

## ***In vitro* rumen fermentation of complete feed based on local feed ingredients with different ratios of protein degradability to non-fibrous carbohydrate**

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*Received March 01, 2026; Accepted May 11, 2026*

### **ABSTRACT**

The objective of this study was to clarify *in vitro* ruminal fermentation of complete feeds with different ruminal degradable protein (RDP) - non-fibrous carbohydrate (NFC) ratios. The four complete feeds were formulated using local feed ingredients. Four feed treatments of RDP-NFC ratios were T1 = 0.32 (10.35:32.70); T2 = 0.29 (9.89:34.49); T3 = 0.26 (9.61: 37.45) and T4 = 0.23 (9.36:39.78). The parameters assessed were *in vitro* gas production (IVGP), feed rumen fermentability and microbial protein synthesis (MPS). The results showed that cumulative IVGP in T1 and T2 were higher than in T3 and T4 ( $P < 0.05$ ). However, the IVGP efficiency in T3 did not differ significantly from that observed in T1 and T2, nor was there a significant difference in T4. The molarities of individual VFAs were not differ significantly between treatments ( $P > 0.05$ ), other than isobutyrate. The isobutyrate molarity in T2 was lower than the others ( $P < 0.05$ ). The concentration and efficiency of MPS in T2, T3, and T4 were higher than in T1 ( $P < 0.05$ ). In conclusion, an RDP-NFC ratio of 0.26 (9.61:37.45) in complete feed has optimal effects on ruminal fermentation and MPS.

*Keywords: Rumen gas production, Feed fermentability, Local feeds, Non-fibrous carbohydrate, Ruminal degradable protein*

### **INTRODUCTION**

The last findings of the study recommend the importance of considering ruminal protein degradability (RDP) in ruminant feed formulation (Pormalekshahi *et al.*, 2020; Putri *et al.*, 2021; Valizadeh *et al.*, 2021; Kim *et al.*, 2025). A deficiency of RDP within the diet may lead to ruminal ammonia levels below optimal thresholds, which hinders microbial proliferation, diminishes nutrient digestibility, and suppresses

dry matter intake (Bach *et al.*, 2005). In contrast, RDP provision in excess without commensurate availability of readily fermentable carbohydrates constitutes a wasteful practice, as unused nitrogen is excreted via urine.

Ruminal degradable protein is a fraction of dietary protein; it's hydrolysed by bacterial proteolytic enzymes into peptides, amino acids, and ammonia (NH<sub>3</sub>) within the rumen (Schwab and Broderick, 2017). The ammonia serves as the primary nitrogen source for ruminal microbes,

particularly cellulolytic bacteria, thereby facilitating the synthesis of their own cellular protein, known as “microbial protein” (Wang *et al.*, 2018). Non-fibrous carbohydrates (NFC) constitute readily fermentable carbohydrates, which supply the requisite energy and carbon skeleton required to assimilate ammonia from RDP degradation into their cellular protein, known as “microbial protein”. The microbial protein synthesis (MPS) proceeds inefficiently in the absence of sufficient and temporally synchronised RDP and NFC supplies. An appropriate dietary balance of RDP and NFC represents a strategic approach to maximise overall MPS and fermentability of diets (ruminal fermentation)

Ruminal degradable protein supplementation has been employed to enhance the nutritive value of low-quality roughages (Fauzyah *et al.*, 2017; Pormalekshahi *et al.*, 2020; Dorri *et al.*, 2021; Valizadeh *et al.*, 2021). However, formulations of rations that precisely calibrate for optimal RDP levels and their balance with NFC remain scarce, particularly for indigenous sheep breeds. Previous studies have explored the critical role of synchronising RDP and NFC within in cattle rations. For instance, Syamsu *et al.* (2024) and Tanuwiria *et al.* (2025) recommended specific NFC and RDP levels to optimise VFAs and NH<sub>3</sub>, and included them to optimise MPS in dairy cows (Rosmalia *et al.*, 2022). Research involving sheep has likewise indicated that energy-protein synchronisation improves nitrogen use efficiency from feeds (Valizadeh *et al.*, 2021). Nevertheless, the present study diverges fundamentally from prior research by implementing RDP-NFC ratios in complete feeds for fattening indigenous sheep, utilising combinations of locally sourced agricultural wastes and agro-industrial byproducts—materials characterised by unique degradability profiles that remain largely undocumented. This investigation specifically targets the RDP and NFC balance most compatible with the ruminal ecosystem of local sheep fed agriculture- and industry-based waste diets.

