

PJN

ISSN 1680-5194

PAKISTAN JOURNAL OF
NUTRITION

ANSI*net*

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***In vitro* Nutrient Digestibility and Fermentation Characteristics of King Grass Combined with Concentrate-Containing Mixed Microbes**

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Abstract: The main feed for ruminants is forage, which is composed of polysaccharides. Feed digestion in the rumen is mediated by microbes, thus the type and makeup of rumen microbes are an important factor that affects nutrient digestibility. This study aimed to evaluate the *in vitro* nutrient digestibility and fermentation characteristics of king grass combined with a concentrate that contained mixed microbes. *Lactobacillus plantarum*, *Saccharomyces cerevisiae* and two strains of cellulolytic bacteria (i.e., *Acinetobacter baumannii* and *Pseudomonas aeruginosa*) were added to the concentrate. Cellulolytic bacteria were isolated from waste from either rice straw or palm oil seeds. The concentrate was mainly composed of agricultural and food industry wastes, such as cassava waste, tofu waste and rice bran. The following four concentrates were made: A, concentrate without microbe; B, concentrate containing *L. plantarum* and *S. cerevisiae*; C, concentrate containing *L. plantarum*, *S. cerevisiae* and *P. aeruginosa* and D, concentrate containing *L. plantarum*, *S. cerevisiae* and *A. baumannii*. Bacteria and yeast were added to the concentrate at 10^6 - 10^7 cfu/g. *In vitro* nutrient digestibility assays were conducted using 250 mg substrate composed of king grass and concentrate (70: 30, DM). Our findings revealed that concentrate contained 7.2×10^6 cfu/g *L. plantarum*, 3×10^8 cfu/g *S. cerevisiae* and 8.6×10^7 cfu/g *A. baumannii* and *P. aeruginosa*. The OM digestibility was greater ($p < 0.01$) for the grass substrate with concentrate containing *L. plantarum*, *S. cerevisiae* and cellulolytic bacteria than for the concentrate that only contained *L. plantarum* and *S. cerevisiae*. Moreover, NDF digestibility was greater ($p < 0.01$) for the grass substrate combined with concentrate that contained mixed microbes compared with the concentrate without microbes. The addition of cellulolytic bacteria increased NH_3 -N and acetic acid concentrations ($p < 0.05$). We concluded that the addition of mixed microbes to the concentrate improved fermentation activity and the digestibility of nutrients *in vitro*.

Key words: By-products, concentrate, digestibility, rumen, cellulolytic

INTRODUCTION

There is growing interest in studying the potential uses of natural products as feed additives instead of chemical compounds, such as ionophores or antibiotics, as manipulators of rumen fermentation to improve fermentation activity. The usage of growth-promoting antibiotics in animal feed is banned in Europe because of potential risks that include the spread of antibiotic resistance genes or contamination of milk or meat with antibiotic residues (Hong *et al.*, 2005).

The main feed for ruminants is forage, which is composed of polysaccharides. Feed digestion in the rumen is carried out by microbes, thus the type and population of microbes are important factors that affect the digestibility of nutrients. Recently, probiotics have been frequently evaluated for use as replacements for antibiotics. Probiotics are live microbial feed supplements that may beneficially affect the host animal upon ingestion by improving the balance of intestinal microbes (Fuller, 1989). Seo *et al.* (2010) reported that micro-organisms, such as *Lactobacillus*, *Streptococcus* and *Enterococcus*, are

commonly used as probiotics for ruminants. Furthermore, *Saccharomyces cerevisiae* and *Aspergillus oryzae* are two primary fungal direct-fed micro-organisms (DFMs) that have been used as diet supplements in ruminants. Seo *et al.* (2010) also found that propionibacteria ferment lactic acid to yield propionic acid. Because propionic acid is the major precursor for gluconeogenesis, increasing propionic acid production in the rumen increases hepatic glucose production. Additionally, increased amounts of propionic acid may reduce the amount of hydrogen that is available for CH_4 production in the rumen. Newbold (1995) and Lila *et al.* (2004) found that the addition of *S. cerevisiae* to the ruminant microflora could improve animal production by promoting bacterial viability, reducing the amount of lactate and slightly reducing levels of CH_4 and hydrogen. In another study, Santoso *et al.* (2014) concluded that the addition of *L. plantarum*, *L. acidophilus* and *S. cerevisiae* in feed concentrate increased the amount of propionic acid, promoted *in vitro* nutrient digestibility and reduced *in vitro* CH_4 production. In previous *in vivo* studies, most of researchers directly fed probiotics to animals. However,

