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

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ABSTRACT

The aim of the experiment was to evaluate the effect of probiotic addition in concentrate on fermentation characteristics, methane (CH4) production and in vitro nutrient digestibility. Two strains lactic acid bacteria (LAB) i.e. Lactobacillus plantarum and Lactobacillus acidophilus, 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 CH4 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).

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

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 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 such as Lactobacillus, Streptococcus and Enterococcus are commonly used in probiotic for ruminants. Furthermore, Saccharomyces cerevisiae and Aspergillus 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, increments 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 purpureopoides 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 materials 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
weighed into 100 ml glass syringes (Model Fortune, Händerle 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 CH₄ 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)

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

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Concentrates</th>
<th>G</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cassava waste</td>
<td></td>
<td>-</td>
<td>40</td>
<td>36</td>
<td>36</td>
<td>36</td>
</tr>
<tr>
<td>Tofu waste</td>
<td></td>
<td>-</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Rice bran</td>
<td></td>
<td>-</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Salt</td>
<td></td>
<td>-</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Urea</td>
<td></td>
<td>-</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>L. plantarum</td>
<td></td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td></td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>L. acidophilus</td>
<td></td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

Chemical composition

<table>
<thead>
<tr>
<th></th>
<th>Concentrates</th>
<th>G</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry matter</td>
<td></td>
<td>16.7</td>
<td>82.0</td>
<td>81.0</td>
<td>83.0</td>
<td>84.1</td>
</tr>
<tr>
<td>Organic matter</td>
<td></td>
<td>91.8</td>
<td>93.9</td>
<td>93.8</td>
<td>92.8</td>
<td>93.1</td>
</tr>
<tr>
<td>Crude protein</td>
<td></td>
<td>7.1</td>
<td>19.4</td>
<td>17.9</td>
<td>17.5</td>
<td>17.0</td>
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<tr>
<td>NDF</td>
<td></td>
<td>80.3</td>
<td>50.8</td>
<td>42.7</td>
<td>44.1</td>
<td>42.1</td>
</tr>
<tr>
<td>ADF</td>
<td></td>
<td>54.0</td>
<td>21.2</td>
<td>20.2</td>
<td>23.7</td>
<td>20.9</td>
</tr>
<tr>
<td>Hemicellulose</td>
<td></td>
<td>26.3</td>
<td>29.6</td>
<td>22.5</td>
<td>20.4</td>
<td>21.2</td>
</tr>
<tr>
<td>L. plantarum</td>
<td></td>
<td>-</td>
<td>-</td>
<td>5.4 × 10⁶</td>
<td>-</td>
<td>8.1 × 10⁷</td>
</tr>
<tr>
<td>L. acidophilus</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.9 × 10⁵</td>
<td>-</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td></td>
<td>-</td>
<td>-</td>
<td>6.2 × 10⁶</td>
<td>7.4 × 10⁶</td>
<td>-</td>
</tr>
</tbody>
</table>

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
determination. A further 2 ml of sub-samples were added to 2 ml of 20 g/l (w/v) NaCl and for NH\textsubscript{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 milliliters 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\textsubscript{3}, 9.3 g NaHPO\textsubscript{4}.12H\textsubscript{2}O, 0.47 g NaCl, 0.57 g KCl, 0.04 CaCl\textsubscript{2}, 0.12 g MgSO\textsubscript{4}.7H\textsubscript{2}O per 1000 ml distilled water. After gassing CO\textsubscript{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 $5 \times 10^9$ cfu/g as used by Lila *et al.* (2004).

**Fermentation Characteristics**
The pH value, concentrations of NH\textsubscript{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 substrate. 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 $5 \times 10^9$ 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. cerevisiae* 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\textsubscript{3}-N. Concentration of NH\textsubscript{3}-N in substrate concentrate without
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 NH3-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 NH3-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. Furthermore, lactic acid to be converted by lactic acid utilizing bacteria such as Megasphaera elsdonii 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 CH4 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 CH4 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 CH4 production. The decrease CH4 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 pcentrated increased
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 CH₄ production compared to other probiotic. Decreasing CH₄ from ruminants may have positive impact for the environmental sustainability.

### ACKNOWLEDGMENT

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