When evaluating efficacy of feed formulation, *in vivo* experimentation persists as the primary benchmark; however, such methods entail substantial costs, extended durations, and intensive labor requirements. An *in vitro* experiment has been widely used to evaluate feed before be-

ing tested *in vivo*, which offers a more precise measurement of digestibility and feed utilisation efficiency (Scicutella *et al.*, 2025). *In vitro* gas production, feed fermentability and microbial protein synthesis will provide a more comprehensive and measurable picture in evaluating feed as well as its impact on environmental management (Hasanah *et al.*, 2020; Li *et al.*, 2025). Accordingly, *in vitro* investigations to clarify the effect of complete feeds with different ruminal degradable protein (RDP) - non-fibrous carbohydrate (NFC) ratios on ruminal fermentation are critically needed, particularly for indigenous sheep breeds, as evaluated through gas production, feed fermentability, and microbial protein synthesis (MPS) parameters

## MATERIALS AND METHODS

Forage feed materials were obtained from Polbangtan Yoma’s grass and forage planting lands and forage planting lands of farmer’s group in the Magelang Regency area. The agricultural industry residues were purchased from producers and distributors in the Central Java region. Rumen fluid as the inoculant of *in vitro* experiment was obtained from slaughtering thin tailed lambs. McDougall solution was used as a buffer solution of *in vitro* feed experiments. The composition of McDougall solution was used as described by Syamsu *et al.* (2024).

### Determination of RDP and NFC Ingredient of Feedstuffs

All feed ingredients except molasses were evaluated for their RDP value. Ruminal degradable protein value was effectively CP degraded and determined based on CP that has disappeared for 3, 6, 12, 24 and 48 hours incubated by the culture batch technique (Tilley & Terry, 1963).

In a single *in vitro* trial, there were 55 anaerobic tubes incubated (40 tubes of sample and 15 blank tubes). Every fermenter tube for sample-contained feed sample (0.55-0.56 g) and incubation media (solution of 10 ml rumen fluid and 40 ml of buffer) were anaerobically incubated in a water bath with a constant temperature of 39°C. The *in vitro* trial was repeated three times. *In vitro* experimental residues of each fermenter tube were filtered and collected

for crude protein analysis.

Effectively CP degraded was determined by fitting data of crude protein percentage that disappeared during the incubation times tested to a linear regression program to obtain the values of degradability parameters (a, b, and c) and then inserting them into the formula of Ørskov and Shand (1997).

$$ED = a + \left( \frac{bc}{c + k} \right)$$

Where:

ED = Effective degradability protein (%)

a = Soluble protein fractions (%)

b = Protein fractions that are insoluble but can be degraded (%)

c = Degradation rate of fraction b (% h<sup>-1</sup>)

k = Rate of digesta flow out from the rumen (assumed 6% h<sup>-1</sup>)

Non-fiber carbohydrate corrected by protein and ash value of experimental diets was calculated following the instructions of Detmann and Filho (2010). The value of NFC was calculated based on nutrient ingredient data of the diet formula.

### Formulation of Feed with Different RDP to NFC Ratios

The experimental diets were composed of nine local feed ingredients consisting of agricultural and agro-industrial waste and legume forage with the composition presented in Table 1. The fresh local feed (tofu waste, *Calliandra calothyrsus*, and *Gliricidia sepium*) was dried (minimum 85% of DM), ground, then mixed with other feedstuffs to get complete feed in accordance with the formulation.

