

MICROBIOLOGICAL CHARACTERISTIC AND FERMENTABILITY OF KING GRASS (*Pennisetum hybrid*) SILAGE TREATED BY LACTIC ACID BACTERIA-YEAST INOCULANTS CONSORTIUM COMBINED WITH RICE BRAN ADDITION

A. Sofyan¹, L.M. Yusiati², Y. Widyastuti³ and R. Utomo²

¹Research Unit for Processes Development and Chemical Engineering (BPPTK),
Indonesian Institute of Sciences (LIPI),
Jl. Jogja-Wonosari Km. 31, Gading, Playen, Gunungkidul, DIY 55861 - Indonesia

²Faculty of Animal Sciences, Gadjah Mada University,
Jl. Fauna No.3, Bulaksumur, Yogyakarta 55281 - Indonesia

³Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI)
Jl. Raya Bogor Km. 46, Cibinong, Jawa Barat 16911 - Indonesia
Corresponding E-mail: sofyan_lipi@yahoo.co.id

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ABSTRAK

Penelitian ini dilakukan untuk mengevaluasi pengaruh konsorsium inokulum yang terdiri dari *Lactobacillus plantarum* (Lp) yang dikombinasikan dengan *Saccharomyces cerevisiae* (Sc) yang dikombinasikan dengan penambahan dedak padi terhadap karakteristik mikrobiologi dan fermentabilitas silase rumput raja (*Pennisetum hybrid*). Efektivitas penambahan inokulum (kontrol, Lp, Lp+Sc) dan dedak padi (0, 5, 10%) disusun dalam Rancangan Acak Lengkap pola Faktorial (3x3). Peubah yang diamati terdiri dari jumlah koloni mikroba (bakteri asam laktat, yeast dan clostridia), produksi gas, asam lemak volatil (VFA) dan amonia (NH₃). Hasil penelitian menunjukkan bahwa jumlah koloni clostridia pada silase yang diberi perlakuan Lp dan Lp+ Sc berkisar 37,5% dan 68,7% lebih rendah dari kontrol. Penambahan inokulum dan dedak padi nyata (P<0,05) meningkatkan fermentabilitas, namun tidak berpengaruh nyata (P>0,05) terhadap produksi VFA dan NH₃. Produksi gas dari fraksi silase terfermentasi yang diberi perlakuan inokulum Lp (49,5 ml) dan Lp+Sc (47,9 ml) lebih tinggi dibandingkan dengan silase tanpa perlakuan (46,6 ml). Dapat disimpulkan bahwa penggunaan konsorsium inokulum *L. plantarum* dan *S. cerevisiae* dengan penambahan dedak padi meningkatkan fermentabilitas dan menurunkan koloni clostridia pada silase rumput raja.

Kata kunci: clostridia, fermentabilitas, L. plantarum, S. cerevisiae, silase

ABSTRACT

This research was conducted to evaluate the effect of inoculants consortium consisted of *Lactobacillus plantarum* (Lp) and *Saccharomyces cerevisiae* (Sc) and combined with rice bran addition on microbiological characteristic and fermentability of king grass (*Pennisetum hybrid*) silage. Effectivity of treatments was assessed by addition of inoculants (control, Lp, Lp+Sc) and level of rice bran (0, 5 and 10%) which were arranged on Completely Randomized Design with Treatments Factors (3x3). The variables measured were total colonies of microbes (lactic acid bacteria, yeast and clostridia), total gas production, volatile fatty acids (VFA) and ammonia (NH₃). Results showed that clostridial colonies counted on silage treated by Lp and Lp+Sc around 37.5% and 68.7% in which lower than control. Addition of inoculants and rice bran increased fermentability of silage significantly (P<0.05), however treatment had no affected (P>0.05) on production of VFA and NH₃. Gas production from silage fermentable fraction with inoculation of Lp (49.5 ml) and Lp+Sc (47.9 ml) higher than untreated silage (46.6 ml). It was concluded that the use of inoculants consortium consisted of *L. plantarum* and *S. cerevisiae* with rice bran addition improved fermentability and reduced clostridial colony in king grass silage.

