Evaluation of urea-nitrate supplement made with Maillard reaction for delaying ruminal ammonia formation

Dedy Nanda Kurniawan[1], *Vita* Apriani[1], *Yulianri Rizki* Yanza[2], Nahrowi[1], and *Anuraga* Jayanegara[1*]

[1]Department of Nutrition and Feed Technology, Faculty of Animal Science, IPB University, Bogor, West Java, Indonesia
[2]Department of Animal Nutrition and Feed Technology, Faculty of Animal Science, Universitas Padjadjaran, Jatinangor, West Java, Indonesia

Abstract. Ruminants have a stomach that can convert NPN compounds as amino acids. This ability has not been effectively utilised. The present study aimed to determine the influence of urea-nitrate supplementation on rumen fermentation profiles *in vitro*. The urea-nitrate was prepared by employing the Maillard reaction (MR) using two different sugar sources, i.e., molasses and liquid waste of palm juice. The present study's randomized complete block design consisted of five treatments and four replications. The treatments are T0 = control diet; T1 = T0 + urea; T2 = T0 + urea-nitrate; T3 = T0 + urea-nitrate MR with molasses; T4 = T0 + urea-nitrate MR with liquid waste of palm juice, where urea and urea-nitrate supplementation treatments were expected to enhance dietary crude protein by 2% of the control diet. Results revealed that T3 treatments enhanced ammonia concentration up to 11.4% compared to T0, T1, and T4 ($P<0.05$). However, treatments were similar in terms of rumen pH, volatile fatty acids, microbial protein synthesis, total gas production, methane production, dry matter digestibility, and organic matter digestibility. In conclusion, the slow release urea-nitrate supplementation in ruminant feed may inhibits ammonia formation in the rumen in vitro.

1 Introduction

Ruminant has an extraordinary digestive system that rapidly degrades nutrients from the uptake feed, thanks to the rumen accommodating various microbes that play a crucial role in converting feed nutrients. Moreover, microbes in the rumen are also affordable for converting nitrogen from feed into proteins or amino acids; hence, ruminants can be fed with non-protein nitrogen (NPN) as alternative protein sources. Urea is the only organic NPN-rich source commonly supplemented in ruminant feed rations containing 46.7% nitrogen [1]. Incorporating urea as an NPN supplementation in feed ration has several advantages, such as enhanced dry matter digestibility [2], increased microbial protein synthesis efficiency, and consequently, greater production of nitrogen by rumen microbes [3]. However, the level of urea supplementation in ruminants should be at most 1% of the total feed in dry matter [4].

* Corresponding author: anuraga.jayanegara@gmail.com

Exceeding urea supplementation in ruminants may exceed ruminal ammonia concentration, poisoning the animal and leading to the exceeded ammonia absorption in the ruminant's liver beyond its capacity. Consequently, high blood ammonia levels can damage normal liver cells, impairing the breakdown of harmful compounds in the body.

On the other hand, nitrate can be employed as an alternative source of NPN composed of nitrogen and oxygen molecules. A previous study revealed that a low concentration of nitrate supplementation in feed ration was favourable as a defaunation agent to suppress the growth of protozoa there. The nitrate denaturation effect benefits the environment by reducing ruminant enteric methane production [5]. Due to urea susceptibility to rapid hydrolysis by rumen microbes, urea-nitrate supplementation in ruminant feed can be considered a proper treatment for ruminants to slow released urea-nitrate hydrolysis in the rumen. Urea-nitrate synthesis can be obtained by the Maillard reaction's coating technique between reducing sugars and amino acids [6]. Therefore, this study aimed to identify the most effective coating material to protect urea-nitrate from rumen microbial degradation. The addition of slow-release urea-nitrate is expected to provide an alternative protein source in feed and reduce methane gas production in livestock.

2 Material and Methods

The materials used in the present study were urea, urea-nitrate, molasses, liquid waste of palm juice, and rumen fluid. *In vitro* treatments were separated; a control group used only a basal diet, and an experimental group used a basal diet supplemented with urea and urea-nitrates. A basal diet composed of forage and concentrate (40:60). The present experiment was carried out in two stages: the slow-release urea-nitrate preparation stage and the *in vitro* analysis stage. The nutrient composition of the basal diet used is shown in Table 1.

Table 1. Nutrient composition of basal diet.

Nutrient content (% DM)	
Ash*	7.04
Crude protein*	14.93
Crude fat*	2.69
Crude fiber*	15.19
Nitrogen free extract*	48.48
Total digestible nutrient**	59.73

DM = dry matter.
*Laboratory of Animal Feed Science and Technology (2023) results.
**Results calculated according to Wardeh [7].