The different RDP-NFC ratios in complete feed treatments are namely T1 = 0.32 (10.35:32.70), T2 = 0.29 (9.89:34.49), T3 = 0.26 (9.61:37.45) and T4 = 0.23 (9.36:39.78). The decreasing ratios of RDP-NFC of complete feed from 0.32 to 0.23 were achieved by decreasing the RDP ingredients from 103.47 to 93.58 g/kg of DM and increasing the NFC ingredients from 326.96 to 397.81 g/kg of DM. The chemical ingredients of complete feed are presented based on dry matter condition (Table 1). The diets contained 137.67 ± 2.73 g/kg of DM (14%) of crude

protein, 668.71 ± 2.66 g/kg of DM (67%) of total digestible nutrients (TDN) and 414.95 ± 9.3 g/kg of DM (42%) fiber (NDF).

### In Vitro Gas Production

*In vitro* gas production were carried out in glass syringes (Menke *et al.*, 1979; Hasanah *et al.*, 2020). First, a 0.20 g sample was inserted into each syringe whose ends had been connected with a rubber tube that could be folded and clamped so that the gas could not escape. The glass syringes contained samples (five replications per treatment) and controls were placed in a water bath for pre-incubation. Rumen fluid of lamb and McDougall solution (1:2 of 30 ml) were added to each syringe glass and infused with CO<sub>2</sub> gas.

All glass syringes were randomly allocated and incubated in a water bath for 48 h at 39°C and shook periodically. Gas production readings were recorded at 0, 2, 4, 8, 12, 16, 24, 36 and 48 h of incubation. Cumulative IVGP was obtained by subtracting the corrected gas volume readings at subsequent times from the initial gas volume at the start of incubation. When 48 h incubation has finished and the fermentation is stopped, the residue of each syringe was collected for organic matter analysis. The *in vitro* organic matter degradability (IVOMD) was determined as OM which disappeared from the initial weight added into the syringe using the following equation:

IVOMD (%) =

$$\frac{OM \text{ of sample} - (OM \text{ of residue-blank})}{OM \text{ of sample}((g))} \times 100 \%$$

### In vitro Fermentability and Microbial Protein Synthesis

*In vitro* fermentability and microbial protein synthesis were conducted by the culture batch technique (Tilley and Terry, 1963) using glass tubes 100 ml. The rumen fluid of the lamb and the McDougall ratio was 1:4 (50 ml). The anaerobic tubes were placed randomly in a water bath for 3 h at 39°C. The pH was immediately measured after incubation stopped and then centrifuged (3000 × g, 15 min, 4°C).

The supernatant resulting from centrifugation was retrieved and further filtered. The filtrate's pH was lowered to <2 with 0.1 N HCl, followed by subdivision into three small bottles

Table 1. Composition And Chemical Ingredients of Experimental Diets with Different RDP to NFC Ratios

Item (g/kg of DM)	Treatments <sup>1</sup>			
	T1	T2	T3	T4
Rice bran	52.50	27.00	101.00	128.50
Cassava meal	111.50	150.00	160.00	161.00
Coconut cake	173.30	140.00	64.00	14.00
Tofu waste	125.00	170.00	162.50	155.00
<i>Calliandra calothyrsus</i>	43.70	116.00	142.50	261.50
<i>Gliricidia sepium</i>	210.00	117.00	121.00	62.00
Water Spinach	210.00	195.00	81.70	20.00
Coffee husk	37.00	48.00	130.00	161.00
Molasses	20.00	20.00	20.00	20.00
Premix	17.00	17.00	17.30	17.00
	<b>100.00</b>	<b>100.00</b>	<b>100.00</b>	<b>100.00</b>
TDN <sup>2</sup>	669.36	669.12	666.95	669.39
CP	141.28	138.25	135.90	135.24
NDF	422.80	422.50	410.50	404.00

TDN = total digestible nutrients, CP = crude protein, RDP = rumen degradable protein, RUP = rumen undegradable protein, NDF = neutral detergents fiber, NFC = nonfibrous carbohydrate by protein and ash corrected.