Keywords: clostridia, fermentability, L. plantarum, S. cerevisiae, silage

INTRODUCTION

Livestock development is significant contribution for supplying foodstuff from animal product. However, limitation of forage supply implies the low productivity of ruminant. Preservation of forage using ensilage method is one attempt to maintain the forage stock. In contrast, silage technology has not been widely applied by farmers because the lacking of knowledge in silage technology transfer (Mannetje, 2000) and high level of spoilage due to failure to achieve anaerobic conditions.

The process of silage to produce lactic acid can be constrained by the presence of oxygen, so that the density and anaerobic conditions could not be maximized in silo. This because of the anaerobic conditions are not achieved, a high level of damage at silage occurred by indication of increasing clostridia colonies and butyric acid content in silage (Vissers *et al.*, 2007).

Efforts to improve the anaerobic conditions necessary for quality silage can be maintained. One effort that might be done to improve the anaerobic conditions in the silage by addition of *Saccharomyces cerevisiae*. According to Chaucheyras *et al.* (1995), *S. cerevisiae* can reduce aerobic conditions because its activities require oxygen. The presence of *S. cerevisiae* can support the growth of lactic acid bacteria (LAB) (Gobbetti, 1998).

In addition, *S. cerevisiae* on silage has the potential to support the growth of LAB which has been used as silage inoculant. Therefore, research on the use of *S. cerevisiae* and its interaction with LAB as silage inoculant needs to be done. The objective of this study was to evaluate effect of inoculants consortium consisted of *L. plantarum* and *S. cerevisiae* (Sc) and combination of addition of soluble carbohydrates (rice bran) on microbiological characteristics and fermentability of king grass silage.

MATERIALS AND METHODS

Forage and Inoculant Preparation

King grass (*P. hybrid*) for silage making was harvested at the age of 60 days and obtained from the field laboratory at UPT. BPPTK- LIPI Yogyakarta. Inoculants consisted of lactic acid bacteria (*L. plantarum*) and yeast (*S. cerevisiae*) which were isolated and identified in previous study (Sofyan *et al.*, 2011). Inoculants had been

prepared by pre-culturing of *L. plantarum* on MRSB (*deMann Rogossa Sharpe* Broth) and yeast on MEB (Malt Extract Broth) medium which was incubated during 24 hours at 37°C and 30°C respectively for LAB and yeast. The number of colonies of *L. plantarum* was 10⁸ cfu/ml and *S. cerevisiae* 10⁷ cfu / ml in medium.

Treatment

The effectiveness of inoculant on silage quality was evaluated by adding inoculants and rice bran as a source of soluble carbohydrate (water soluble carbohydrate / WSC) in king grass silage. The treatments were arranged on completely randomized design with factorial treatment were inoculants (control, Lp, Lp+Sc) and addition of rice bran (0, 5, and 10%) with 3 replication as mentioned on Table 1.

The silage making process consisted of 3 stages, were: 1) preparation of materials, 2) mixing processes referred to the formula and treatment, 3) packaging and incubation. Preparation of feed material was conducted by chopping the grass with shredded size 1-3 cm. Previously, grass wilted during 24 hours in order to increase dry matter content.

Inoculant 1% (v/w) added into silage and addition of water in order to adjust moisture content up to 75%. After all the ingredients mixed homogeneous, packed in plastic containers (5 kg/pack) and incubated for 21 days. Samples for in vitro digestibility analysis were prepared by freeze-drying method using a freeze dryer Leybold-Heraeus GT Lyovac type-2 (Peterswan Ltd., Edinburgh) at -20°C for 20 hours. Then, samples were sieved by 1.0 mm screening.

