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Effects of Ensiling, Urea Treatment and Autoclaving on Nutritive Value and *In-vitro* Rumen Fermentation of Rice Straw

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Abstract | The study was conducted to investigate the effects on the in-vitro rumen fermentation characteristics of rice straw of ensiling, urea treatment, and autoclaving . The treatments consisted of untreated rice straw (P1) compared to the ensiling of rice straw using anaerobic conditions for 30 days (P2), treatment with 3% urea solution (P3), autoclaving at 150° C and 1.2 atm for 60 minutes (P4), combination of ensiling with urea (P5), ensiling with autoclaving (P6), and combination ensiling, urea treatment, and autoclaving (P7). None of the treatments showed significant differences in neutral detergent fibre (NDF) or volatile fatty acid (VFA) content, but did produce significant differences ($P < 0.05$) in dry matter (DM), organic matter (OM), acid detergent fibre (ADF), pH, NH_3, gas production, methane gas, in-vitro dry-matter digestibility (IVDMD) and in-vitro organic-matter digestibility (IVOMD). The ensiling process had no effect on protein value, IVOMD, or methane gas production. Gas production from all the treatment samples was low up to 12 h but increased at 24 h and 48 h of in-vitro incubation. Rice straw samples ensiled in combination with urea (P5) and autoclaving (P6) had the highest gas production at 48 h. Methane gas production was higher ($P < 0.05$) in P2, P3, P5, and P6 samples than the rest. Overall, this study concludes that the ensiling process can maintain the quality of rice straw, including in combination with added urea and autoclaving.

Keywords | Rice straw, Ensiling, Urea, Autoclaving, *In-vitro* characteristics

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INTRODUCTION

The utilization of agricultural residues for livestock feed needs to be improved through the development of processing techniques that can increase nutritional value and digestibility. Rice straw is the residue from rice plants produced when harvesting after four months' growth. In Indonesia, rice straw is particularly used by farmers as an alternative feed for livestock such as cattle and sheep. It can be used to substitute for grass, availability of which is increasingly limited today. Indonesia has many rice fields in almost all provinces, and rice is produced throughout the year as the main food of the population. Alam et al. (2016) reported the chemical composition of rice straw on a dry matter basis as 94.6% dry matter, 4.6% crude protein, 14.4% crude ash, 31.9% crude fibre, 73.1% NDF, 41.7% ADF, and 2.4% lignin. There are three main limiting factors in the utilization of rice straw as a feedstuff: a) cell walls are covered with silica crystals which are difficult for enzymes in the rumen to hydrolyse; b) cell walls contain lignin, which forms complex compounds with cellulose. The structure of the cellulose is therefore not amorphous and the glucose molecules are strengthened by hydrogen bonds which are difficult for microbes to digest; and c) it

has a low protein content of about 3–5% (Van Soest, 2006).

Numerous physical, chemical, and biological treatments have been investigated together with other feedstuffs or components to improve the utilization of rice straw by ruminants. Because rice straw is high in lignocellulose and low in insoluble protein, it cannot be given as a single feed to meet all the nutritional requirements of ruminants. Most studies of rice straw processing have looked at combinations of two or three techniques at a time, such as physical and chemical, physical and biological, or physical, chemical, and biological techniques. Sarnklong et al. (2010) describe strategies that can be used to improve rice straw utilization such as the application of physical, chemical, and biological pre-treatments.

Liu et al. (1999) reported that steam treatment in high-pressure vessels at different pressures and for different treatment times increased in-vitro degradation in rumen fluid after 24 hours and increased the rate of degradation, but did not increase the potential for degradation of the fibrous fraction (NDF, ADF, and hemicellulose). Physical treatments of crop residues have received an appreciable amount of research. Jayanegara et al. (2017) reported that urea treatment increased total VFA concentration compared to controls and suggested that combining 1% urea and autoclave treatment for 30 minutes might replace the conventional urea treatment (4 weeks' incubation) used to improve the nutritional value of rice straw.