<sup>1</sup>Treatments of different RDP-NFC ratio in complete feeds: T1 = 0.32 (10.35:32.70); T2 = 0.29 (9.89:34.49); T3 = 0.26 (9.61: 37.45) and T4 = 0.23 (9.36:39.78); <sup>2</sup>TDN calculated based on the percentage of nutrient content proximate components.

and allocation to distinct tubes (designated for VFA, N-NH<sub>3</sub>, and MPS assays). These filtrate-containing tubes were preserved at -20°C pending analysis. Before MPS evaluation, the defrosted filtrate was subjected to additional centrifugation (10,000 × g, 15 min, 4°C) (Hasanah *et al.*, 2020). The efficiency of MPS was calculated from the concentration of MPS per unit of energy available in the rumen, expressed commonly in units of organic matter or fermented feed carbohydrates (Bach *et al.*, 2005).

### Chemical Analysis

Analysis of proximate component and fiber contents of feedstuffs and samples was carried out following the procedure of AOAC (2005). Concentrations of N-NH<sub>3</sub> and individual VFA by the Conway method (Conway, 1958) and GC-FID VFA test method (Adorno *et al.*, 2014), respectively. The Lowry method (Lowry *et al.*, 1951) was employed to estimate MPS.

### Experimental Design and Data Analysis

Treatments that were arranged on a completely randomised design consisted of 4 treatments (RDP-NFC ratios): T1 = 0.32 (10.35:32.70); T2 = 0.29 (9.89:34.49); T3 = 0.26

(9.61: 37.45); and T4 = 0.23 (9.36:39.78). Each treatment was conducted in 5 replications. Data were analysed based on the one-factor approach using the general linear model of the Statistical Package for the Social Sciences (SPSS) 26.0 version program (George and Mallery, 2019). Analysis of variance at a significance level of 5% was performed and proceeded by Duncan's multiple range test to determine the difference among means when the treatment effect showed significantly different  $p < 0.05$ .

## RESULTS AND DISCUSSION

### *In vitro* organic Matter Degradability and Cumulative Gas Production (IVGP)

The results showed that the difference in RDP-NFC ratio significantly affected ( $P < 0.05$ ) the accumulation and efficiency of IVGP, but it did not significantly affect ( $P > 0.05$ ) IVOM degradation and IVOMD (Table 2). Both T1 and T2 produced cumulative IVGP that was equally higher (64.8 ml and 64.00 ml) compared to T3 (55.96 ml), and T4 (44.20 ml) was the lowest one. This implies that rumen microbial fermentation was more extensive for substrates in treatments T1 and T2 than in T3, with T4 exhibiting

Table 2. In Vitro of Organic Matter Degradability and Cumulative Gas Production of Diets with Different RDP to NFC Ratios

Parameters	Treatments <sup>1</sup>				SEM	<i>P</i> -value
	T1	T2	T3	T4		
IVOMD (%)	58.17	59.82	57.69	55.96	1.08	0.69
IVOM degraded (mg)	92.32	92.45	92.16	89.82	1.63	0.94
IVGP (ml)	64.8 <sup>a</sup>	64.0 <sup>a</sup>	55.6 <sup>b</sup>	44.2 <sup>c</sup>	2.12	0.00
IVGP efficiency (ml/mg) <sup>2</sup>	0.71 <sup>a</sup>	0.70 <sup>a</sup>	0.60 <sup>ab</sup>	0.50 <sup>b</sup>	0.03	0.01

IVOMD = in vitro organic matter degradability, IVGP = in vitro gas production; <sup>1</sup>Treatments of different RDP-NFC ratio in complete feeds: T1 = 0.32 (10.35:32.70); T2 = 0.29 (9.89:34.49); T3 = 0.26 (9.61: 37.45) and T4 = 0.23 (9.36:39.78); <sup>2</sup>calculated from IVGP per IVOM degraded.

