Effect of urea treatment of cocoa pod on rumen fermentation characteristics in vitro

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Abstract. Indonesia is a third largest country in the world for cocoa production. A cocoa pod could be utilized as alternative feeds due to their sufficient quantity and availability throughout the year. On the other hand, low nutritional quality such as highly fibrous materials and low protein content usually characterized in agricultural and plantation by-products as it appears on cocoa pod. Ammoniation treatment using urea improve the nutritional quality of feedstuff. The objective of this study was to evaluate the effect of ammoniation treatments on a cocoa pod on in vitro feed fermentation and gas production on ruminal fluid. KA treatment gave highest gas production than other treatment. Total gas production during 48 hours of the cocoa pod was significantly affected by treatments (P<0.05). Total volatile acid (VFA), acetate (C2), propionate (C3), butyrate (C4) total VFA and A/P ratio indicated no significant difference among treatments. KA and KD treatments have tendency effect on IVDMD than control (KS) treatment. In this study ammoniation treatment using 5% of urea had a positive and effect on the best degradability and ruminal fermentation of cocoa pod.

1. Introduction
Increasing in feed cost will lead a decrease in performance of farm business especially in the small farm business. Grasses and legumes are the main sources of feeds to satisfy ruminant animals nutritional requirements, for maintenance, production or reproduction. In farm business feed cost needs 60-70% of total production cost [1], based on this condition; it is necessary to explore alternative feed to reduce feed cost and also to avoid competition with human needed. Some agricultural and plantation by-products have the potential as alternative feeds due to their sufficient quantity and availability throughout the year. On the other hand, highly fibrous materials and low protein content that indicated low nutritional quality usually characterized in agricultural and plantation by-products. This condition often leads the by-products to be treated, either physically, chemically and biologically before feeding to animals [2-4].

Indonesia a third largest country in the world for cocoa production after Ivory Coast and Ghana with total planting area in 2008 was 1,473,258 ha, total production of 792,791 tons [5]. In 2013 the area of cocoa was recorded at 1,745,789 ha, with cocoa seed production of 938.8 thousand tons. Comparison between cocoa pod: cocoa beans: fresh placentas are 74%: 24%: 2% of cocoa [6] respectively. Based on the ratio of cocoa beans with the cocoa pod, the potential of drying cocoa pod is 872,3 thousand tons/year [5]. When it used as an alternative feed fiber source to replaces forage (50% dry matter in rations), it can meet the need for 635,305 animal units (1 AU = 250 kg) or equivalent to 5,310,873 small...
ruminants [7]. However, the Indonesian people are still little bit utilized cocoa pod as an alternative feed. It is usually just thrown away, without any use and treatment. Cocoa pod can be used as animal feed cause it contains crude protein 6.8-11.71%, crude fiber 20.79%, fat 11.80%, BETN 34.90% NDS 55.30-73 , 90% and ADS 38.31-58.98% [6]. Behind the potential of the cocoa pod; it contained anti nutrients such as theobromine, lignin, and tannins. Theobromine is a harmless alkaloid but it can be destroyed by heating or drying, however continuous feeding of theobromine can reduce growth of animal [8]. The high content of lignin (between 12% to 19% dry matter higher than rice straw content) and silica, also causes low digestibility in animal [9-10]. Lignin is composed of a network of phenolic polymers that attach cellulose and hemicellulosic fibers, so the structure becomes very strong which it makes hard to digest [11]. While the presence of tannins performs complex bonds with proteins and carbohydrates that cause a reduction in rumen microbial activity in degrading protein and carbohydrates that decreasing the digestibility [6]. The cocoa pod can not be stored for long periods due to their high water content, which easily decomposed and moldy, resulting in the cocoa pod not palatable for livestock [7,12].

Therefore, to minimize the effects of antinutrients and maximize the use of cocoa pod and extend the shelf life it is necessary to do one of treatment with nitrogen sources, chemical and physical treatment [13-14]. Urea treatment of rice straw could increase its nutritive value [15-16]. Alkali source like urea has been reported to be effective in improving fibrous feed quality [17-18]. The urea also supplies N for rumen microbial growth besides improves fiber utilization. Volatile fatty acid (VFA), gas production and microbial biomass are the main product of feed organic matter fermentation in ruminant [19]. The objective of this research was to evaluate the effect of ammoniation treatments using urea on a cocoa pod on in vitro feed fermentation and gas production on ruminal fluid.