Techniques of combining chemical (urea) and physical (heating temperature and pressure) processing on rice straw and various agricultural and plantation by-products, such as oil palm by-products, cocoa pods, corn cobs, corn hay, sugarcane dregs, and coffee skins, have shown an increase in in-vitro digestibility while not decreasing the fibre fraction content. The addition of 1% urea is not sufficient to break down the lignocellulose complex; however, ammonia can be absorbed into the cell walls of rice straw and may break down the linkages between lignin and cellulose or hemicellulose (Van Soest, 2006; Sarnklong et al., 2010). However, Jayanegara et al. (2017) reported that this was not the case in their experimental treatment with 1% urea, since the NDF and ADF content of rice straw did not decrease after four weeks' incubation with the urea solution. The addition of 1% urea is not sufficient to break down the lignocellulose complex. Furthermore, they found that urea treatment together with autoclaving for 30 minutes might replace conventional urea treatment to improve the nutritional value of rice straw, but an extension of the incubation period in the autoclave from 30 to 60 min did not further improve the digestibility of the material.

Fermentation is applied to rice straw by storing it in anaerobic conditions, with the aim of maintaining shelf life and reducing fibre fraction through the activity of lactic acid bacteria (LAB) during the ensiling process. Biological treatment with certain species of fungi can be used to metabolize lignocelluloses in feedstuffs to improve their nutritional value by selective delignification. However, it is currently too early to apply this method in developing countries because of practical difficulties, such as a lack of available technology to produce the large quantities of fungi or enzymes required. Given that rice straw is very abundant in Indonesia, it is necessary to store it as silage to extend its period of use for animal feed. This study's objective was to observe the effect on the in-vitro rumen fermentation characteristics of rice straw of combining the ensiling process without additives with added urea and autoclaving.

MATERIALS AND METHODS

Sample preparation and treatment

A sample of rice straw were collected from a paddy rice field in Limapuluh Kota District, West Sumatra. This material was chopped and oven dried at 60°C for 48 h, then ground in a hammer mill and passed through a 1 mm sieve. The material derived was then used in further experimental steps in which it was ensiled combined with urea and processed in an autoclave in the following experimental combinations:

T1 : rice straw (untreated)
T2 : T1 + ensiling
T3 : T1 + urea
T4 : T1 + autoclaving
T5 : T1 + urea + ensiling
T6 : T1 + autoclaving + ensiling
T7 : T1 + urea + autoclaving + ensiling

Samples for ensiling were maintained in anaerobic conditions for 30 days. Autoclaving was used to generate high temperature and pressure, i.e. 150°C and 1.2 atm, for 60 minutes, while samples with urea treatment were prepared by adding 3% urea solution (dry matter basis of rice straw = 1:25 g/mL) and incubated for one hour. Each treatment was replicated three times.

The rice straw samples were then analysed for DM, OM, and CP using the AOAC (2005) proximate standard procedure, and for NDF and ADF using Fibertec™ 2010 (FOSS).

In-vitro rumen fermentation

The treatments were subjected to in-vitro analysis according to the process as described by Theodorou et al. (1994). A 0.5 g sample from each treatment was inserted into a 125 ml serum bottle followed by 100 ml of rumen fluid:buffer mixture (1:2 v/v). Rumen fluid was obtained from two fistulated Peranakan Ongole (PO) grade cattle before morning feeding at the Research Centre for Biotechnology, LIPI Cibinong Bogor. Incubation was conducted in