this method was less efficient when applied to a large number of animals. Therefore, this present study aimed to evaluate the *in vitro* nutrient digestibility and fermentation characteristics of king grass combined with a concentrate that contained a mixture of microbes.

MATERIALS AND METHODS

Concentrate preparation and treatments: Rice bran, tofu waste and cassava waste were obtained from small-scale food industry located in the Manokwari and Prati Districts, Manokwari Regency, Indonesia. Tofu and cassava waste were dried in an oven at 60°C for at least 48 h and were ground to pass through a 1 mm sieve in a Wiley mill. *Lactobacillus plantarum* was isolated from *Pennisetum purpureophoides* that had been used in a previous study by Santoso *et al.* (2012). *L. plantarum* were cultured using MRS broth at 30°C for 48 h (Santoso *et al.*, 2013a), while *S. cerevisiae* was cultured using malt extract broth at 30°C for 48 h (Newbold, 1995). Cellulolytic bacteria were isolated from rice straw or palm oil seed waste and were cultured using CMC. Solid concentrate materials were manually mixed by hand and then sprayed on top with a culture of LAB, yeast and cellulolytic bacteria at 10^6 - 10^7 cfu/g. The four following concentrates were generated: (A) concentrate without microbes; (B) concentrate containing *L. plantarum* and *S. cerevisiae*; (C) concentrate containing *L. plantarum*, *S. cerevisiae* and *P. aeruginosa* and (D) concentrate containing *L. plantarum*, *S. cerevisiae* and *A. baumannii* (Table 1). A total of 5 treatments were used in this study as follows: (G) grass, (G+A) grass+concentrate A, (G+B) grass+concentrate B, (G+C) grass+concentrate C and (G+D) grass+concentrate D.

Donor animals: A total of two ruminally fistulated Ongole crossbreed cattle were used as rumen liquor donors. Animals were fed 6.8 kg DM king grass to meet their maintenance requirements and were adapted for 3 weeks prior to rumen liquor collection. 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 CH₄ measurements:** *In vitro* gas production was determined according to the method of Menke and Steingass (1988), as previously described by Hariadi and Santoso (2010) and Santoso *et al.* (2013b). Briefly, oven-dried samples of $\sim 300 \pm 5$ mg were weighed into 100 ml glass syringes (Model Fortune, Habertechnik, Germany) with pistons that were lubricated with Vaseline. Additionally, three parallel syringes that contained mixtures of rumen liquor-buffer without substrate served as blanks. The buffer solution contained carbonate buffer, macromineral solution and micromineral solution. Syringes were pre-warmed at 39°C overnight, before the addition of 30 ± 1.0 ml of rumen liquor-buffer

mixtures into each syringe. Each syringe was incubated in a water bath at 39°C for 48 h and were gently shaken every 8 h. The volume of gas that was released from each syringe was recorded before incubation (0 h) and at 1, 2, 4, 6, 8, 12, 24, 36 and 48 h of incubation.

To facilitate CH₄ measurements, glass syringes were fitted with an extra outlet containing a gas-tight septum for gas sampling, as described by Hariadi and Santoso (2010) and Santoso *et al.* (2013b). At 24 and 48 h of incubation, 100 ml gas was sampled from the headspace of an airtight syringe. Methane was determined based on the injection of 100 μ l gas into a gas chromatograph (GC model 263-50, Hitachi Ltd., Ibaraki, Japan).