2. Materials and methods

2.1. Sample preparation

The cocoa pod was obtained from Nglanggeran village, Gunungkidul regency, Yogyakarta, Indonesia. The fresh cocoa pod was chopped manually around 1-2 cm thickness as homogenous as possible. Three kg of chopped cocoa pod was used for each treatment. All treatment kept for one month at room temperature. Each treatment was conducted in three replicates. The treated cocoa pod was analyzed for dry matter (DM) by following the procedure from [20].

2.2. Treatment and experimental design

This research was conducted based on Completely Randomized Design (CRD) with three treatments and three replications. Treatments described as follows:

KS: Cocoa pod without added urea.
KA: Cocoa pod ammoniation treatment used 5% urea in simple open clip packed.
KD: Cocoa pod ammoniation treatment used 5% urea in aerated treatment.

KA, treated cocoa pod with adding 5% urea were placed in an unused plastic banner that packed in simple open clip method. KD treated cocoa pod that puts in unused plastic banner without packed process only aerated a treatment. KS treated cocoa pod without adding urea placed in an unused plastic banner that packed in simple open clip method. All treatment incubated for 30 days at room temperature.

2.3. Fermentability and in vitro degradability assessment

The sample of cocoa pod each treatment and rumen liquid were prepared before in vitro assessment. Two ruminally fistulated Ongole crossbreed cattle used as rumen liquor donor, those adapted by feeding consisted of forage (P. hybrid) and concentrate (80:20 in dry matter basis). Rumen fluid was taken using aspirator, and immediately transported in pre-warmed vacuum flask (39 °C water temperature) and filtered.
In vitro gas production technique according to [21] was used to evaluate in vitro fermentability. The exponential equation according to [22] was used to calculate gas production kinetics. The exponential function is \( P = a + b \times (1 - e^{-ct}) \) with describing \( P \) is cumulative total gas production, \( a \) is shared gas production from soluble fraction, \( b \) is the gas production from insoluble fraction, \( c \) is the rate of gas production, \( t \) is the time of incubation and \( e \) is Euler’s constant (2.7183…). Fitting curve method using Neway Software [23] use to estimated value of \( a \), \( b \) and \( c \). 100 mL syringe glass (Fortuna model, Poulten and Graft Gmbh Germany) used for fermentation. Three syringes are containing rumen-buffer without sample (blank) used in the experiment. All of the syringes consisted of samples and blank were randomly incubated for 48 hours in an incubator at 39°C [24].

Gas production cumulative recorded at 0, 2, 4, 8, 12, 24, 36 and 48 hours incubation. Gas was released after 48 h incubation and the fluid contained in the syringe taken for analysis of VFA, and in vitro dry matter degradability. Substrate from each syringe measured according to [20] method to get dry matter (DM) value. Percentage of DM differences between initial and after incubation and corrected with blank were calculated as in vitro degradability as followed the formula as previously describe by [25]:

\[
IVDMD = \frac{[DMf - (DMr - DMb)]}{DMf}
\]  

Where: IVDMD in vitro dry matter degradability. DMf: dry matter of feed, DMb: dry matter of blank, DMr: dry matter of residue.

Volatile fatty acid (VFA) product from fermentation measured according to [26]. Meta-phosphoric acid added at the sample and stored at -20°C before analysis. Gas chromatography (Shimadzu type 8A) was used to analyzed VFA content using GP10% SP-1200/1% H3PO4 column with 80/100 Chromosorb WAW (Supelco, Bellefonte, PA).

2.4. Data Analysis
Variables measured were in vitro degradability (IVDMD), fermentability (gas production kinetics, \( a \), \( b \) and \( c \)), and individual volatile fatty acids (VFA). Analysis of variance (ANOVA) was used to evaluated data, and post hoc test of Duncan’s Multiple Range Test was used to analyzed differences among mean treatments that performed by the CoSTAT statistical software [27].