four replicates and each treatment per run was represented by two fermentation tubes. The buffer solution was prepared by mixing 9.8 g $NaHCO_3$, 4.63 g $Na_2HPO_4.2H_2O$, 0.57 g KCl, 0.47 g NaCl, 0.12 g $MgSO_4.7H_2O$, and 0.053 g $CaCl_2.2H_2O$ with 1000 ml of distilled water (McDougall, 1948). The anaerobic condition of the in-vitro system was maintained by continuous treatment of the incubation medium with CO_2. Serum bottles were sealed with butyl rubber stoppers and aluminium crimp seals shortly before the incubation commenced. The bottles were then placed in a water bath at 39°C for 48 h and gas production from each serum bottle was vented and recorded at 6, 8, 10, 12, 24, and 48 h of incubation. Methane (CH_4) production was measured at 24 and 48 h incubation with a methane analyser (Riken Keiki RX415). The incubation was performed in four runs (replicates) and each treatment per run was represented by four incubation bottles. The pH of the mixed buffer rumen fluids was measured using a pH meter (Jenway Model 3505, UK). Before measurements, the pH of the Cyberscan pH 310 Eutech equipment was calibrated using a pH 7 buffer solution.

The supernatant was taken for pH, total VFA, and NH_3 analysis as described by Jayanegara et al. (2016). The residue was added to 20 ml of 0.2% pepsin-HCl solution, incubated for another 48 h, and filtered through Whatman paper no. 41 under vacuum conditions. It was then oven dried at 130°C for 8 h and then burned in a furnace at 600°C for 3 h to obtain the DM and OM values of the residues. In-vitro dry-matter digestibility (IVDMD) and in-vitro organic-matter digestibility (IVOMD) were determined by subtracting DM and OM amounts in the residue from their initial amounts prior to incubation. Two tubes containing rumen fluid and buffer only but without sample substrate (i.e. blanks) were incubated as described above and served as correction factors for the VFA, NH_3, DM, and OM content of the residuals.

Statistical analysis

Data were analysed by analysis of variance (ANOVA) according to a completely randomized design. Significance of an effect of a particular parameter was assumed at the probability level of $P < 0.05$. Comparison among different treatments was performed by applying Duncan's multiple range test. All the statistical analyses were performed using IBM SPSS software version 22.0 (IBM Corp., Armonk, 2013).

RESULTS AND DISCUSSION

Chemical composition

The various rice straw treatments showed significant differences ($P < 0.05$) in DM, OM, and CP and a tendency to differ for ADF ($P < 0.1$); however, there was no effect on NDF (Table 1). Ensiling, added urea, autoclaving, and combinations of these treatments increased the DM of rice straw compared to the untreated sample. The OM value of rice straw was lower in T6 (78.7%) and T7 (79.3%). Ensiled rice straw showed the same value for CP as untreated rice straw; this result indicates that the ensiling process did not change CP during 30 days' of anaerobic incubation (T2, T6, and T7). The highest CP value (x.x%) was found in the sample with 3% urea solution added (T3), followed by the autoclaved T4 sample (4.8%). The high N content in urea causes an increase in CP content. The addition of 3% urea solution + ensiling (T5) tended to reduce the ADF content in the rice straw. The combination treatment of 3% urea solution + ensiling with high pressure and temperature from autoclaving (T7) also tended to reduce the ADF content of the rice straw (Table 1). Urea treatment was a form of chemical processing (ammoniation) technique in which the compound releases ammonia after being dissolved in water. Ammonia causes a breakdown in the structure of lignocellulose because it can be absorbed into the cell walls of the rice straw and break down the linkages between lignin and cellulose or hemicellulose (Jayanegara et al., 2017; Sarnklong et al., 2010; Van Soest, 2006). However, the combination of urea with high temperature and pressure using autoclaving triggers a faster chemical reaction for ammonia degradation of the lignocellulose complexes in the rice straw. This finding is in accordance with previous research which reported that the addition of 5% urea + fibre cracking technology using high temperatures and pressures resulted in the lowest NDF, ADF, cellulose, and lignin contents in empty fruit bunches of oil palm (Jayanegara et al., 2019a). High temperature and pressure can promote the release of acetyl groups from the fibre structure, leading to an increase in substrate acidity and fibre solubility (Jayanegara et al., 2019a; Thomsen et al., 2015). Short-term ensiling for a few weeks can be considered as being a biological pre-treatment that increases holocellulose convertibility and so effectively enhances biogas production (Ambye-Jensen et al., 2013; Gallegos et al., 2017; Gao et al., 2012).

