Research Article

Effect of Rumen-Degradable Protein (RDP) to Non-Fiber Carbohydrate (NFC) Ratio in Cattle Feed on NH3 Production, Volatile Fatty Acids (VFA), and Protozoal Populations: An in Vitro Study

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Abstract | This study investigates how the ratio of rumen-degradable protein (RDP) to non-fiber carbohydrates (NFC) in cattle feed affects the production of ammonia (NH3), total volatile fatty acids (VFA), and protozoa. The experimental design was completely randomized (CRD) with six treatments and five replications. The treatments consisted of varying RDP to NFC ratios: R1 (60:35), R2 (60:40), R3 (65:35), R4 (65:40), R5 (55:39), and R6 (55:41). The research data were analyzed for variance, followed by Duncan's Multiple Range Test. The results indicated that the six treatments had a significant effect ($P < 0.05$) on NH_3 production and total VFA but no significant impact ($P > 0.05$) on protozoa populations. This study concluded that the ratio of rumen degradable protein (RDP) to non-fiber carbohydrates (NFC) significantly increased the production of ammonia (NH_3) and total volatile fatty acids (VFA). However, it did not affect the protozoan population. The treatments R1, R3, and R4 exhibited the highest levels of NH_3 and total VFA production.

Keywords | RDP, NFC, NH_3, Total VFA, Protozoa, Rumen

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INTRODUCTION

The productivity of ruminants, such as cattle, is influenced by several factors, particularly the quality and quantity of feed provided. Typically, livestock feed is supplied in rations, which are mixtures of various feed ingredients like forage and concentrates. These rations are designed to meet the livestock's nutritional needs for 24 hours. Feeding serves not only to meet the needs of the animals but also to support the rumen microbes. This is important because microbes provide amino acids to ruminants, and it is not just that microbial protein fulfills the requirement. High-quality feed is defined by its complete nutrient profile, which enhances the genetic potential of cattle and improves production outcomes to meet established targets. In ruminants, feed digestion occurs in three stages, including

mechanical digestion in the mouth, re-mastication, and rumination (Pazla *et al*., 2023; Purwanti *et al*., 2024), fermentation by microbes in the rumen (degradation by microbes), and chemical digestion by digestive enzymes in the abomasum and intestine (Tanuwiria *et al*., 2023).

Fermentative digestion involves the transformation of complex compounds by rumen microbes into simpler compounds that can be absorbed in the small intestine. Measuring how much feed can be fermented by these microbes is known as fermentative capacity. This concept is closely linked to the activity and population of microorganisms in the rumen. In vitro methods, conducted in a laboratory setting, can measure rumen fermentative capacity. The final products of the fermentation process in the rumen include ammonia (NH_3) and volatile fatty acids (VFAs). Protein and carbohydrates are vital factors when considering the nutrient requirements of livestock. Optimal utilization of feed protein occurs when there is a balanced supply of energy. Feed protein quality is determined by its rumen digestibility, degradability, and amino acid content (Mushawwir *et al*., 2022). Protein in feed is essential for the growth of specific bacteria, including cellulolytic, amylolytic, and proteolytic bacteria, which serve as a source of microbial protein and help meet the protein needs of the host animal (Firmansyah *et al*., 2024; Setiawan *et al*., 2024; Adriani *et al*., 2024). Protein requirements comprise various sources, notably degraded protein and bypass protein.

Research results indicate that in vitro studies on the balance of Rumen-Degradable Protein (RDP) to Non-Fibre Carbohydrates (NFC) can influence total gas production. Similar findings were reported by Setiawan *et al*. (2024), showing that non-fiber carbohydrates can increase total and partial volatile fatty acid (VFA) levels. Additionally, in vivo experiments demonstrated that butyrate levels were absorbed into blood plasma (Tanuwiria and Mushawwir, 2020). RDP is the portion of protein broken down into amino acids, peptides, and ammonia by microbes in the rumen. This breakdown process produces ammonia (NH_3), a nitrogen source for the microbes involved in microbial protein synthesis (Mushawwir *et al*., 2022; Kharazi *et al*., 2022). The amount of ammonia produced reflects the activity level and population of the rumen microbes.

