Effect of urea coating by chitosan on the dynamics of ammonia concentration and rumen fermentation in vitro

S Nayohan*, K G Wiryawan and A Jayanegara
Nutrition and Feed Technology Department, IPB University, Jalan Raya Dramaga, Bogor-Jawa Barat 16680, Indonesia

*Email: nayohansandy5@gmail.com

Abstract. The aim of this study was to determine the effect of coating urea by chitosan at graded levels on ammonia concentration and rumen fermentation in vitro. This study used Factorial Randomized Complete Block Design (RCBD) to test ammonia parameter and Randomized Complete Block Design (RCBD) for pH, microbial protein synthesis, dry matter and organic matter digestibility, and Volatile Fatty Acid (VFA). The treatments tested were: P0 = addition non coating urea 1%; P1 = coating urea by chitosan 1%; P2 = coating urea by chitosan 2%; P3 = coating urea by chitosan 3%. The data obtained were analysed by using ANOVA and continued with Tukey HSD test with SPSS version 25. The results of this study showed that the coating of urea chitosan had no significant effect on pH, dry matter and organic matter digestibility, microbial protein synthesis, and ammonia concentration in the rumen. However, it significantly reduced (P <0.05) total VFA concentration. It can be concluded that the application of urea coating by chitosan does not affect on the degradation of urea in the rumen.

1. Introduction
Urea is used in the rumen as a source of Non Protein Nitrogen (NPN) in place of protein sources, because urea contain 46.7% nitrogen [1]. Urea can be given as supplementation, especially for ruminants who get basal feed with low nutritional quality [2]. Furthermore, the NPN in the rumen will be converted into protein by rumen microbes. NPN in the rumen will be converted into protein by rumen microbes. NPN has a high potential to be used as protein source at relatively lower price than protein from the feed. It can be used as an effort to improve nutritional conditions to increase livestock productivity in Indonesia.

Several positive impacts from urea as a source of NPN have increased digestibility and dry matter intake, increased protein content, increased milk production, and increased body weight [3]. In addition, the use of urea increases the efficiency of microbial protein synthesis, increases N production by rumen microbes, decreases methane gas production, increases cellulolytic, proteolytic, and amylolytic bacteria [4]. However, the negative impact using urea can occur when the excess dose of urea. Indiscriminate urea causes decrease in palatability, disruption of the fermentation process [5], poisoning [6], and high N excretion to the environment [7].

According to [8], urea is considered less efficient than other feed ingredients rich in protein [9,10], because urea in the rumen is rapidly degraded to ammonia. This causes high ammonia levels in the rumen especially in the first hour after consumption. Carbohydrate breakdown and microbial
development occur considerably more slowly in the rumen than urea degradation. The two must be in sync. This synchronization can increase the efficiency of incorporation of NPN into microbial protein and increase the efficiency of the overall N utilization. Therefore, it is necessary to treat urea from being rapidly degraded in the rumen.

There are various methods to reducing the degradation of urea in the rumen. One of them is by coating method with biopolymer from chitosan. According to [11], chitosan is well known in agriculture as a growth-inducing and anti-bacterial agent and controls the rate of release of easily lost nutrient elements. Based on this, this research was conducted to evaluate the effect of chitosan biopolymer coating on urea which is expected to reduce urea degradation and reduce the formation of ammonia concentration in the rumen.

2. Materials and methods

2.1. Research design
This study used Randomized Complete Block Design (RCBD) for parameters of Dry Matter Digestibility (DMD), Organic Matter Digestibility (OMD), pH, total VFA, and Microbial Protein Synthesis (MPS) and Factorial Randomized Block Design (RCBD), with factor A are treatments consist of P0 = 1% addition of non-coating urea; P1 = 1% chitosan urea coating; P2 = 2% chitosan urea coating; P3 = 3% chitosan urea coating and factor B is time for ammonia concentration.

2.2. Urea coating by chitosan
The process of making chitosan solution as a coating refers to the results of [12], namely chitosan solution derived from chitosan powder and 2% acetic acid with a ratio of 1:2. Furthermore, urea coating method used in this study refers to the results of [13]. 23.8 g of chitosan was put in a glass beaker, 2.4 g of 2% acetic acid solution was added. The solution was homogenized with homogenizer at 8000 rpm for 5 minutes. In the last step, added 30 g of urea, maltodextrin in a ratio of 1:2 to 47.6 g of chitosan, and 600 mL of alcohol in turns. Homogenization between the coating material and urea is carried out until all ingredients are evenly mixed. The homogenization process was carried out for ±15 minutes. The mixture of coating material and urea is then dried using a spray dryer with inlet temperature of 120°C and outlet temperature of 80°C.