3. Result and discussion
Cocoa pod ammoniation treatment used 5% urea in simple open clip packed was generated the highest gas production compared to the others (Figure 1).

Figure 1. Cumulative gas production of cocoa pod ammoniation treatment used 5% urea in simple open clip packed (KA), cocoa pod ammoniation treatment used 5% urea in aerated treatment (KD), cocoa pod without added urea (KS) incubated during 48 hours.

Figure 1 showed that KA treatment gives highest gas production than other treatment. Kinetic gas production parameters used as an indication of fermentability evaluation by in vitro gas production [26]. Based on a gas production kinetic curve, treatment KA and KD began to show the different than KS.
treatment up to 2 hours of incubation. But, after 12 hours of incubation gas production of the cocoa pod was significantly affected by treatment. The highest cumulative gas production found at KA treatment followed by KD and KS. It can say that ammoniation treatments using urea 5% (KA) produce the best cocoa pod degradability than other treatment. Urea treatment showed to be effective in improving the nutritive value of cocoa pod. The reason is that urea supplement may act as nonprotein nitrogen source that rumen microbes could utilize it and then passed as microbial protein for the host animal [4]. Five percent of urea using in this treatment is still safe and not produce toxic ammonia effect on microbes in the in vitro gas test. This result according to [28] that high level of urea used on in vitro gas production decrease in U3 treatments but increased from treatments U0 to U2. Added by [29] explained that urea would toxic to the rumen if present in large amount.

Table 1. Gas production kinetic, volatile fatty acid production and in vitro degradability of cocoa pod ammoniation treatments

| Variable                        | Treatments   |
|---------------------------------|--------------|
|                                 | KA          | KD | KS |
| **Fermentability/ Gas production kinetic parameters** |             |    |    |
| a (mL)                          | 0.95        | 0.52 | 0.29 |
| b (mL)                          | 18.50       | 16.70 | 14.72 |
| c (mL/h)                        | 0.04<sup>b</sup> | 0.04<sup>b</sup> | 0.01<sup>a</sup> |
| a+b (mL)                        | 19.45       | 17.22 | 15.01 |
| Gas (48 h) (mL)                 | 17.19<sup>b</sup> | 15.09<sup>b</sup> | 4.09<sup>a</sup> |
| **Volatile fatty acids**        |             |    |    |
| Acetate (mM)                    | 19.00       | 13.45 | 11.65 |
| Propionate (mM)                | 5.76        | 4.22 | 3.28 |
| Butyrate (mM)                   | 3.13        | 2.07 | 1.64 |
| Total VFA (mM)                  | 27.89       | 19.74 | 16.57 |
| A/P ratio                       | 3.29        | 3.15 | 3.55 |
| **In vitro degradability**     |             |    |    |
| IVDMD (%)                       | 35.47<sup>b</sup> | 31.84<sup>ab</sup> | 24.24<sup>a</sup> |

KA = ammoniation treatment used 5% urea in simple open clip packed (KA) urea in simple open clip packed, KD = cocoa pod ammoniation treatment used 5% urea in aerated treatment, KS = cocoa pod without added urea (KS). Different superscript in the same row indicates significantly different (p<0.05)

Ruminal fermentability characteristic productions are shown in Table 1. Total gas production 48 hours of the cocoa pod was significantly affected by treatments (P<0.05) and gas production from soluble fraction (a) or total fraction (a+b) from cocoa pod had tendency affected by treatments. During the first 24 hours of incubation, fermentation is relatively intensive (lag phase), after which it reaches a stationary phase. The kinetics of gas production emerge to be determined by two distinct phases; the first one insoluble but potentially fermentable fraction and second suitable to the degradation of the soluble fraction of the tested mixtures. Positive results on gas production generated from the soluble fraction (a) indicated ruminal microbes already done adaptation time (lag phase) before degrading the soluble particle this results contrast with the previous study reported by [29], [26] that possibility there was negative "a" value arising from lag time of soluble fraction degradation activity by ruminal microbes and then to adhere to cellulosic fraction. Short-chain fatty acids (acetate, propionate, and
butyrate), gases, and microbial cells were the product of carbohydrates fermentation when a feedstuff incubated with buffered rumen fluid in vitro. Gas production from protein fermentation is relatively small as compared to carbohydrate fermentation [25,30].