In-vitro gas production and methane emission

Total gas production during *in-vitro* incubation will indicate the digestibility of the sample substrate. Gas production on all treatments showed significant difference from 6 h to 48 h of incubation (Table 2). Ensiling gas production was 19.1 mL/g at 24 h and 24.6 mL/g at 48 h, but in combined ensiling with urea and autoclaving (T7), gas production decreased by 21% at 24 h and 48 h of in-vitro incubation. However, the T7 treatment resulted in a tremendous amount of gas production from the 2 h to t 48 h. This occurred because ensiling with 3% urea solution in combination with high temperature and pressure caused an increase in the degradation of complex carbohydrates in the rumen, so that gas production was higher than in the

Table 1: Chemical composition of treatments (% dry matter basis)

Treatment	DM	OM	CP	NDF	ADF
T1	95.6^{a}	81.5^{bc}	3.5^{a}	72.2	54.8^{b}
T2	96.4^{d}	82.4^{c}	3.9^{a}	74.4	56.0^{c}
T3	95.7^{ab}	82.7^{c}	6.1^{c}	69.3	52.5^{abc}
T4	95.9^{c}	81.3^{bc}	4.8^{b}	72.7	53.3^{abc}
T5	95.8^{bc}	81.5^{bc}	4.1^{ab}	70.3	49.5^{a}
T6	95.7^{ab}	78.7^{a}	3.6^{a}	71.0	52.6^{abc}
T7	96.0^{c}	79.3^{ab}	3.8^{a}	73.1	51.4^{ab}
SEM	0.60	0.40	0.20	0.66	0.64
P-value	0.00	0.023	0.00	0.479	0.078

Different superscripts in the same column are significantly different at P<0.05 and tend to be different P<0.1.
T1, rice straw (untreated); T2, T1 + ensiling; T3, T1 + urea; T4: T1 + autoclaving; T5, T1 + urea + ensiling; T6, T1 + autoclaving + ensiling; T7, T1 + urea + autoclaving + ensiling; DM, dry matter; OM, organic matter; CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre, SEM, standard error of mean.

Table 2: Gas production of rice straw in various treatments

Treatment	Gas production (mL/g)					
	6 h	8 h	10 h	12 h	24 h	48 h
T1	2.6^{b}	2.5^{b}	2.8^{bc}	0.9^{a}	14.5^{a}	20.0^{a}
T2	0.0^{a}	0.5^{a}	3.1^{c}	0.0^{a}	19.1^{b}	24.6^{d}
T3	2.75^{b}	3.5^{bc}	3.0^{bc}	2.75^{b}	18.1^{b}	23.0^{c}
T4	0.0^{a}	2.4^{b}	2.1^{b}	0.0^{a}	14.3^{a}	21.3^{b}
T5	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	21.8^{c}	26.6^{e}
T6	0.0^{a}	2.8^{bc}	2.1^{b}	2.9^{b}	22.8^{c}	27.5^{e}
T7	4.5^{c}	4.1^{c}	4.4^{d}	4.0^{c}	30.1^{d}	23.8^{cd}
SEM	0.37	0.31	0.26	0.32	0.98	0.50
P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Different superscripts in the same column are significantly different at P<0.05.
T1, rice straw (untreated); T2, T1 + ensiling; T3, T1 + urea; T4: T1 + autoclaving; T5, T1 + urea + ensiling; T6, T1 + autoclaving + ensiling; T7, T1 + urea + autoclaving + ensiling; SEM, standard error of mean.