2.3. In vitro fermentation
This research was conducted in vitro using the method of [14] as described in [15]. The study utilized elephant grass concentrate, conventional urea, and urea coated with chitosan. The sample used as substrate was oven-dried at 60°C and ground. 0.75 g of substrate made of ration with a ratio of forage and concentrate 60: 40 and 1% coating treatment; 2%; and 3% was put into a 100 mL injection vial. Furthermore 50 mL of Mc Dougall solution and 25 mL of rumen fluid were added as an incubation medium that had been saturated with CO2 gas. The tube was prepared and then shaken with CO2 flowing for 30 seconds, closed with a rubber cap, and sealed with a crimper. It was incubated using a water bath to create conditions resembling the rumen conditions at 39 °C for 24 hours. To stop the fermentation process, open the rubber cap and dripping with two drops of HgCl2.

2.4. Dry matter and organic matter digestibility
The Tilley and Terry technique was used to determine the digestibility of dry matter and organic matter [16]. The sample from the fermenter tube was dripped with HgCl2 and centrifuged at a speed of 2500 rpm for 20 minutes. The precipitate and supernatant were separated. The precipitate resulting from the centrifuge was added to 50 mL of 0.2% pepsin-HCl solution, and the mixture was incubated for 48 hours. The precipitate mixture was filtered through Whatman filter paper No. 41 with a vacuum pump. The filter results were placed on a porcelain dish, and the dry materials were dried for 24 hours at 105 °C. The material in the crucible was ashed in an electric furnace for 6 hours at 600 °C.
2.5. *Fermentability (pH)*

The pH measurement was carried out on the fermented samples for 24 hours using a pH meter. The supernatant was put into a beaker, and the pH meter electrode was dipped into the sample.

2.6. *Volatile fatty acid (VFA)*

The method used to measure the total VFA concentration is the steam distillation method [17]. Total of 5 mL of supernatant liquid was put into a distillation flask heated with water vapour, 1 mL of 15% H₂SO₄ solution was added to the distillation flask. An Erlenmeyer flask filled with 5 mL of 0.5 N NaOH solution is prepared to hold up to 250 mL of hot water vapour condensation. The Erlenmeyer flask was dripped with phenolphthalein indicator and titrated with a 0.5 N HCl solution.

2.7. *Ammonia concentration*

Ammonia measurement was carried out using the method referred to from the research results of [18]. Prepare a sample of 1 mL and put it in a test tube. 0.1 mL of Nessler reagent was added and calibrated to 10 mL with distilled water. The standard solutions in test tubes using 0.1; 0.2; 0.3; 0.4; 0.5; 0.6; 0.7 mL of standard ammonia solution, followed by 0.1 mL of Nessler reagent and calibrated to a volume of 10 mL with distilled water. The absorbance of the sample standard solution was measured at 425 nm. The ammonia concentration in the sample was calculated by plotting the absorbance level of the sample against the standard NH₃ curve.

2.8. *Microbial protein synthesis*

Measurements with the Lowry method [19] were carried out by storing some reagents, such as a mixture of 2% sodium carbonate in 0.1 N NaOH solution and 0.5% copper sulfate in 1% NaK tartrate solution as reagent (1), phenol reagent, and BSA (Bovine Serum Albumin) solution. Standard curve was made in a test tube with a size of 0; 0.1; 0.2; 0.4; 0.6; 0.8; and 1 mL of standard protein and added water 4 mL. Another test tube, 1 mL of the sample was added and added water until it reached 4 mL. Furthermore 5.5 mL of reagent (1) was added to all standard curve tubes and samples, homogenized and allowed to stand for 15 minutes. In the last step, 0.5 mL of phenol reagent was added to each test tube homogenized and allowed to stand for 30 minutes. The final step is to measure the absorbance at 650 nm.

2.9. *Data analysis*

The fermentation parameters data were statistically analysed using Analysis of Variance or ANOVA. Tukey HSD will further test significant differences between treatments. To analyse the data, the SPSS 25 version was utilized.

### 3. Results and discussion

#### 3.1. Dynamics of ammonia concentration

There was a significant difference in ammonia concentration from the effect of incubation time (P<0.05), the increase in ammonia starting at the fourth hour of incubation (Table 1). However, ammonia concentration was not significantly different between treatments, and the interaction between ammonia and time was also not significantly different.