<sup>1</sup>Treatments of different RDP-NFC ratio in complete feeds: T1 = 0.32 (10.35:32.70); T2 = 0.29 (9.89:34.49); T3 = 0.26 (9.61: 37.45) and T4 = 0.23 (9.36:39.78); <sup>2</sup>TDN calculated based on the percentage of nutrient content proximate components.

<sup>a,b,c</sup> Means with different superscripts in the row indicate significant difference ( $P < 0.05$ ).

the least fermentation.

Among the dietary factors influencing the quantity of gas production is the proportion and characteristics of the substrate (Fan *et al.*, 2025; Li *et al.*, 2025). The elevated IVGP observed in treatments T1 and T2 is likely attributable to their neutral detergent fiber (NDF) content, which is predominantly composed of cellulose and hemicellulose, in conjunction with comparatively lower NFC levels relative to T3 and, especially, T4. Substrates enriched with hemicellulose and cellulose furnish an ample substrate for microbial fermentation (Miranda-Sosa *et al.*, 2024). Materials featuring elevated hemicellulose and cellulose concentrations, coupled with reduced lignin content and diminished NFC levels, promote superior utilisation of fibrous components by rumen microbiota, thereby augmenting IVGP. This effect stems from the lack of VFA accumulation, which could otherwise suppress the functionality of cellulolytic bacteria.

Gas production serves as an indicator of rumen microbial fermentation activity (Ahmed *et al.*, 2021). Accordingly, the findings of this study demonstrate that rumen microbial fermentation activity in diets with RDP-NFC ratios of 0.32 and 0.29 is comparably elevated, surpassing those observed at a ratio of 0.26, with the lowest activity at a ratio of 0.23. The increased proportion of coffee husk and Calliandra incorporation in complete feed formulations, consistent with the RDP-NFC ratio reduction treatments, is presumed to be closely associated with the diminished rumen microbial fermentation activity ob-

served in T3 and T4.

The high level of coffee husk composition (16.10%) contributed to increasing the lignin and tannin of T4 (de Souza Rodrigues *et al.*, 2013). The combination high-level composition of coffee husk (16.10%) and Calliandra *calothyrsus* (26.15%) in the T4 diet contributed to elevated condensed tannins (Messi *et al.*, 2020); it progressively diminishes microbial fermentation activity. Lignin and tannin compounds in the diet obstruct the physical and chemical accessibility of microbial enzymes to reach the substrate (Holik *et al.*, 2025). Amanzougarene and Fondevila (2020) stated that feed components such as tannins determine the volume of gas production which affects the rate of fermentation by covering part of the carbohydrate structure.

The decline in ruminal fermentation activity can be ascribed not only to reduced availability of fermentable substrates but also, potentially, to a decreased proportion of cellulolytic bacteria in treatments T3 and T4. Rumen-degradable protein constitutes the principal nitrogen source for the synthesis of cellulolytic bacteria; consequently, a reduction in dietary RDP may hinder their proliferation. It is postulated that the RDP-NFC ratio, which decreased to 0.26 in T3, induces a reduction in the proportion of cellulolytic bacteria, thereby redirecting fermentation pathways toward the glucogenic pathway.