Microbiological Characteristics Evaluation

Microbiological characteristics assessed by counting microbes (LAB, yeasts and clostridia) colonies. Each of microbes was grown on selective media MRSA, MEA and RCA (Reinforced Clostridial Agar) respectively for LAB, yeasts and clostridia. Silage sample was prepared for enumeration of microbial colonies which was conducted by taking 50 g samples from each replication, added by 50 ml of sterile distilled water and stirred for 5 minutes in aseptic condition. Amount of 3 ml supernatant and homogenized in aseptic conditions. Supernatant obtained at each treatment was grown on selective

Table 1. Inoculant and Rice Bran Addition into King Grass Silage

Inoculants	Level of Rice Bran		
	0%	5%	10%
Control (A)	A0	A5	A10
Lp (B)	B0	B5	B10
Lp+Sc (C)	C0	C5	C10

Lp (*L. plantarum*), Sc (*S. cerevisiae*)

medium with serial dilution at 10^2 , 10^3 , 10^4 , 10^5 and 10^6 of LAB (MRSA) and Clostridia (RCA) in which incubated at 37 °C while yeasts (MEA) incubated at 30°C with the same incubation time (24 hours).

Fermentability Evaluation

Evaluation of silage fermentability, VFA (Volatile Fatty Acids) and NH₃ (ammonia) production measured by the total gas production. Silage samples that had been freeze-dried, ground with a mortar and sieved by a filter with a hole size of 1 mm. Measurement of gas production refers to Menke *et al.* (1979) and Blümmel *et al.* (1997) modified by Jayanegara *et al.* (2009a). Silage samples 380 mg (dry matter 86.4%) was placed into the syringe to the pre-incubation for 24 hours at a temperature of 39°C.

Rumen fluid (10 ml) and buffer solution (20 ml) inserted into syringe with saturated CO₂. Composition of buffer solution per 100 ml rumen fluid consisted of macrominerals (23.7 ml), micro-minerals (0.012 ml), bicarbonate buffer solution (23.7 ml), resazurin 4% (0.122 ml), reducing solution (4.96 ml) and distilled water (47.5 ml) (Menke *et al.*, 1979). Rumen fluid was taken from fistulated beef cattle (Ongole crossbred) in which was conditioned by feeding standard (feed composition consisted of 60% forage and 40% concentrate).

Evaluation of fermentability and in vitro digestibility were arranged in factorial completely randomized design with 2 factors of treatments. Each treatment consisted of 3 replications with 2 sub samples. Pangola grass (*Digitaria decumbens*) was used as standard sample and each syringes containing silage, standard samples and blank were randomly allocated in the incubator. Incubation was carried out for 48 hours and gas

production was observed at 0, 1, 2, 4, 6, 8, 12, 18, 24, 36 and 48 hours after incubation.

Gas production kinetics was calculated based on the exponential equation according to Ørskov and McDonald (1979). The estimated value of a, b, c were calculated by a fitting curve method using Neway Software program (Rowett Research Institute, Aberdeen, UK) that installed at Microsoft Office Excel 2007[®] and developed by Chen (1997).

Production of VFA and NH₃ were measured after sample was incubated in a syringe containing rumen fluid-buffer. Analysis of VFA was performed by gas chromatography method as followed by Frigens *et al.* (1998) and NH₃ analysis using spectrophotometric method (Broderick and Kang, 1980). Sample preparation was conducted by taking samples of rumen fluid-buffer after 48 hours incubation which were centrifuged at 3000 rpm for 15 minutes followed to centrifugation at 10,000 rpm for 10 minutes. A total of 0.2 ml supernatant was added into a microtube and added 1 ml meta-phosphate acid 25% (w/v). Samples of 1 µl injected into the packed column type GP10% SP-1200/1% H₃PO₄ on 80/100 Chromosorb WAW (Supelco, Bellefonte, PA) on GC (Shimadzu 8-A) and equipped with FID (Flame Ionization Detector).

Sample preparation for NH₃ analysis was performed by taking 0.4 ml of rumen fluid-buffer after 48 h incubation, added 0.2 ml solution A (10% sodium tungstate) and 0.2 ml solution B (H₂SO₄ 1 N), then centrifuged at 3000 rpm for 15 minutes and followed centrifugation at 10,000 xg for 10 minutes. Amount of 10 µl of supernatant was diluted with 10 µl distilled water, added 2.5 ml of solution C (phenol solution) and 2.5 ml of solution D (sodium hypo-chloride 5%). Mixture solution was heated at 40°C for 30 minutes and then read the sample absorbance with the spectrophotometer in the wavelength (λ) 630 nm.