2.1 Slow Release Urea-Nitrate Preparation

The combination of urea and KNO3 salt granules formed the urea-nitrate. The preparation of slow-release urea-nitrate molasses followed the Rafleliawati et al. [8] protocol by adding urea and nitrated with 50 grams of molasses that were heated until caramelization occurred. Moreover, urea-nitrate molasses was then reheated in a 105oC oven for 24 h before being smashed into powder form. The same protocol was used to make the urea nitrate MR liquid waste of palm juice.

2.2 In Vitro Experiment

The present study was performed using an experimental design of 5x4 (treatments x bottles) (20 bottles in total) and was completed in two runs. Experimental treatments were: A control

group that used a basal diet (T0), and experimental treatments that used basal diet + urea group (T1); basal diet + urea-nitrate (T2); basal diet + urea-nitrate MR with molasses (T3); basal diet + urea-nitrate MR with liquid waste of palm juice (T4). The control group were inserted with 0.75 g as fed basal diet consisting of elephant grass: concentrate 40:60 w/b, while the experimental groups basal diet (also 0.75 g as fed in each bottle) were added with 6.9 g/kg DM urea (T2), 12.2 g/kg DM urea-nitrate (T3), 30.8 g urea-nitrate MR with molasses (T3), and 27.5 g/kg DM urea-nitrate MR with liquid waste of palm juice (T4).

The in vitro experiment followed Theodorou et al. [9] protocol. Each experimental diet (T0, T1, T2, T3, and T4) was inserted in a 100 mL bottle. Each bottle was flowed with CO2, and approximately 25 mL of rumen fluid and 50 mL of McDougall's solution were flowed with CO2 gas. Each bottle was directly sealed with a rubber cap and aluminium, and a crimper was used. All bottles were then directly put into the conditioned water bath at 39°C.

Gas production of each bottle was measured at 2, 4, 6, 8, 10, 12, and 24 h. A methane gas sample was collected from the wages previously transferred in the vacuum bottle. Each buffered rumen fluid in the bottle was then transferred into 50 ml falcon and was centrifuged at 2500 rpm for 10 minutes. A large amount of buffered rumen fluid supernatant was used to analyze ammonia (NH3) microbial protein synthesis and total volatile fatty acid (VFA). Digestibility and fermentability samples were carried out after buffering rumen fluid, and the precipitate residue was reincubated for 24 hours after adding the pepsin reagent. After another 24 hours, the precipitate residue was analyzed for its dry matter digestibility (DMD) and organic matter digestibility (OMD).

3 Results and Discussion

The Addition of urea-nitrate to the basal diet had no significant effect ($p>0,05$) on pH, total VFA, and SPM. However, adding urea-nitrate had a significant effect ($p<0,05$) on ammonia concentration, with the highest concentration in T3 (Table 2). The rumen pH value was still in a good range [10], which means that the addition of urea-nitrate did not interfere with rumen microbial activity to ferment feed. This can be seen in the fermentability value, which is still in the normal range. The range of feed fermentability values is MPS 10-50 mg N/kg [11], total VFA 70-150 mM [12], NH3 3.24 - 7.14 mM [13].

Table 2. Effect of SRUN on rumen fermentability.

Treatment	Parameter			
	pH	**NH3 (mM)**	**MPS (mg/10 mL)**	**VFA (mM)**
T0	6.59	3.94^{b}	17.77	100.96
T1	6.61	4.13^{b}	15.89	90.96
T2	6.58	4.24^{ab}	15.59	86.60
T3	6.61	4.60^{a}	17.42	89.65
T4	6.60	3.95^{b}	17.32	94.51
SEM	0.05	0.12	0.77	4.54
p-Value	0.36	0.03	0.29	0.57

T0 = control ration; T1 = T0 + urea; T2 = T0 + urea-nitrate; T3 = T0 + urea-nitrate MR with molasses; T4 = T0 + urea-nitrate MR with date juice liquid byproduct.
NH3: ammonia concentration; VFA: volatile fatty acids; MPS: microbial protein synthesis.
Different superscripts in the same row with different letters showed significant differences ($P<0.05$).