At the end of the incubation period, ~ 10 ml of syringe contents were sampled. The pH of the medium was immediately recorded using a pH digital meter (Hanna, Hi 8520, Ronchi di Villafranca, Italy). Subsequently, 0.2 ml sub-samples were pipetted into 1.5 ml micro centrifuge tubes containing 1 ml of 25 g/100 ml (w/v) metaphosphoric acid and centrifuged at $9000 \times g$ for 10 min for volatile fatty acids (VFA) determination. For NH₃-N analysis, an additional 2 ml of sub-samples were added to 2 ml of 20 g/l (w/v) NaCl.

***In vitro* nutrient digestibility:** Measurements of DM, organic matter (OM) and neutral detergent fiber (NDF) digestibility were conducted using the *in vitro* procedure of Tilley and Terry (1963). A total of 25 ml rumen liquor-buffer mixtures in a 1:4 (v/v) ratio were dispensed in 100 ml glass tubes that contained 250 mg dry sample, which consisted of grass and concentrate (70:30, DM). Triplicate blank (with no feed sample) and standard (Pangola grass) samples were also included in each run. Rumen liquor was collected in the morning prior to feeding and strained through four layers of cheesecloth into a pre-warmed thermos flask. The buffer solution contained 9.8 g NaHCO₃, 9.3 g NaHPO₄·12H₂O, 0.47 g NaCl, 0.57 g KCl, 0.04 CaCl₂ and 0.12 g MgSO₄·7H₂O per 1000 ml distilled water. After gassing CO₂ into the tube, corks were tightly placed over the tubes, which were incubated in a water bath at 39°C for 48 h. After 48 h of microbial incubation, samples were incubated at 39°C for 48 h with acid-pepsin. Thereafter, 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) values. The remaining residue was ashed at 550°C to determine the *in vitro* OM digestibility (IVOMD).

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

Statistical analysis: Data were analyzed following a completely randomized design with the GLM procedure of the SAS software package (SAS Institute Inc., Cary, NC). Duncan's multiple range test was used to identify significant differences between means.

RESULTS AND DISCUSSION

Chemical composition of feeds: The chemical compositions of the king grass and concentrate that we used in this study are presented in Table 1. The grass had 11.4% of CP, which is above the threshold value of 7%. Minson and Milford (1966) reported that digestibility declines when animals are fed herbage with a CP content below 7% because microbial activity in the rumen becomes depressed by the lack of nitrogen. Dry matter content of the concentrates showed a trend to be reduced by the addition of microbes to the culture because the added microbes were in a liquid form. Organic matter contents in all of the concentrates used in this experiment were similar, which varied from 93.8 to 94.7%. The addition of 1% urea in concentrates increased the CP content to 15%. The NDF and ADF contents in the concentrates were similar, as they varied from 59.4 to 61.9% and 16.0 to 17.8%, respectively. The population of LAB, yeast and cellulolytic bacteria in the concentrate ranged from 10^6 to 10^8 cfu/g, which was lower than concentration of probiotic of 5×10^9 cfu/g, which was used by Lila *et al.* (2004).

Fermentation characteristics: The pH value as well as the concentrations of $\text{NH}_3\text{-N}$ and VFA are presented in Table 2. The pH values in the substrates that consisted of grass and concentrate containing *L. plantarum*, *S. cerevisiae* and *P. aeruginosa* (G+C) and concentrate containing *L. plantarum*, *S. cerevisiae* and *A. baumannii* (G+D) were lower ($p < 0.01$) than the control substrate. The lower pH value could be a consequence of the higher total VFA concentration, which was a result of cellulolytic bacteria activity that suppressed the pH value. However, the pH values in all treatments ranged from 6.82 to 6.91, which were in the optimal pH range of 6.7 ± 0.5 required to maintain normal cellulolysis (Van Soest, 1994) and were above 6.0, which is the threshold for microbial protein synthesis (Russell *et al.*, 1992). In a previous *in vitro* study, Lila *et al.* (2004) noted that the addition of probiotic that contained 5×10^9 *S. cerevisiae* cells/g had no significant effect on the pH value. By contrast, Mwenya *et al.* (2004) reported that supplementation of 4 g/d of *S. cerevisiae* significantly increased sheep rumen pH values. The ammonia concentration represents a balance between degradation of feed protein and the uptake of ammonia for the synthesis of microbial proteins. During fermentation in the rumen, feed protein can be degraded by microbes to $\text{NH}_3\text{-N}$. Concentrations of $\text{NH}_3\text{-N}$ in grass combined with concentrate without microbe (G+A) were