Data Analysis

Silage microbial colonies characteristic data were statistically analyzed descriptively. Data of gas production, VFA and NH₃ were analyzed with analysis of variance (ANOVA) and if among the treatments showed significant differences (P <0.05) followed by orthogonal contrast test

(Gomez and Gomez, 1984).

RESULTS AND DISCUSSION

Microbiological Characteristics of Silage

Microbiological characteristics of silage which includes the number of colonies of lactic acid bacteria (LAB), yeasts, and Clostridia are shown in Table 2. In general, the number of colonies (colony forming unit/cfu) of LAB grown on MRSA at 6.5×10^6 to 3.7×10^7 cfu /g, while the number of yeast colonies were counted MEA medium in the range 2.5 to 6.5×10^6 cfu/g, where the number of colonies of LAB and yeast does not show difference between treatments (Table 2).

Effect of inoculant on the number of LAB colonies was significant influenced by the type isolate. Addition of LAB inoculant (*L. species casei*) had no effect on the number of LAB and yeast colonies in silage, while the inoculant of *L. buchneri* increased LAB colonies 50.7% and reduce yeast colonies up to 65.6% compared with controls in which the population of LAB and yeasts on silage respectively 5.2×10^5 cfu /g and

4.2×10^5 cfu /g (Nishino *et al.*, 2004).

Referred to Filya (2003), mentioned that the number of colonies of LAB, yeasts and fungi in silage influenced by the type of bacterial inoculant, length of incubation and type of materials used in silage making. A longer time of incubation affected increasing the BAL and declining yeasts and fungal colonies in silage. This is related to the accumulation of lactic acid which increase in length of incubation. Furthermore, it was followed by decreasing pH which implied inhibiting growth of yeasts and fungi.

The number of clostridia in silage was grown on RCA medium showed that clostridial colonies was ranging from 5.3×10^6 cfu /g on control, it colonies decreased into 3.0×10^5 cfu/g and 1.8×10^5 cfu/g were found at silage treated by *L. plantarum* and combination *L. Plantarum* + *S.cerevisiae*, respectively.

The highest decreasing number of clostridial colonies were found at silage treated LAB+yeast inoculant consortium combined with rice bran addition. This indicated that *S. cerevisiae* in silage had contributing in clostridial inhibition. Furthermore, effect of adding rice bran in a silage inoculant had enhancing production of lactic acid. The presence of lactic acid in silage had an impact in declining clostridial colonies. Due to clostridia growth would be inhibited by decreasing pH (pH approximately 4) in which in lower pH clostridia difficult to grow. The pH optimum for clostridia growth around at neutral range pH 7.0-7.4 (McDonald *et al.*, 1991).

Lactic acid bacteria had ability to produce bacteriocin which was a secondary metabolite could inhibits pathogenic bacteria such as clostridia. It can synthesize antibacterial compounds 'bacteriocin' which has been shown to inhibit the pathogenic gram-positive bacteria (*Micrococcus luteus*) and gram-negative bacteria (*Pseudomonas aeruginosa*) (Gollop *et al.*, 2005) and *Clostridium tyrobutyricum* (Thuault *et al.*, 1991). Bacteriocin that produced by LAB was widely used as bio-preservative agent to prevent spoilage of food or feed by *Clostridium botulinum* (Montvile and Winskowski, 1997).