The addition of urea-nitrate increases the concentration of rumen ammonia. This is because urea-nitrate contains nitrogen that will be converted by rumen microbes into protein and ammonia as a precursor in forming new rumen microbes [14]. Using date palm juice

effluent as a urea-nitrate coating is better than using molasses, where the coating of date palm juice effluent can reduce ammonia concentrations by 11.4% compared to treatments T1, T2 and T3. However, the slow-release treatment of urea-nitrate with Maillard reaction dressing has not protected urea-nitrate from being degraded by rumen microbes. Similarly, Nayohan study [15–17] made slow-release urea with chitosan dressing and could not reduce the rumen ammonia concentration due to the urea-nitrate dressing, which is readily hydrolysed by rumen microbes. Feggie study [16] using tannin dressing as a urea protector can reduce ammonia concentration.

Table 3. Results of measurements of dry matter digestibility and organic matter digestibility.

Treatment	Parameter	
	DMD (%)	**DMO (%)**
T0	47.26	40.34
T1	50.86	44.48
T2	49.48	42.90
T3	49.21	42.73
T4	49.58	40.98
SEM	0.87	1.15
p-Value	0.47	0.49

T0 = control ration; T1 = T0 + urea; T2 = T0 + urea-nitrate; T3 = T0 + urea-nitrate MR with molasses; T4 = T0 + urea-nitrate MR with date juice liquid byproduct. DMD: dry matter digestibility; OMD: organic matter digestibility.

The DMD and OMD of the treatment diets are shown in Table 3. Based on the analysis of variance, the treatment diets showed no statistically significant effect ($p>0.05$). This is in line with study [15,18] which states that the provision of slow-release urea has no significant effect on the digestibility of dry and organic matter. According to Schneider [18], the value of dry matter digestibility of good feed is 50.7-59.7%. Based on the results obtained, the value of feed digestibility is still below average; this is due to the use of feed ingredients such as elephant grass, rice bran, and pollard, which are low-quality agricultural by-products.

Table 4. Total gas production and methane gas production

Treatment	Parameter	
	Total gas production (mL g^{-1} DM^{-1})	**Methane gas (mL g^{-1} DM^{-1})**
T0	96.83	45.68
T1	84.77	48.59
T2	91.33	47.68
T3	87.16	47.74
T4	88.58	47.64
SEM	3.58	5.20
P-*Value*	0.17	0.43

T0 = control ration; T1 = T0 + urea; T2 = T0 + urea-nitrate; T3 = T0 + urea-nitrate MR with molasses; T4 = T0 + urea-nitrate MR with date juice liquid byproduct.

Gas production is one of the results of rumen microbial fermentation, which describes rumen activity [19]. The addition of urea-nitrate supplements in the ration did not affect total gas production and methane gas production ($p>0.05$). Nitrate addition in basal diet has yet to reduce total gas and methane gas production significantly. This is due to the nitrate content in the product being in a small concentration, so it has been unable to lyse methane gas-producing microbes. Zijderveld study [20] added nitrate and sulphate in rations with a

concentration of 2.6%, which can significantly reduce methane gas production. Data on total and methane gas production are presented in Table 4.

4 Conclusion

The addition of slow-release urea-nitrate has not reduced rumen ammonia levels or methane gas production. However, the addition of slow-release urea-nitrate did not disturb rumen fermentability.