In addition to nitrogen, these microbes also require energy sources from feed, specifically Non-Fiber Carbohydrates (NFC). NFC includes readily fermentable food energy sources, such as sugars and starches, which are crucial for the performance of ruminants (Tanuwiria and Mushawwir, 2020, 2022a). The fermentation of carbohydrates produces volatile fatty acids (VFAs) and Adenosine Triphosphate (ATP). VFAs and ATP provide energy for the rumen microbes, aiding their digestion of feed (Tanuwiria *et al*., 2022b; Purwanti *et al*., 2024). To optimize microbial growth and fermentation production, it is essential to balance the availability of protein and carbohydrates in ruminant diets, ensuring that the synchronization between nitrogen, VFAs, and ATP is achieved.

The relationship between RDP and NFC has not been studied with a focus on local Indonesian cattle, particularly regarding the effects of protozoa and VFA metabolism in the rumen. This study aimed to determine the impact of the rumen-degradable protein to non-fiber carbohydrate ratio on the production of NH_3, total VFA, and protozoa. We hypothesized that synchronizing the ratio of rumen-degradable protein to non-fiber carbohydrates could enhance the production of NH_3, total VFA, and protozoa.

MATERIALS AND METHODS

Diet and Treatment

The research was conducted at the Laboratory of Ruminant Nutrition and Feed Chemistry at Universitas Padjadjaran from July to September 2023. This study employed a completely randomized design (CRD) of six treatment ratios, each repeated five times. This resulted in a total of 30 experimental units. The list of treatments is provided in Table 1.

Table 1: Nutrient content of the research ration.

No	Nutrient	Treatment diet					
		R1	R2	R3	R4	R5	R6
		(%)					
1	Dry matter	91,42	92,01	90,97	91,67	91,27	91,34
2	Ash	9,74	9,66	11,86	10,59	8,42	8,23
3	Crude protein	16,00	16,00	16,00	16,00	14,00	14,00
4	fiber	16,72	16,40	17,15	18,77	17,49	17,35
5	Ether extract	3,99	4,72	2,99	3,28	4,74	4,75
6	Nitrogen free extract	53,38	52,28	51,43	50,81	55,47	55,74
7	Total digestible Nutrient	65,00	65,00	65,00	65,00	65,00	65,00
8	RDP	60,00	60,00	65,00	65,00	55,00	55,00
9	NFC	35,00	40,00	35,00	40,00	39,00	41,00

Each material used in the ration preparation was sun-dried for one week and ground using a hammer mill machine. The components were mixed until they were homogeneous, according to the specifications of each treatment ration. The rations were tested in vitro using Theodorou *et al*.'s method (Theodorou *et al*., 1994). This in vitro testing evaluated the fermentative capacity of the experimental rations by measuring the protozoa population, as described by Michalowski (2005), and the fermentation products formed in the rumen fluid, which included volatile fatty acids and ammonia.

Rumen Liquid

The rumen fluid used came from two Brahman crossbred cattle obtained from a local slaughterhouse, RPH Ciroyom, Indonesia. Each ruminal fluid was placed in a different thermos flask, filled to the brim, and sealed.

In Vitro Implementation

Samples weighing up to 0.5 g were placed in an incubation vessel preheated in a water bath at 39-40°C. McDougall's solution (Artificial Saliva) was added to bring the total volume to 40 mL, along with 10 mL of bovine rumen fluid. CO_2 gas was introduced into the incubation bottle to create a more anaerobic atmosphere. The bottle was closed and sealed with silicone and incubated for 24 hours, with agitation every 2 hours.

After 24 hours of incubation, 20 µL of the contents from the incubation vessel were collected and transferred to a 10 mL test tube filled with Formalin to calculate the total protozoa under a microscope. The contents of the incubation bottle were centrifuged at 4000 rpm for 10 minutes, and the resulting supernatant was analyzed for volatile fatty acids and ammonia.

Measurement of Ammonia (NH_3)

A supernatant of up to 1 mL was placed in the left compartment of the Conway cup, and 1 mL of NaOH was added to the right compartment. The center of the Conway cup was filled with 1 mL of boric acid containing methyl red and bromocresol green indicators. The Conway cup was then covered with a greased lid using Vaseline to prevent air from entering. The cup was stirred to mix the supernatant and the NaOH solution thoroughly. It was left at room temperature for 24 hours. After this period, titration was performed using 0.005 N H_2SO_4 until a color change to pink was observed. Calculation of ammonia using the formula: NH_3= (V H_2SO_4 x N H_2SO_4 x 1,000) mM.