Lowering number of clostridial colonies in silage inoculated LAB+yeast consortium indicated that *S. cerevisiae* ability to produce antibacterial substances. *S. cerevisiae* produced oxylipin that inhibit clostridia growth (Strauss *et al.*, 2005). Presence of those active compounds

Table 2. The Number of Microbial Populations in King Grass

Inoculant Treatment	Level of Rice Bran			Average
	0%	5%	10%	
LAB	----- x 10^7 cfu/g -----			
Control	3.7	0.9	0.8	1.8
Lp	1.5	0.7	1.6	1.3
Lp+Sc	2.0	1.1	0.6	1.2
Average	2.4	0.9	1.0	
Yeast	----- x 10^6 cfu/g -----			
Control	4.0	2.5	3.3	3.3
Lp	4.8	3.3	5.0	4.4
Lp+Sc	5.3	6.5	5.3	5.7
Average	4.7	4.1	4.5	
Clostridia	----- x 10^5 cfu/g -----			
Control	11.0	3.0	2.2	5.3
Lp	4.5	1.9	2.8	3.0
Lp+Sc	3.3	1.0	1.2	1.8
Average	6.2	2.0	2.1	

Lp (*L. plantarum*); Sc (*S. cerevisiae*)

Table 3. Total Gas Production (p), Potentially Soluble Fraction (a+b) and Gas Production Rate (c) of King Grass Silage Incubated for 48 Hours

Treatment	Parameter	Rice Bran Level			Average
		0%	5%	10%	
Control	p (ml)	34.18	38.44	36.46	36.36
	a+b (ml)	47.93	47.71	44.22	46.62 ^A
	c (ml/h)	0.027	0.036	0.039	0.034
Lp	p (ml)	34.18	36.80	38.13	36.37
	a+b (ml)	48.91	50.59	41.03	49.51 ^C
	c (ml/h)	0.026	0.029	0.045	0.030
Lp+Sc	p (ml)	33.23	37.71	39.16	36.70
	a+b (ml)	47.44	48.85	47.47	47.92 ^B
	c (ml/h)	0.026	0.033	0.040	0.033
Average	p (ml)	33.86 ^a	37.65 ^b	37.91 ^b	
	a+b (ml)	48.09	49.05	46.90	
	c (ml/h)	0.027 ^a	0.033 ^b	0.038 ^c	

Lp (*L. Plantarum*); Sc (*S. Cerevisie*)

implied to support the role of bacteriocin produced by LAB in inhibiting clostridia. Synergism of LAB and *S. cerevisiae* for inhibiting growth of clostridia in silage was supported clostridial colonies data that silage treated with LAB+yeast which tend to be lower than single inoculant (LAB) or control.

The number of clostridial colonies was lower in the inoculated silage, in which had implications for minimizing the silage deterioration. Clostridia in silage converted protein fraction into ammonia by proteolysis enzymes (McDonald *et al.*, 1991), and capable of converting lactic acid into butyric acid accompanied by the formation of hydrogen and carbon dioxide (Stefanie *et al.*, 2000)

Gas Production Kinetics

Fermentability parameters and digestibility in vitro of silage evaluated by analyzing the kinetics of gas production during incubation, production of VFA and NH₃. Kinetics of gas production and fermentability of silage during incubation of 48 hours which included gas production from potentially soluble fraction (a+b), rate of gas production (c) were shown at Table 3.

The highest gas production (39.2 ml) obtained from silage received inoculant consortium treatment with addition of 10% rice bran, while it was without rice bran addition showed that lowering in gas production (33.2 ml). Inoculant treatment significantly (P<0.05) increased gas production from the total fraction of silage (a + b) with gas production rate was relatively similar (0.029 to 0.034 ml/h). The highest of gas production was found from silage treated by *L. plantarum* (50.0 ml), and followed by *L. plantarum* + *S.cerevisiae* (48.2 ml) which was significantly higher than control (46.9 ml).

Addition of rice bran were significant difference (P<0.05) on increasing rate of gas production during fermentation of silage. Silage added by rice bran (5-10%) had a gas production rate around 0.032 to 0.037 ml/h in which higher than without rice bran. It mean rice bran as a source of readily digestible carbohydrates that supply of nutrients for silage inoculant in optimizing fermentation process. It was indicated by the increased gas production.

