical compounds *i.e*. ionophores and antibiotics as manipulators of rumen fermentation to decrease CH_4 production. The use of growth promoting antibiotics in animal feeds is banned in Europe due to potential risks such as spread of antibiotic resistance genes (Hong *et al*., 2005) or the contamination of milk or meat with antibiotics residues. Recently, probiotics have been increasingly evaluated to replace in the use of antibiotics.

Probiotic is a live microbial feed supplement that may beneficially affect the host animal upon ingestion by improving its intestinal microbial balance (Fuller, 1989). Seo *et al*. (2010) stated that microorganisms sush as *Lactobacillus, Streptococcus* and *Enterococcus* are commonly used in probiotic for ruminants. Furthermore, *Saccharomyces cerevisiae* and *Aspergilus oryzae* are two primary fungal direct-fed microorganism (DFM) that have been supplemented to diet in ruminants. Seo *et al*. (2010) stated that propionibacteria ferments lactic acid to propionic acid. Since propionic acid is the major precursor for gluconeogenesis, increaments propionic acid production in the rumen increases of hepatic glucose production. In addition, increased propionic acid may reduce hydrogen available for CH_4 production in the rumen. Newbold (1995) revealed that addition of *S. cerevisiae* in ruminant could improve animal production through increasing mechanism of bacteria viability. Mwenya *et al.* (2004) reported that adding of yeast culture containing 21% of *S. cerevisiae* in sheep reduced CH_4 production by 10% as compared to control sheep. In *in vitro* study, Lila *et al*. (2004) concluded that *S. cerevisiae* stimulated mixed ruminal fermentation with decreased lactate, and a small decrease of CH_4 and hydrogen. In the previous *in vivo* study, most of researchers directly fed probiotic to the animal. However, this method is less efficient when it is applied to a number of animal. Therefore, the objective of the present study was to evaluate the effect of different concentrate containing probiotics on *in vitro* fermentation characteristics, CH_4 production and nutrient digestibility.

MATERIALS AND METHODS

Concentrate Preparation

Rice bran, tofu waste and cassava waste were obtained from small-scale food industry located at Manokwari and Prafi Districts, Manokwari regency. Tofu waste and cassava waste were dried in the oven 60 °C at least 48 h and ground to pass a 1 mm sieve in a Wiley mill. *Lactobacillus plantarum* was isolated from *Pennisetum purpureophoides* that has been used in the previous study by Santoso *et al*. (2012). *L. plantarum* and *L. acidophilus* were cultured using MRS broth at 30 °C for 48 h (Santoso *et al*. 2013a), meanwhile *S. cerevisiae* was cultured using malt extract broth at 30 °C for 48 h (Newbold *et al*., 1995). The solid meterials of concentrate were manually mixed by hand and then sprayed on top with culture of LAB and yeast (Table 1).

Donor Animal

Two ruminally fistulated Ongole crossbreed cattle were used as rumen liquor donor. Animals were fed at 6.8 kg DM of king grass to meet their maintenance requirement and adapted for 3 weeks before rumen liquor collection. The feed was offered twice daily at 08:00 and 16:00 h. Rumen liquor was collected before the morning feeding and strained through four layers of cheesecloth into a pre-warmed thermos flask.

In vitro Gas Production and CH_4 Measurement

In vitro gas production was determined in line with the method of Menke and Steingass (1988) previously described by Hariadi and Santoso (2010); Santoso *et al*. (2013). Briefly, oven-dried samples of about 300 mg were

Table 1. Ingredients of Concentrate and Chemical Composition of Grass Silage and Concentrates (%)

	G	Concentrates			
		A	B	C	D
Ingredients					
Cassava waste	-	40	36	36	36
Tofu waste	-	20	20	20	20
Rice bran	-	35	35	35	35
Salt	-	2	2	2	2
Urea	-	3	3	3	3
L. plantarum	-	-	2	-	2
S. cerevisiae	-	-	2	2	-
L. acidophilus	-	-	-	2	2
Chemical composition					
Dry matter	16.7	82.0	81.0	83.0	84.1
Organic matter	91.8	93.9	93.8	92.8	93.1
Crude protein	7.1	19.4	17.9	17.5	17.0
NDF	80.3	50.8	42.7	44.1	42.1
ADF	54.0	21.2	20.2	23.7	20.9
Hemicellulose	26.3	29.6	22.5	20.4	21.2
L. plantarum	-	-	5.4×10^6	-	8.1×10^7
L. acidophilus	-	-	-	1.9×10^5	
S. cerevisiae	-	-	6.2×10^6	7.4×10^6	-