Total VFA Measurement

A set of Markham steam distillation apparatus was used to measure the flying fatty acids. 5 mL of supernatant was put into a steam distillation tube and then heated with water vapor. The tube was filled with 1 mL of 15% H_2SO_4 and sealed. Flying fatty acids carried by water vapor will pass through the cooling tube and undergo a condensation process, then be accommodated in an Erlenmeyer tube containing a 5 mL solution of 0.5 N NaOH, and then left until it reaches a tube volume of ± 300 ml. Into the Erlenmeyer tube, two drops of phenolphthalein solution were added and then titrated with 0.5 N HCl solution until the color changed from red to transparent. For the blank, titration was carried out on a solution of 5 ml of 0.5 N NaOH. Calculation of total flying fatty acids using the formula:

$$Total\ VFA\ (mM) = \frac{(a-b) \times N\ HCl \times 1{,}000}{5}$$

Measurement of Protozoan

Counting the number of protozoa was conducted using the method outlined by Michalowski (2005). A dilution was prepared by mixing 100 mL of 37% formalin with 900 mL of distilled water in a 1:10 ratio. The first step involved combining the formalin with sterile water. Then, 6 mL of the prepared solution was added to a 10 mL test tube. Next, 1 mL of the incubated rumen fluid was added to the test tube, bringing the total volume to 7 mL. After that, perform the calculations using a light microscope (Binocular XSZ-107BN; Yazumi, China). Protozoan were counted with a specified volume of 10 µL for Entodiniomorphida microscope magnification 10 with a magnification of 160/0.17, 100 µL for Diplodinium microscope magnification 10 with a magnification of 160/0.17, and 100 µL for Holotricha with microscope magnification 5 with magnification 160/0.17. To calculate the protozoan population, the following formula was used:

- Holotricha

$$Protozoan = \frac{Holotricha\ Count \times 7 \times 1000}{100}$$

- Diplodiniumorpha

$$Protozoan = \frac{Diplodiniumorpha\ Count \times 7 \times 1000}{100}$$

- Entodiniomorpha

$$Protozoan = \frac{Entodiniomorpha\ Count \times 7 \times 1000}{10}$$

Statistical Analysis

The data were analyzed statistically using Analysis of Variance (ANOVA) with IBM SPSS Statistics software (version 25.0; IBM Corp., NY, USA, 2017). The ANOVA test showed a significant effect, followed by a comparison test. Duncan's Multiple Range Test was employed to determine significant differences between treatments, with statistical significance set at $P<0.05$.

RESULTS AND DISCUSSION

NH_3 Production

The effect of the study on the ratio of rumen-degradable protein to non-fiber carbohydrate on NH_3 production is shown in Table 2.

The results showed that NH_3 production ranged from 2.52 mM to 5.97 mM. Based on Pazla *et al.* (2023), the optimal NH_3 concentration for rumen microbial growth is 3.57-7.14 mM. The highest results were obtained by treatments

R1 (60:35), R3 (65:35), and R4 (65:40) with NH_3 production of 5.94 mM, 5.97 mM, and 5.42 mM, respectively, with no significant differences observed between treatments (P>0.05). The lowest NH_3 production was found in treatment R5 (55:39) with 2.52 mM, which was significantly different (P<0.05) from R6 (55:40) with 3.50 mM and R2 (60:40) with 4.53 mM. Previous studies on the relationship between NH_3 production and rumen protozoa population showed mixed results, while the current study showed a decrease in NH_3 concentration with increasing protozoa population (r = -0.68) (Figure 1).

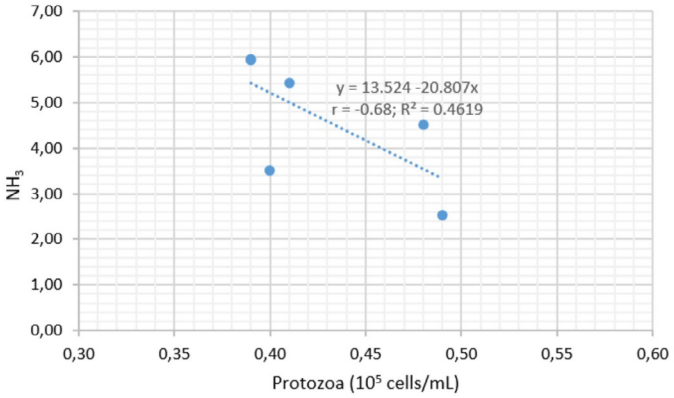

Figure 1: The correlation between protozoan population and NH3 concentration.