Production of Volatile Fatty Acids (VFA) and Ammonia (NH₃)

Besides the *in vitro* digestibility parameters, production of VFA, mainly acetate, propionate and butyrate, and NH₃ were very important in supporting the needs of nutrients for animal. Synchronization of VFA and NH₃ production was significant affect on animal performance. Due to it was a precursor for microbial protein and fat body synthesis of animal. Production of VFA and NH₃ from silage during 48 h incubation is presented in Table 4.

In general, VFA and NH₃ production from silage between treatments was not significantly different ($P < 0.05$). The average production of acetate, propionate and butyrate 105.7, 36.2, and 13.8 mM respectively with the average ratio of acetate (C₂) of propionate (C₃) was 2.9. Ammonia production was generally ranges from 22.2 to 25.1

mM or ranged from 37.7 to 42.7 mg/100 ml (Table 4). There was tendency that the higher the addition of bran to produce NH₃.

Levels of ammonia in silage with 10% rice bran showed that NH₃ production 4% higher than silage treated with 0% and 5% rice bran. This is related to protein content in silage due to the addition of rice bran. Based on the results of chemical analysis, rice bran contains about 13% protein so that addition of 10% rice bran silage resulted in a 1.3% increase protein content. This results supported by Santoso and Hariadi (2009) stated that concentration of ammonia produced was significant influenced by the chemical composition of feedstuffs especially protein content. Ammonia production from straw corn (CP 11.9%) resulted higher than rice straw (CP 6.8%) (23.0 vs 8.9 mg/100 ml).

Volatile fatty acids concentration of rumen fluid produced during the fermentation process is an indicator of energy availability for animal

Table 4. Volatile Fatty Acids (VFA) and Ammonia (NH₃) Production *In vitro* from Silage Incubated for 48 Hours

Treatment	Parameter	Rice Bran Level			Average
		0%	5%	10%	
----- (mM) -----					
Control	Acetate	115.56	113.74	123.12	117.47
	Propionate	37.83	42.87	38.63	39.77
	Butyrate	12.18	13.57	21.46	15.74
	Ammonia	23.95	23.62	22.49	23.35
Lp	Acetate	104.02	112.03	101.03	105.69
	Propionate	40.67	29.82	32.45	34.31
	Butyrate	15.74	12.54	12.11	13.46
	Ammonia	22.36	22.22	25.09	23.22
Lp+Sc	Acetate	110.02	100.67	71.65	94.11
	Propionate	39.53	36.01	27.99	34.51
	Butyrate	12.66	13.90	9.73	12.10
	Ammonia	23.06	23.23	24.55	23.61
Average	Acetate	109.87	108.81	98.60	
	Propionate	39.34	36.23	33.02	
	Butyrate	13.53	13.34	14.43	
	Ammonia	23.12	23.02	24.04	

Lp: *L. plantarum*; Sc: *S. cerevisie*

(Jayanegara *et al.*, 2009b). Its components consisted of acetate, propionate and butyrate absorbed through the rumen wall and used as a source of energy in various organs of cattle through oxidation of tricarboxylic acid cycle (Hungate, 1966). Concentrations of VFA in the rumen was influenced by type of substrate / feed consumed by ruminants. Total VFA in the rumen of cattle consuming grass silage about 108 mM with 74% the proportion of acetate (79.9 mM), propionate 17% (12.6 mM) and butyrate 7% (1.2 mM).

Production of VFA and the proportion of acetate, propionate and butyrate might be changed with supplementation of feed concentrate (McDonald *et al.*, 2002). According to Owens and Goetsch (1988), stated that total VFA in rumen of cattle consumed concentrations higher than cattle consumed hay (150 vs. 100 mM). Further stated that the proportion of acetate: propionate: butyrate in cattle rumen that consumed concentrate was 50:40:10, while the cattle consumed hay was 65:25:10.

CONCLUSION

The use of lactic acid bacteria - yeast consortium inoculant consisted of *L. plantarum* and *S. cerevisiae* in making of grass silage increased *in vitro* digestibility and fermentability, reduce contamination of clostridia in silage without negative effect on production of VFA and NH₃.

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