G: grass silage; A: concentrate without probiotic; B: concentrate containing *L. plantarum* and *S. cerevisiae*; C: concentrate containing *L. acidophilus* and *S. cerevisiae*; D: concentrate containing *L. plantarum* and *L. acidophilus*

weighed into 100 ml glass syringes (Model Fortune, Häberle Labortechnik, Germany) with pistons lubricated with vaseline. Three parallel syringes that contained rumen liquor-buffer mixtures without substrate served as blanks. Buffer solution contained carbonate buffer, macromineral solution, and micromineral solution. The syringes were pre-warmed at 39 °C overnight, before the addition of 30 ± 1.0 ml of rumen liquor-buffer mixtures into each syringe. The syringes were incubated in a water bath at 39 °C for 48 h and were gently shaken every 8 h. The volume of gas released from each syringe was recorded before incubation (0 h) and 1, 2, 4, 6, 8, 12, 24, 36 and 48 h of incubation.

To facilitate CH_4 measurement, glass syringes fitted with an extra outlet containing gas-tight septum for gas sampling as previously demonstrated by Hariadi and Santoso (2010); Santoso *et al*. (2013). One hundred micro litter of gas was sampled from the headspace of syringe in an airtight syringe at 24 and 48 h of incubation. Methane was determined by injection 100 ml of gas into a chromatograph gas (GC model 263-50, Hitachi Ltd., Ibaraki, Japan).

At the end of the incubation period, about 10 ml of syringe contents were sampled. The pH of medium was recorded immediately using a digital pH meter (Hanna, Hi 8520, Ronchi di Villafranca, Italy). Subsequently, 0.2 ml of sub-samples were pipetted into 1.5 ml micro centrifuge tube containing 1 ml of 25 g/100 ml (w/v) metaphosphoric acid and centrifuged at 9000 *g* for 10 min for volatile fatty acids (VFA)

determination. A further 2 ml of sub-samples were added to 2 ml of 20 g/l (w/v) NaCl and for NH_3-N analysis.

***In vitro* Nutrients Digestibility**

Determinations of DM, organic matter (OM) and neutral detergent fiber (NDF) digestibilities were conducted using *in vitro* procedure of Tilley and Terry (1963). Twenty five milliliter of rumen liquor-buffer mixtures in a 1 : 4 (v/v) ratio were dispensed in 100 ml glass tubes containing 250 mg of dry sample consisted of grass silage and concentrate (70 : 30, DM). Triplicates of blank (with no feed sample) and standard (Pangola grass) were included in each run. Rumen liquor was collected in the morning before feeding and strained through four layers of cheesecloth into a pre-warmed thermos flask. Buffer solution contained 9.8 g $NaHCO_3$, 9.3 g $NaHPO_4.12H_2O$, 0.47 g NaCl, 0.57 g KCl, 0.04 $CaCl_2$, 0.12 g $MgSO_4.7H_2O$ per 1000 ml distilled water. After gassing CO_2 in the tube, corks were tightly placed over the tubes and were incubated in a water bath at 39 °C for 48 h. After 48 h of microbial incubation, the samples were incubated at 39 °C for 48 h with acid-pepsin. Therefore, the contents were filtered through pre-weighed Gooch crucibles and dried at 105 °C for 24 h. The percent loss in weight was determined and presented as *in vitro* DM digestibility (IVDMD) and *in vitro* NDF digestibility (IVNDFD). The residue left was ashed at 550 °C for determination of *in vitro* OM digestibility (IVOMD).

Chemical Analysis

Dried samples were used to determine DM, OM and CP according to procedure of AOAC (2005). The fiber content *i.e.* NDF and acid detergent fiber (ADF) were analyzed using Van Soest *et al*. (1991) method with some modification *i.e*. NDF was determined without the use of μ-amylase and sodium sulfite.