Based on the treatment data, the application of urea coating with chitosan did not reduce the ammonia degradation process in the rumen. It is in line with the research results conducted by [20], which showed the utilization of Slow-Release Urea (SRU) Optigen was at the level 0%; 0.4%; 0.8%; and 1.2% did not affect reducing the concentration of ammonia in the rumen. This study is also supported by the results of other studies by [21]. In this study, the utilization of SRU had the same ammonia concentration as conventional urea.
Table 1. Effect of coating urea by chitosan on dynamics of ammonia concentration in vitro.

| Time (h) | Treatment (mM) | Average (mM) | P value |
|---------|----------------|--------------|---------|
| 0       | 0              | 4.56 ± 0.04  | 4.80 ± 0.04 | 4.80 ± 0.05 | 4.91 ± 0.15 | 4.64 ± 0.27 | 0.001 |
| 1       | 1              | 4.63 ± 0.04  | 4.34 ± 0.05 | 4.34 ± 0.06 | 4.86 ± 0.04 | 4.54 ± 0.25 |       |
| 2       | 2              | 4.56 ± 0.07  | 4.82 ± 0.10 | 4.52 ± 0.08 | 4.67 ± 0.11 | 4.64 ± 0.14 |       |
| 4       | 3              | 4.94 ± 0.05  | 5.29 ± 0.06 | 5.13 ± 0.04 | 4.94 ± 0.03 | 5.08 ± 0.17 |       |
| 8       | 4              | 4.89 ± 0.04  | 5.60 ± 0.04 | 5.21 ± 0.03 | 5.41 ± 0.03 | 5.28 ± 0.30 |       |
| 12      | 5              | 5.47 ± 0.09  | 5.22 ± 0.13 | 5.86 ± 0.07 | 6.04 ± 0.11 | 5.65 ± 0.37 |       |
| Average | 0              | 4.84 ± 0.35  | 5.01 ± 0.45 | 4.90 ± 0.61 | 5.14 ± 0.50 | 4.97 ± 0.44 |       |

P0 = 1% addition of non-coating urea; P1 = 1% chitosan urea coating; P2 = 2% chitosan urea coating; P3 = 3% chitosan urea coating

Coating urea by chitosan does not affect slowing down the hydrolysis process of urea in the rumen, because coating chitosan cannot protect the degradation process from microbes. It can happen because the bonds between chitosan and urea are hydrogen bonds. According to [22], the presence of high temperatures can cause the destabilization of noncovalent interactions (hydrogen bonds, electrostatics, and Van Der Walls forces). The use of inlet temperature 120ºC and outlet temperature 80ºC allows the existing hydrogen bonds to be weakened and break these bonds by rumen microbes.

According to [8], urea in the rumen is more likely to be rapidly degraded to ammonia. Ammonia level in the rumen becomes high, especially in the first hour after consumption. However, there is no spike in ammonia levels in this study, non-coated urea or coated urea. In this treatment, the ratio given to livestock has a reasonably low protein content. The proportion of forage used was higher than concentrate. 60% elephant grass used in this research. According to [23], the crude protein content of elephant grass is 6.26%.

Additionally, the concentrate utilized is believed to have a low protein level. When the protein in the concentrate is digested in the rumen to produce ammonia, the rumen accumulates ammonia slowly during the first hour. Another aspect that may contribute to this ammonia result is rumen microbial activity. The energy supply and the concentration of ammonia in the rumen to produce microbial protein were synchronized throughout the first hour. Findings of investigation corroborate those of [24], which showed that the provision of 0.95% urea supplementation in the ration did not significantly affect ammonia concentration in the rumen. According to [25], the ammonia concentration in the rumen is influenced by protein content in the feed, rumen pH, protein solubility of feed ingredients, and feeding time. However, the ammonia concentration in the rumen was still in the normal range. According to [26], the average concentration of ammonia in the rumen, which can support rumen microbial growth is 3.27-7.14 mM.

3.2. Rumen fermentation

Treatment of coating urea by chitosan with level of 1%; 2%; and 3% in vitro had a significant effect (P<0.05) on the concentration of rumen VFA (Table 2). Treatment P1 resulted in a higher total VFA than P2 and P3 (P<0.05), but not different from P0. The treatment of urea coated with chitosan did not significantly affect the acidity levels in the rumen, DMD, OMD, and microbial protein synthesis.