Productions of total volatile acid there is acetate (C2), propionate (C3), butyrate (C4) total VFA and A/P ratio showed no significant difference (P>0.05) among treatments (Table 1). Total VFA varied between 16-28 mM per mL of rumen fluid. VFA production was not sufficient condition for optimal rumen microbial protein synthesis because VFA range required for rumen microbial growth was 80-160 mM [31], added by [32] that total VFA production ranged from 106.67-165.81 mM. Other studies stated that the range of VFA in the rumen was 60 mM acetate, 20 mM propionate and ten mM butyrate [33]. Increased VFA concentration correlated with increasing microbial production [32]. An increase in the number of VFA shows quickly or not the feed is degraded by rumen microbes. The composition of VFA at rumen changes with the difference of physical form, the composition of feed, level and feeding frequency, and also feed processing. High VFA production is the energy sufficiency for ruminant. Production of VFA from KA 68.28% and KD 19.12 higher than KS. The low value of total VFA (Table 1) may be because sample measurement was done in another laboratory based on this condition less precise handling during sample preparation which caused volatile acid disappear before analysis. Although the low value of VFA, we assured all samples kept in similar condition.

A/P ratio seemed constant in all treatments with average value 3.33. In Table 1, cocoa pod treatment using urea (KA) and aerated (KD) resulted in the ratio of acetate to propionate (A/P) lower than the control (KS). A/P ratio of control was 3.52 while the A/P ratio KA and KD treatment were 3.29 and 3.15 respectively. It has been suggested that there is an increased proportion of propionate in the rumen compared to acetate. Urea treatment plays a role in glucose metabolism affected the production of propionate that was glucogenic. This result in line with [32] that supplementation of H. tiliaceous leaves led to a lower ratio of A/P. The proportion of C2: C3: C4 was constant about 69:21:10 for all treatments. Based on the ruminal fermentation stoichiometry, individual VFA proportion consisting of C2, C3, and C4 were 60-70%, 20-30% and 10-15% respectively [33]. Event among treatments indicated no significant difference, but in KA treatment show highest total VFA than other treatments. Urea treatment could improve the nutritive value of cocoa pod; it will reduce fiber content and increase the protein content of the cocoa pod. Production of VFA mostly depends on the fermentation of the carbohydrate feed, a fraction of the protein [35-36]. A lot of nutrient supply also provides more substrate for rumen microbes to grow and to yield more microbial protein from their biomass [37-38], which also observed in this study. When the microbes are abundant and active, an elevated VFA concentration expected as the end product of microbial fermentation in the rumen [39].

In vitro dry matter digestibility (IVDMD) was present in table 1. KA and KD treatments have tendency effect on IVDMD than control (KS) treatment. Urea treatment emerged to be effective in improving the nutritive value of cocoa pod by reducing ADF content of cocoa pod [4]. Zain [40] observed that treatment using 6% urea on the cocoa pod and save for 21 d decreased ADF by 8.2%. Urea provided ammonia after being hydrolyzed to produce stepwise ammonium carbamate and ammonium carbonate [41]. Such treatment reduces physical strength of primarily fibrous feed, disrupts the silicified cuticular barrier and cleavages of some lignin-carbohydrate bonds [41-42]. As a consequence of this, the degradability of urea-treated cocoa pod increased as shown by higher IVDMD values compared to the control. Apart from modifying structural carbohydrate of the cocoa pod, urea addition could contribute to feeding the microbes an additional nitrogen source for microbial protein synthesis purposes and subsequent utilization of the protein by the host animal; it will give better digestibility than other treatment [4]. Gas production and degradation rate of particle affected the IVDMD. A significant correlation between gas production and degradability contributed by many factors such as the composition of nutrient in which associated with microbial ability to adhere and degrade the fraction of feed materials [43].

4. Conclusion
In vitro degradability indicated nutrient utilization in the rumen which attributed by gas production kinetics parameters and VFA. In this study ammoniation treatment using 5% of urea had a positive and effect on the digestibility and ruminal fermentation of cocoa pod.

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