similar to those of a concentrate that contained *L. plantarum* and *S. cerevisiae* (G+B). Our present findings are supported by Lila *et al.* (2004) and Mwenya *et al.* (2004) who found that the concentration of ammonia N did not change after the addition of probiotic, i.e., *S. cerevisiae* or LAB. However, a higher $\text{NH}_3\text{-N}$ concentration was observed in substrate grass combined with concentrate containing cellulolytic bacteria (G+C and G+B) compared with grass alone (G) or a combination of grass and concentrate without microbes (G+A). The concentration of $\text{NH}_3\text{-N}$ in this present study ranged from 25.9 to 29.6 mg/100 ml, which was above the threshold value for maximum fiber digestion that was recommended by Abdulrazak *et al.* (2000).

The proportions of acetic acid (C2) and total VFA concentration were higher ($p < 0.05$) in concentrate in which cellulolytic bacteria had been added (G+C and G+D). This finding is in accord with those of Lila *et al.* (2004) who found that the addition of *S. cerevisiae* increased the proportion of propionic acid and total VFA. Additionally, Mwenya *et al.* (2004) reported that proportion propionic acid and the concentration of total VFA in the rumen were similar between sheep fed either LAB or *S. saccharomyces* and control sheep. The increased proportion of propionic acid could be a consequence of increased lactic acid production by LAB. Furthermore, lactic acid can be converted by lactic acid-utilizing bacteria, such as *Megasphaera elsdenii*, to propionic acid. Increasing the proportion of propionic acid (C3), however, reduced the C2/C3 ratio in treatment groups G+B, G+C and G+D. Increasing the total VFA in concentrate by adding probiotic is supported by IVDMD data for treatments G+B, G+C and G+D.

Total gas production during a 48 h incubation was higher ($p < 0.05$) in substrate grass combined with concentrate (G+B, G+C, G+D and G+E) compared with grass alone (G). Gas production can be used as an indicator of feed degradation in the rumen. Beuving and Spoelstra (1992) concluded that there was a significant correlation between organic matter digestibility, VFA concentrations and gas production. However, our measurements of total gas production in this study are supported by IVDMD values, as presented in Table 3.

Nutrient digestibility: Table 3 shows the *in vitro* DM, OM and NDF digestibility of grass and concentrate-containing mixed microbes. IVDMD in the substrate that consisted of grass and concentrate was higher ($p < 0.01$) compared with that of control feed. The addition of LAB and yeast in concentrate (G+B) increased the IVDMD value by 11% when compared with concentrate without mixed microbe (G+A). A combination of LAB, yeast and cellulolytic bacteria in concentrates (G+B, G+C and G+D) enhanced ($p < 0.01$) IVNDFD by an average of 21.45% compared with concentrate without mixed microbes. This finding was comparable with those of Lila *et al.* (2004) who found that

Table 1: Ingredients of the concentrate and chemical composition of the grass and concentrates (%)

	Concentrates				
	G	A	B	C	D
Ingredients					
Cassava waste	-	42	38	36	36
Tofu waste	-	25	25	25	25
Rice bran	-	30	30	30	30
Salt	-	2	2	2	2
Urea	-	1	1	1	1
<i>L. plantarum</i>	-	-	2	2	2
<i>S. cerevisiae</i>	-	-	2	2	2
<i>P. aeruginosa</i>	-	-	-	2	-
<i>A. baumannii</i>	-	-	-	-	2
Chemical composition					
Dry matter	84.3	89.9	86.1	83.3	83.3
Organic matter	91.8	94.3	93.8	94.7	94.1
Crude protein	11.4	15.0	13.5	14.0	13.9
NDF	79.3	61.7	61.4	61.9	59.4
ADF	38.9	16.2	16.0	17.8	16.5
Hemicellulose	39.1	45.5	45.4	44.1	42.9
<i>L. plantarum</i> (cfu/g)	-	-	7.2×10 ⁶	7.4 × 10 ⁶	7.0 × 10 ⁶
<i>S. cerevisiae</i> (cfu/g)	-	-	3.0×10 ⁸	3.1 × 10 ⁸	3.0 × 10 ⁸
<i>P. aeruginosa</i> (cfu/g)	-	-	-	8.6 × 10 ⁷	-
<i>A. baumannii</i> (cfu/g)	-	-	-	-	1.9 × 10 ⁸