Based on the data from the research conducted, the treatment had significant effect on the concentration of VFA in the rumen. This follows the results of study by [27], which stated the utilization of urea-zinc, urea-zeolite, and urea-zinc-zeolite in the diet slow-release urea had a significant effect in controlling the concentration of VFA. The treatment of urea chitosan coating with 1% showed the highest VFA concentration compared to other treatments. After that, further tests were carried out using the Tukey HSD test. Data were obtained that urea coating by chitosan with a level of 1% showed the highest VFA concentration compared to other treatments. It happens because in the first treatment, the initial energy availability was high. When synchronization occurred in forming microbial protein, the
reduction in VFA concentration was lower relative to other treatments. It is also supported by the high concentration of SPM in the first treatment. It shows that in the first treatment, there was synchronization between ammonia and the energy source from the VFA so that the microbes in the rumen were more optimal in forming the microbial protein. According to [28], the normal range of VFA production in the rumen is 80-160 mM.

Table 2. Effect of coating urea by chitosan on pH, DMD, OMD, MPS, and VFA in vitro.

| Variable | Treatment | 0   | 1          | 2          | 3   | P   |
|----------|-----------|-----|------------|------------|-----|-----|
| pH       |           | 7.19±0.05 | 7.06±0.08  | 7.07±0.13  | 7.01±0.09 | 0.147 |
| DMD (%)  |           | 49.7±4.56 | 46.2±2.71  | 49.4±1.71  | 48.8±2.00 | 0.430 |
| OMD (%)  |           | 49.2±5.20 | 45.6±3.17  | 49.0±2.39  | 48.9±1.70 | 0.358 |
| MPS (mg/10 mL) |       | 12.9±8.16 | 14.4±8.99  | 13.8±9.04  | 13.4±10.57 | 0.897 |
| VFA (mM) |           | 98.2±0.03ab | 108±6.69b | 88.6±10.6a | 86.8±6.39a | 0.042 |

\[a,b,c\] in the column shows the superscript has a significant effect (P<0.05) using Tukey HSD Test

P0 = 1% addition of non-coating urea; P1 = 1% chitosan urea coating; P2 = 2% chitosan urea coating; P3 = 3% chitosan urea coating

SRU treatment using chitosan did not significantly affect rumen fermentability. This follows the results of study conducted by [20], which showed the utilization of SRU did not affect pH. The pH ranges in the P1, P2, and P3 treatments were still in the normal rumen pH range. However, in the P0 treatment with conventional urea, the pH level was slightly higher. According to [29], the average pH range in the rumen is 6-7. This indicates that the addition of chitosan-coated urea coating treatment did not interfere with rumen fermentability.

According to [30], rumen pH affects the process of rumen ammonia absorption. When the pH decreases, the absorption of ammonia by the rumen wall will also decrease. In addition, high or low rumen pH will affect rumen microbial activity [31]. When rumen pH is not in the normal range, it will decrease rumen microbial activity.

The treatment of urea coated by chitosan did not significantly affect the digestibility of dry and organic matter. It is in line with the research conducted by [20], which showed that giving Slow-Release Urea (SRU) to sheep had no significant effect on dry matter and organic digestibility. According to [32], the normal range of dry matter digestibility of feed ingredients is 50.7-59.7%. Based on these data, the range of dry matter digestibility values from the treatment was below the normal range. Several factors affect digestibility, such as the composition of feed ingredients, comparison of compositions between feed ingredients, treatment, enzyme supplementation, and feeding level [33]. In addition, high or low dry matter and organic digestibility were also influenced by urea supplementation. According to [34], supplementation of urea in the diet, which is converted into ammonia, can loosen the lignocellulosic bonds to experience swelling. Bacteria and fungi can utilize this condition to penetrate enzymes to increase the digestibility of dry and organic matter.

Microbial protein synthesis is a protein produced by microbes in the rumen. The results showed the application of urea coating by chitosan had no significant effect on microbial protein synthesis. It shows there is synchronization between ammonia and energy sources in this treatment. Microbial protein synthesis can run quite well. According to [30], microbial protein synthesis also depends on the rate of nitrogen breakdown in the feed, the rate of absorption of ammonia and amino acids, and the type of fermentation influenced by the type of feed.

4. Conclusions

Effect of coating urea by chitosan with in vitro study at level 1%; 2%; and 3% were unable to reduce urea degradation and inhibit the formation of ammonia in the rumen. It is because the bonds formed between urea and chitosan are not able to protect urea from rumen microbes.
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