The study indicates a negative association between protozoan populations and NH_3 levels, with a correlation coefficient of r = -0.68. As protozoan populations increased, NH_3 levels consistently decreased. Phagocytes, which act as predators of rumen bacteria, mainly target amylolytic bacteria. These amylolytic bacteria attach to starch granules, and protozoa consume these starch particles, ingesting the amylolytic bacteria along with the starch. This consumption decreases the bacterial population (Wijayanti *et al.*, 2022; Pazla *et al.*, 2023), reducing the levels of ammonia (NH_3) (Mosoni *et al.*, 2011; Lee *et al.*, 2020). The findings suggest that 46.19% of the decrease in NH3 levels can be attributed to the rise in the protozoan population, as shown by the linear equation y = 13.524 - 20.807x. Three main factors influence microbial protein synthesis: the type of nutrients, particularly carbohydrates; the pH level in the rumen; and the composition of the microbial population (Franzolin *et al.*, 2010). Increased availability of ammonia (NH_3) and carbon resulting from carbohydrate fermentation enhances microbial protein synthesis. The high levels of NH_3 observed in treatments R1, R3, and R4 are believed to result from optimal synchronization between ruminal degradable protein (RDP) and non-fiber carbohydrates (NFC). Higher levels of NH_3 are associated with fermentation processes (Rahmania *et al.*, 2022; Tanuwiria *et al.*, 2023). The energy required for fermentation comes from readily fermentation-capacity non-fiber carbohydrates (NFC), such as starch, sugars, and pectin. Maintaining a balanced ratio of RDP to NFC is crucial, as an imbalance can lead to NH_3 accumulation that microbes cannot fully utilize. In treatments R1, R3, and R4, the RDP to NFC ratios of 1.7, 1.8, and 1.6 indicate an efficient fermentation combination. An increased RDP availability boosts nitrogen for microbial protein synthesis (Aritonang *et al.*, 2024), enhancing microbial activity. Conversely, the low RDP to NFC ratio in treatment R5 resulted in the lowest NH_3 levels due to the imbalance of RDP and NFC utilized.

Table 2: Effect of RDP: NFC on NH3 Production, Total VFA and protozoa population.

Treatment	NH_3	VFA	Protozoa ($\times 10^5$) cells/mL
R1	5,94 ± 0,50^{d}	179 ± 0,87^{b}	0,39 ± 0,17^{a}
R2	4,53 ± 0,42^{c}	152 ± 0,13^{a}	0,48 ± 0,21^{a}
R3	5,97 ± 0,56^{d}	177 ± 0,10^{b}	0,39 ± 0,17^{a}
R4	5,42 ± 0,26^{d}	190 ± 0,10^{b}	0,41 ± 0,17^{a}
R5	2,52 ± 0,43^{a}	138 ± 0,12^{a}	0,49 ± 0,20^{a}
R6	3,50 ± 0,83^{b}	149 ± 0,57^{a}	0,40 ± 0,17^{a}

Notes: Different superscript indicates significantly different (P<0.05).

Total VFA Production

The effect of the study on the ratio of rumen-degradable protein to non-fiber carbohydrates on total VFA production is shown in Table 2. Based on the ANOVA and Duncan test, the highest VFA production was obtained in treatments R1 (60:35), R3 (65:40), and R4 (65:35) at 179 mM, 177 mM, and 190 mM, respectively, which were not significantly different (P>0.05) from each other. Still, it was significantly different (P<0.05) from the other treatments. The lowest VFA production was achieved in treatments R5 (55:39), R6 (55:41), and R2 (60:40) with values of 138 mM, 149 mM, and 152 mM, respectively, which were also not significantly different from each other. The optimal concentration of volatile fatty acids (VFAs) for supporting rumen microbial growth ranges from 80 to 160 mM. This study demonstrates that VFA production meets these requirements for microbial growth in the rumen (138 to 190 mM). Variations in VFA concentrations are affected by the carbohydrate and protein content of the diet, as well as by increased microbial fermentation activity (Denton *et al.*, 2022).

Non-structural carbohydrates, such as starch, pectin, and simple sugars, ferment more quickly than structural carbohydrates like cellulose and hemicellulose (Izzatulallh *et al.*, 2019). Soluble carbohydrates enhance microbial activity in the rumen, which increases the production of volatile fatty acids (VFAs). Additionally, rumen-degradable protein (RDP) provides the nitrogen necessary for microbial protein synthesis. This process boosts the bacterial population

and fermentation activity, further contributing to the overall production of VFAs (Tanuwiria *et al*., 2022; Setiawan *et al*., 2024). The treatments R1, R3, and R4 exhibited the highest VFA production due to the optimal ratios of RDP to non-fiber carbohydrates (NFC), which were 1.7, 1.8, and 1.6, respectively. This study emphasizes the importance of balancing RDP and NFC to support efficient fermentation. Easily degradable organic matter enhances rumen microbial kinetics, resulting in higher VFA production.