other treatments. Gas production appears to be related to the chemical composition of the fibre content of feed (Elghandour et al., 2014). According to the results of previous studies, the increase in cell wall content due to increasing the ratio of maize silage was thought to reduce microbial activity, causing gas production to decrease (Elghandour et al., 2015). However, other studies have reported that vapour pressure plus ammonia at 13–15 atm and an incubation period of 5–10 minutes increased total gas production in vitro by 27% compared to controls (Weimer et al., 2003). From Table 3 it can be seen that rice straw treated with autoclaving and ensiling (T6) had higher methane gas production at 24 h and 48 h. In autoclave processed rice straw (150°C, 1.2 atm, 60 minutes) it is possible that microbes in rumen fermentation find it easier to degrade the substrate. Meanwhile, methane gas production was lower in the untreated sample (T1), the autoclaved sample (T4), and the combined ensiling, urea and autoclaving sample (T7), at 6% at 48 h of *in-vitro* incubation. This is because the autoclaving and ensiling treatment may have stretched or damaged the lignocellulose bonds of the rice straw so that rumen microbes could more easily degrade fibre components. This can be seen in the increased gas production and degradability of autoclaved rice straw compared to untreated rice straw. Rumen ammonia concentration increased in the incubation of the untreated rice straw, indicating that urea was converted into ammonia and subsequently dissolved in the rumen. According to McDonald et al. (2010), gas produced from rumen fermentation consist of 40% carbon dioxide (CO_2), 30–40% methane (CH_4), 5% hydrogen, and a small portion of oxygen and nitrogen. Enteric CH_4 formed by fermentation of feed in the gastrointestinal tract of ruminants constitutes a loss of dietary energy to the animal (Astuti et al., 2020). The mechanism of decreasing CF content in the feed by adding urea solution combined with temperature and high pressure causes a decrease in hydrogen production directly through inhibition of methanogen-s (Harahap et al., 2018).

Table 3: Methane gas production of rice straw in various treatments

Treatment	CH_4 (mL)		CH_4 (% gas)	
	24 h	48 h	24 h	48 h
T1	0.5[a]	2.0[a]	1.8[a]	6.0[a]
T2	0.8[b]	2.8[b]	3.5[d]	9.4[b]
T3	1.2[c]	2.9[b]	3.6[c]	8.8[b]
T4	0.4[a]	1.6[a]	2.0[ab]	6.0[a]
T5	0.6[ab]	3.3[b]	2.9[bc]	9.8[b]
T6	1.2[c]	4.3[c]	4.0[d]	11.3[c]
T7	0.4[a]	1.5[a]	2.1[ab]	6.0[a]
SEM	0.07	0.19	0.19	0.41
P-value	<0.001	<0.001	<0.001	<0.001

Different superscripts in the same column are significantly different at P<0.05.
T1, rice straw (untreated); T2, T1 + ensiling; T3, T1 + urea; T4: T1 + autoclaving; T5, T1 + urea + ensiling; T6, T1 + autoclaving + ensiling; T7, T1 + urea + autoclaving + ensiling; SEM, standard error of mean.

Table 4: *In-vitro* ruminal fermentation parameters of rice straw in various treatment

Treatment	pH	VFA (mg/mL)	NH_3 (mg/L)	IVDMD (%)	IVOMD (%)
T1	6.76[ab]	17.9	24.5[ab]	40.2[a]	58.0[b]
T2	6.81[cd]	15.9	38.2[bc]	43.9[b]	53.2[ab]
T3	6.77[ab]	27.3	20.9[a]	45.6[b]	48.9[a]
T4	6.82[c]	33.5	40.8[c]	40.5[a]	52.6[ab]
T5	6.76[ab]	25.8	27.2[abc]	45.3[b]	50.8[ab]
T6	6.75[a]	27.0	22.0[a]	50.8[c]	45.8[a]
T7	6.80[abc]	30.1	39.9[c]	39.8[a]	58.1[b]
SEM	0.01	1.79	2.16	0.77	1.12
P-value	0.031	0.440	0.099	<0.001	0.010

Different superscripts in the same column are significantly different at P<0.05 and tend to be different P<0.1.
T1, rice straw (untreated); T2, T1 + ensiling; T3, T1 + urea; T4: T1 + autoclaving; T5, T1 + urea + ensiling; T6, T1 + autoclaving + ensiling; T7, T1 + urea + autoclaving + ensiling; VFA, volatile fatty acid; NH_3, ammonia; IVOMD, in-vitro organic-matter digestibility; SEM, standard error of mean.