Statistical Analysis

Data were analyzed as completely randomized design using GLM procedure of SAS (SAS Institute Inc., Cary, NC). Duncan's multiple range test was used to identify the significant differences between means.

RESULTS AND DISCUSSION

Chemical Composition of Feeds

The chemical composition of king grass silage and concentrate used in this study are presented in Table 1. Grass silage used in the present experiment had 7.1% of CP, which is comparable with the threshold value of 7%. Minson and Milford (1966) revealed that digestibility can declined when animals are fed herbage with a CP content below 7%, due to microbial activity in the rumen depressed by lack of nitrogen. Dry matter content of king grass silage was lower than the ideal DM content of 20% as recommended by McDonald *et al.* (1991). The lower DM content of silage obtained in this study could be due to high rainfall during silage preparation. Dry matter and OM contents in all concentrates used in this experiment were similar, varied from 81.0-84.1% and 92.8-93.9%, respectively. Addition of 3% urea in concentrates increased CP content up to 15%. The NDF content in concentrate A was slightly higher than other concentrates may be due to cassava waste was replaced by probiotics isolate in concentrate B, C and D. The population of LAB and yeast in the concentrate was lower than concentration probiotic of 5×10^9 cfu/g as used by Lila *et al*. (2004).

Fermentation Characteristics

The pH value, concentrations of NH_3-N and VFA are presented in Table 2. The pH value in substrate consisted of grass silage and concentrate containing *L. acidophilus* and *S. cerevisiae* (R+C) and concentrate containing *L. plantarum* and *L. acidophilus* (R+D) were lower ($P<0.01$) than control subtrate. The lower pH value could be due to higher lactic acid concentration that produced by LAB thus suppressed pH value. However, the pH value in all treatments ranged from 6.82 to 6.91, which is in the optimal range pH 6.7 ± 0.5 required to maintain normal cellulolysis (Van Soest, 1994) and above 6.0, required for microbial protein synthesis (Russel *et al*., 1992). In the previous *in vitro* study, Lila *et al.* (2004) noted that addition of probiotic contained 5×10^9 of *S. cerevisiae* cells/g had no significant effect on pH value. A different result has been reported by Mwenya *et al.* (2004) that supplementation of 4 g/d of *S. cerevisae* significantly increased pH in the sheep rumen.

Ammonia concentration is a balance between degradation of feed protein and uptake of ammonia for synthesis protein of microbial. During fermentation in rumen, feed protein can be degraded by microbe to NH_3-N. Concentration of NH_3-N in substrate concentrate without

Table 2. *In vitro* Fermentation Characteristics in Supernatant after 48 h of Incubation

	Treatments					SEM	P
	G	G + A	G + B	G + C	G + D		
pH	6.91[a]	6.87[a]	6.86[ab]	6.83[b]	6.82[b]	0.01	0.01
NH_3-N (mg/100 ml)	34.4	38.8	37.3	37.6	38.6	3.25	0.33
C2 (mol/100 mol)	58.4	59.1	57.3	58.5	56.7	1.50	0.65
C3 (mol/100 mol)	15.5[b]	15.5[b]	20.4[a]	19.7[a]	18.3[ab]	0.34	0.01
C4 (mol/100 mol)	26.1	25.4	22.3	21.8	25.0	2.39	0.76
C2/C3	3.8	3.8	2.9	3.0	3.1	0.34	0.19
Total VFA (mM)	90.8[b]	103.5[ab]	112.3[a]	113.0[a]	112.1[a]	2.28	0.01
CH_4 (ml)	7.3[a]	6.8[b]	6.4[b]	6.4[b]	6.2[c]	0.08	0.01

G: grass silage; A: concentrate without probiotic; B: concentrate containing *L. plantarum* and *S. cerevisiae*; C: concentrate containing *L. acidophilus* and *S. cerevisiae*; D: concentrate containing *L. plantarum* and *L. acidophilus*

Mean values with different superscript letters within the same row are significantly different (P<0.01)

probiotic (G+A) was similar to concentrate containing probiotic (G+B, G+C and G+D). Result from this study is supported by Lila *et al*. (2004) and Mwenya *et al*. (2004) concentration of ammonia N did not change by addition of probiotic i.e. *S. saccharomyces* or LAB. A slightly higher NH_3-N concentration observed in substrate silage combined with concentrate compared to silage alone could due to concentrate used in this study had higher CP concent. Concentration of NH_3.N in the present study ranged from 34.4 to 38.8 mg/100 ml, and were above the threshold value for both maximum fiber digestion as recommended by Abdulrazak *et al*. (1997).