References

1. F. Kurzer and P. M. Sanderson, Urea in the history of organic chemistry, J. Chem. Educ. **33**, 452 (1956). https://doi.org/10.1021/ed033p452
2. M. Wanapat, P. Gunun, N. Anantasook, and S. Kang, Changes of rumen pH, fermentation and microbial population as influenced by different ratios of roughage (rice straw) to concentrate in dairy steers, J. Agric. Sci. **152**, 675 (2014). https://doi.org/10.1017/S0021859613000658
3. T. Wahyono, M. M. Sholikin, Y. Konca, T. Obitsu, S. Sadarman, and A. Jayanegara, Effects of urea supplementation on ruminal fermentation characteristics, nutrient intake, digestibility, and performance in sheep: A meta-analysis, Vet. World **15**, 331 (2022). https://doi.org/10.14202/vetworld.2022.331-340
4. S. Zhang, L. Cheng, X. Guo, C. Ma, A. Guo, and Y. Moonsan, Effects of urea supplementation on rumen fermentation characteristics and protozoa population in vitro, J. Appl. Anim. Res. **44**, 1 (2016). https://doi.org/10.1080/09712119.2014.978779
5. P. Petropoulos and K. A. Gilbride, Nitrification in activated sludge batch reactors is linked to protozoan grazing of the bacterial population, Can. J. Microbiol. **51**, 791 (2005). https://doi.org/10.1139/W05-069
6. H. Yu, R. Zhang, F. Yang, Y. Xie, Y. Guo, W. Yao, and W. Zhou, Control strategies of pyrazines generation from Maillard reaction, Trends Food Sci. Technol. **112**, 795 (2021). https://doi.org/10.1016/j.tifs.2021.04.028
7. M. F. Wardeh, Models for Estimating Energy and Protein Utilization for Feeds: All Graduate Theses and Dissertations, 1 (1981)
8. P. Rafleliawati, S. Surahmanto, and J. Achmadi, Efek pemanasan pada molases yang ditambahkan urea terhadap ketersediaan NH3, volatile fatty acid dan protein total secara in vitro, J. Ilmu-Ilmu Peternak. **26**, 24 (2016). https://doi.org/10.21776/ub.jiip.2016.026.02.4
9. M. K. Theodorou, B. A. Williams, M. S. Dhanoa, A. B. McAllan, and J. France, A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds, Anim. Feed Sci. Technol. **48**, 185 (1994). https://doi.org/10.1016/0377-8401(94)90171-6
10. F. HF, Monteiro, AP, Ruminal acidosis, bacterial changes, and lipopolysaccharides, Epilepsy Res. **6**, 106192 (2020)
11. M. D. Stern, A. Bach, and S. Calsamiglia, New Concepts in Protein Nutrition of Ruminants, in *21st Annu. Nutr. Manag.* (2006), pp. 23–24
12. P. McDonald, R. A. Edwards, J. F. D. Greenhalgh, C. A. Morgan, L. A. Sinclair, and R. G. Wilkinson, Animal nutrition, in *IGCSE Biol.*, 7th ed. (Cambridge University Press, Cambridge, 2010), p. 714

13. T. Sutardi, N. A. Sigit, and T. Toharmat, Standardization of protein quality in ruminant livestock feed based on metabolic parameters by rumen microbes, *Standardization of Protein Quality in Ruminant Livestock Feed Based on Metabolic Parameters by Rumen Microbes* (Proyek Pengembangan Ilmu dan Teknologi Dirjen Pendidikan Tinggi, Jakarta, 1983)
14. K. Abdoun, F. Stumpff, and H. Martens, Ammonia and urea transport across the rumen epithelium: a review., Anim. Health Res. Rev. **7**, 43 (2006). https://doi.org/10.1017/S1466252307001156
15. S. Nayohan, K. G. Wiryawan, and A. Jayanegara, Effect of urea coating by chitosan on the dynamics of ammonia concentration and rumen fermentation in vitro, IOP Conf. Ser. Earth Environ. Sci. **951**, 012004 (2022). https://doi.org/10.1088/1755-1315/951/1/012004
16. L. J. V. Geron, S. C. De Aguiar, J. T. H. De Carvalho, G. D. Juffo, A. P. Da Silva, E. L. De Sousa Neto, K. S. M. Coelho, J. Garcia, L. C. Diniz, and E. H. De Paula, Effect of slow release urea in sheep feed on intake, nutrient digestibility, and ruminal parameters, Semin. Agrar. **37**, 2793 (2016). https://doi.org/10.5433/1679-0359.2016v37n4Supl1p2793
17. S. B. H and F. WP, The evaluation of feeds through digestibility experiments, *The Evaluation of Feeds through Digestibility Experiments* (University of Georgia press, 1976)
18. F. Feggie, S. Nayohan, K. G. Wiryawan, and A. Jayanegara, Utilization of tannin from chestnut as a protective agent in slow release urea: An in vitro rumen fermentation study, IOP Conf. Ser. Earth Environ. Sci. **1114**, 012108 (2022). https://doi.org/10.1088/1755-1315/1114/1/012108
19. A. Jayanegara, Y. Yogianto, E. Wina, A. Sudarman, M. Kondo, T. Obitsu, and M. Kreuzer, Combination effects of plant extracts rich in Tannins and Saponins as feed additives for mitigating in vitro ruminal Methane and Ammonia formation, Animals **10**, 1 (2020). https://doi.org/10.3390/ani10091531
20. S. M. van Zijderveld, W. J. J. Gerrits, J. A. Apajalahti, J. R. Newbold, J. Dijkstra, R. A. Leng, and H. B. Perdok, Nitrate and sulfate: Effective alternative hydrogen sinks for mitigation of ruminal methane production in sheep, J. Dairy Sci. **93**, 5856 (2010). https://doi.org/10.3168/jds.2010-3281