Increased production of volatile fatty acids (VFA) enhances the digestibility of carbohydrates and proteins in the diet by rumen microbes (Wijayanti *et al*., 2022). Volatile fatty acids (VFAs) play a dual role as an energy source and a carbon skeleton for the formation of microbial protein (Rahayu *et al*., 2023). High production of VFAs indicates that feed substrates are highly fermentable, highlighting the importance of carbohydrates and proteins as energy sources and carbon structure for the animal's body (Pazla *et al*., 2023; Muhammad *et al*., 2023; Setiawan *et al*., 2024). An increase in the total volatile fatty acids was observed when animals were fed digestible protein. (Setiawan *et al*., 2024). Other studies have shown that feeding protein protected with tannins increases acetyl CoA (Tanuwiria *et al*., 2023). Similarly, raising the ratio of non-fiber carbohydrates (NFC) in the diet produces the same effect (Pazla *et al*., 2023).

Total Protozoan Population

Table 2 indicates no significant differences in the protozoan population among the treatments ($P > 0.05$). This suggests that the ratio of Rumen Degradable Protein (RDP) to Non-Fiber Carbohydrates (NFC) does not affect the protozoan population in the rumen. Protozoa primarily source their nitrogen from the bacteria they consume. Although RDP and NFC did not have a measurable impact, the protozoan population remained within the normal range, as Muslim *et al*. (2014) noted, i.e., 10^4-10^6 cells/mL. The study by Rosmalia *et al*. (2022) indicated that protozoan populations could be influenced by energy availability and the physical form of their feed. Grinding and pelleting feed can reduce protozoan populations by increasing acidity (Dudi *et al*., 2023), which inhibits their growth. Franzolin *et al*. (2010) emphasized the importance of maintaining a stable rumen pH for developing protozoa.

Several factors influence microbial populations, including temperature, osmotic pressure, reduction-oxidation potential, and dry matter content. Research conducted by Chena *et al*. (2024) indicated that different balances of non-fiber carbohydrates (NFC) in sheep rations result in changes in protozoa populations. It was suggested that organic materials, particularly NFC, which are easily digestible, enhance the availability of starch in the rumen. This abundance of starch supports the protozoa, which utilize it as an energy source, promoting their proliferation. Decreased protozoan populations may lead to reduced methane production, improved microbial energy and protein efficiency, and less hindrance to fiber digestion (Mosoni *et al*., 2011). Additionally, protozoa minimize the risk of acidosis by slowing the conversion of carbohydrates into lactic acid, which helps prevent a rapid decline in rumen pH (Pazla *et al*., 2023; Setiawan *et al*., 2024).

CONCLUSIONS AND RECOMMENDATIONS

This study concluded that the ratio of rumen degradable protein (RDP) to non-fiber carbohydrates (NFC) significantly increased the production of ammonia (NH3) and total volatile fatty acids (VFA). However, this ratio did not affect the protozoan population. The treatments R1, R3, and R4 demonstrated the highest levels of NH3 and total VFA production. The increases in both NH3 and VFA indicate a strong interaction between carbohydrate metabolism and bacterial activity in protein metabolism. While the results were consistent with expectations, further research is recommended to explore various RDP and NFC ratios and to utilize different in vitro methods. This will help better understand their effects on NH3 production, total VFA levels, and protozoan populations. Additionally, the study aims to identify specific molecular species of protozoa and their unique roles in rumen metabolism.

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NOVELTY STATEMENT

The study examined the ratio of rumen-degradable protein to non-fiber carbohydrates and its effect on the production of ammonia (NH_3), total volatile fatty acids (VFA), and protozoa. The results indicated that the highest ratio of rumen-degradable protein to non-fiber carbohydrates significantly enhanced the production of NH_3 and total VFA. This study also showed that production was within the normal range despite the lower protozoan population. The results of this study are anticipated to serve as a foundation for further investigation into the concentration of partial volatile fatty acids (VFAs) as influenced by the offset of rumen degradable protein (RDP) and non-fiber carbohydrates (NFC) in the diet.

AUTHOR'S CONTRIBUTION

All authors contributed equally to the writing of this manuscript.

Conflict of Interest

The authors have declared no conflicts of interest.

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