In-vitro RUMEN FERMENTATION AND DIGESTIBILITY

The range of pH value for these treatments was 6.75 to 6.81 (Table 4). Meanwhile, the difference in total VFAs produced across the treatments was statistically insignificant. Total VFAs and their individual components did not change with treatments. According to Morvay et al. (2011), increase in gas production and increase in total VFA concentration should match, because these two parameters are the end products of rumen microbial metabolism, particularly of carbohydrates. Statistically, it was found that total VFAs was not significant in this study because there was high variation between replications (Jayanegara et al., 2017). The gas produced in the in-vitro rumen fermentation system comes from two sources, being the end product of microbial metabolism during feed degradation and fermentation and the result of the VFA buffering process of the bicarbonate buffer present in the incubation medium. The production of gas can therefore be considered as an indicator of the digestibility of feed. A number of studies have shown a positive correlation between gas production and feed digestibility (Sebata et al., 2011; Plaizier and Li, 2013). Previous research has shown that the molar percentage of VFA is related to NDF and ruminal starch digestibility, reflecting energy properties of the substrate as well as the place and rate of digestion (Noziere et al., 2011).

The addition of urea solution to rice straw resulted in increasing ammonia production in in-vitro rumen fermentation (Table 4). The urea treatment also added a large amount of nitrogen (in the form of CP) for microbial protein synthesis in the rumen. This substance is naturally confined to rice straw (Polyorach et al., 2019). It is known that NH_3 is an intermediate product of protein metabolism in the rumen, and that its concentration depends on the rate of degradation of protein material, microbial protein synthesis, and absorption through the villi (Sinclair et al., 2014). The production of ammonia by microbes in

the rumen plays an important role in providing microbes as a substrate for microbial protein synthesis and this is a source of protein for optimal ruminant nutrition (Jayanegara, 2019b). The NH_3-N pool in the rumen is relatively small and rotates rapidly. The amount of NH_3-N that enters the pool varies widely, according to the quantity and breakdown of protein in the diet and the rate and method of urea supplementation (Cherdthong et al., 2010).

Based on the results of this present study, it has been shown that T6 has a greater impact on increasing IVDMD than other treatments. However, T7 showed a tendency to increase IVOMD compared to the other treatments (Table 4). This could be explained as reflecting the combination of added urea solution with high temperature and pressure reducing the ADF content and thereby increasing the degradation of rice straw in in-vitro rumen fermentation. Previous studies using XRD to evaluate the crystallinity of cellulose present in agricultural and plantation residues combined with urea solution before and after treatment with high temperature and pressure have shown that amorphous cellulose hydrolyses faster than crystalline cellulose (Dewi et al., 2018). Therefore, change in the structure of cell walls from being crystalline to being more amorphous allows rumen microbes to colonize and degrade components more easily (Mittal et al., 2011; Octavia et al., 2017).

CONCLUSION

Rice straw processed by combining ensiling with added urea and autoclaving and without the addition of silage additives affected levels of pH, DM, OM, CP, ADF, total gas production, methane gas, NH_3, IVDMD, and IVOMD. Therefore the combination between ensiling, urea addition and autoclaving overall enhances the nutritive value of rice straw, although the combination did not affect NDF and total VFA levels. Future research should be directed towards a higher temperature and pressure of autoclaving for treating the rice straw in order to be more effectively break down the lignocellulose component.

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CONFLICT OF INTEREST

All authors declare that there are no conflicts of interest.

AUTHORS CONTRIBUTION

DM, RF and MA carried out the experiment and drafted the manuscript; DM and RPH performed the statistical analysis and data interpretation; MR, EBL, RR and AJ supervised the experiment and revised the manuscript.

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