The proportion of propionic acid (C3) and total VFA concentration was higher (P<0.05) in concentrate with addition of probiotic (G + B, G + C and G + D). This result is supported by Lila *et al.* (2006) that addition of *S. cerevisiae* increased proportion of propionic acid and total VFA. Meanwhile, Mwenya *et al.* (2004) stated that proportion propionic acid and concentration total VFA in the rumen were similar between sheep fed LAB or *S. saccharomyces* and control sheep. Increasing proportion of propionic acid could be due to increased lactic acid production by LAB. Furthemore, lactic acid to be converted by lactic acid utilizing bacteria such as *Megasphaera elsdenii* to propionic acid. Increasing proportion of propionic acid (C3), however, had positive effect to decrease C2/C3 ratio in treatments G+B, G+C, and G+D. Increasing total VFA in concentrate with addition probiotic is supported by data IVDMD in treatments G+B, G+C, and G+D.

Methane production from cattle in the tropics averaged 10–11% of gross energy intake. It has been demonstrated that CH_4 production was suppressed by addition of *S. cerevisiae* (Lila *et al*., 2004; Mwenya *et al*., 2004). Concentrate containing LAB and yeast probiotics (G+B, G+C, G+D) had lower CH_4 concentration (P<0.01) ranged 11-15% than control feed (G) (Table 1). Concentrate containing *L. plantarum* and *L. acidophilus* (G+D) had the greatest effect to suppress CH_4 production. The decrease CH_4 concentration observed in concentrate containing LAB may due to the utilization of metabolic hydrogen by propionibacteria to produce propionic acid.

Nutrient Digestibility

Table 3 shows *in vitro* DM, OM and NDF digestibility of grass silage and concentrate containing probiotic. The IVDMD in substrate consisted of grass silage and concentrate was higher (P<0.01) compared to control feed. Addition of LAB and yeast probiotic increased IVDMD by 19.4% and 5% and IVNDFD when compared to feed without prcentrated increased

Table 3. *In vitro* Digestibility (%) of Dry Matter, Organic Matter and Neutral Detergent Fiber of Feeds

	Treatments					SEM	P
	G	G + A	G + B	G + C	G + D		
IVDMD	48.7[b]	57.4[a]	61.5[a]	62.0[a]	60.1[a]	1.09	<0.01
IVOMD	52.7	62.4	66.0	61.5	64.1	2.56	0.06
IVNDFD	48.8[b]	51.1[b]	56.2[a]	52.8[b]	53.8[ab]	0.98	<0.01

G: grass silage; A: concentrate without probiotic; B: concentrate containing *L. plantarum* and *S. cerevisiae*; C: concentrate containing *L. acidophilus* and *S. cerevisiae*; D: concentrate containing *L. plantarum* and *L. acidophilus*

Mean values with different superscript letters within the same row are significantly different (P<0.01)

IVDMD and IVNDFD by 5.6% and 6.1%, respectively. This result was comparable to study reported by Lila *et al*. (2004) that addition of *S. cerevisiae* increased *in vitro* DM degradability. In other study, Krisnan *et al.* (2009) concluded that addition of probiotic collected from buffalo rumen in catalytic supplement increased NDF digestibility in sheep. Chaucheyras *et al*. (1995) noted that *S. cerevisiae* had ability to provide growth factors, such as organic acids or vitamins, thereby stimulating ruminal populations of cellulolytic bacteria.

CONCLUSION

In the present study, concentrate containing LAB and yeast was effective in modifying ruminal fermentation patterns by increasing the proportion of propionic acid, total VFA concentration, DM and NDF digestibility. Concentrate containing *L. plantarum* and *L. acidophilus* was more effective to reduce CH_4 production compared to other probiotic. Decreasing CH_4 from ruminants may have positive impact for the environmental sustainability.

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