G: Grass, A: Concentrate without microbe, B: Concentrate containing *L. plantarum* and *S. cerevisiae*, C: Concentrate containing *L. plantarum*, *S. cerevisiae* and *P. aeruginosa*, D: concentrate containing *L. plantarum*, *S. cerevisiae* and *A. baumannii*

Table 2: *In vitro* fermentation characteristics in the supernatant after 48 h of incubation

	Treatments					SEM	p-value
	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)	25.9 ^b	26.3 ^b	28.5 ^{ab}	29.6 ^a	29.5 ^a	0.82	0.02
C2 (mol/100 mol)	72.7 ^b	75.3 ^{ab}	80.8 ^{ab}	86.0 ^a	86.5 ^a	3.37	0.05
C3 (mol/100 mol)	29.3	32.0	30.7	32.6	36.4	1.83	0.14
C4 (mol/100 mol)	8.3	9.7	10.7	11.6	9.4	1.15	0.39
C2/C3	2.5	2.4	2.6	2.4	2.6	0.17	0.74
Total VFA (mM)	110.2 ^{ab}	117.1 ^{bc}	122.2 ^{ab}	134.0 ^a	128.6 ^{ab}	4.55	0.03
Total Gas 48 h (ml)	38.3 ^b	42.0 ^a	42.3 ^a	42.7 ^a	42.7 ^a	1.00	0.05

G: Grass, A: Concentrate without microbe, B: Concentrate containing *L. plantarum* and *S. cerevisiae*, C: Concentrate containing *L. plantarum*, *S. cerevisiae* and *P. aeruginosa*, D: concentrate containing *L. plantarum*, *S. cerevisiae* and *A. baumannii*

Mean values with different superscript letters within the same row are significantly different (p<0.05)

Table 3: *In vitro* digestibility (%) of dry matter, organic matter and neutral detergent fiber of feed

	Treatments					SEM	p-value
	G	G+A	G+B	G+C	G+D		
IVDMD	39.2 ^b	44.1 ^a	44.5 ^a	45.4 ^a	46.1 ^a	0.82	<0.01
IVOMD	49.4 ^c	53.8 ^{bc}	59.7 ^a	57.7 ^{ab}	58.2 ^{ab}	1.17	<0.01
IVNDFD	25.5 ^c	35.4 ^b	43.8 ^a	43.4 ^a	45.1 ^a	1.47	<0.01

G: Grass, A: Concentrate without microbe, B: Concentrate containing *L. plantarum* and *S. cerevisiae*, C: Concentrate containing *L. plantarum*, *S. cerevisiae* and *P. aeruginosa*, D: concentrate containing *L. plantarum*, *S. cerevisiae* and *A. baumannii*

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

the addition of *S. cerevisiae* increased *in vitro* dry matter degradability. In another study, Krisnan *et al.* (2009) concluded that the addition of probiotic collected from buffalo rumen in catalytic supplement increased the NDF digestibility in sheep. Chaucheyras *et al.* (1995) noted that *S. cerevisiae* showed an ability to provide growth factors, such as organic acids or vitamins, thereby stimulating ruminal populations of cellulolytic bacteria.

Conclusion: In this present study, the concentrate containing LAB, yeast and cellulolytic bacteria was effective in modifying ruminal fermentation patterns by increasing NH₃-N and total VFA concentrations, along with the proportion of acetic acid. The addition of LAB, yeast and cellulolytic bacteria to the concentrate increased NDF digestibility *in vitro* when compared with the concentrate without added microbes.

ACKNOWLEDGMENTS

This study was funded by the Directorate General of Higher Education of the Indonesian Ministry of National Education via the "Hibah Kompetensi" scheme (Contract No. 45./UN42.2.1/PL/III/2014).

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