Although cumulative IVGP of T3 was lower than both T1 and T2, IVGP efficiency showed no significant differences were observed among them ( $P > 0.05$ ). Similarly, IVGP efficiency in T3

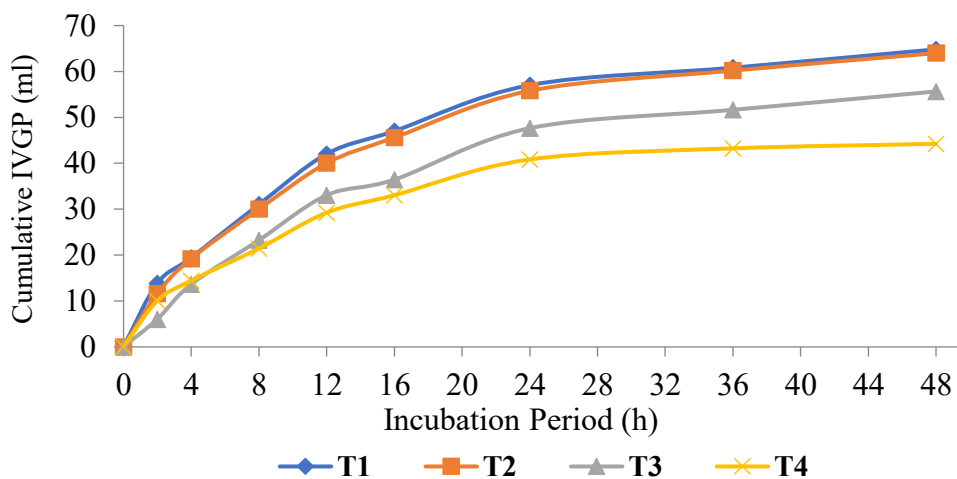


Figure 1. Pattern of *in vitro* gas production (IVGP) of different RDP to NFC ratios diets for 48 h incubation, the RDP and NFC ratios: T1 = 0.32 (10.35:32.70); T2 = 0.29 (9.89:34.49); T3 = 0.26 (9.61: 37.45) and T4 = 0.23 (9.36:39.78).

did not differ from T4 ( $P>0.05$ ), which displayed lower efficiency compared to T1 and T2 ( $P<0.05$ ). *In vitro* gas production efficiency of T3 did not differ significantly from T4 ( $P>0.05$ ); its lower IVGP efficiency than T1 and T2 ( $P<0.05$ ). High IVGP efficiency denotes reduced fermentation efficiency, as a considerable proportion of energy from degraded organic matter is dissipated as gas. Accordingly, the findings of this study indicate that fermentation in feeds with an RDP-NFC ratio of 0.26 is more efficient than those with ratios of 0.32 and 0.29. No significant differences were observed in microbial rumen fermentation efficiency between feeds with RDP-NFC ratios of 0.26 and 0.23. It is highly conceivable, however, that VFA concentrations from 0.26 ratio diet fermentation exhibited superior performance. Nevertheless, it is highly plausible that the primary product from feed fermentation at a 0.26 ratio exhibits superior quality. This improvement is attributable to its lower lignin content, which enhances the potential for greater substrate fermentability.

The T1, T2, and T3 curves still show upward movement until the end of the 48 h of incubation (Figure 1), which means that gas production at T1, T2, and T3 will continue running if the time of incubation is not stopped within 48 h. It indicates that fermentation substrates of T1, T2 and T3 have not been exhausted yet at 48 h incubation; in contrast, T4 has a flat curve at that moment. It implies the low fermentable organic

matter fraction potential of T4.

#### ***In vitro* Fermentation Characteristics and Microbial Protein Synthesis**

The different RDP-NFC ratios in complete feed affected the *in vitro* rumen microbial fermentation characteristics ( $P<0.05$ ), isobutyrate molarity, concentration and efficiency of MPS (Table 3). Although RDP and NFC ratios decreased from 0.32 to 0.23, the pH, N-NH<sub>3</sub> concentration and individual molarity of short-chain VFAs (acetate, propionate, butyrate, isovalerate and n-valerate) were consistent ( $P>0.05$ ). Likewise with the ratio of acetate to propionate.

The pH value of the incubation fluid showed no variation of each other treatments ( $P>0.05$ ). The neutral pH conditions in this study (6.52-6.55) show that the changes in the RDP-NFC ratios from 0.32 to 0.23 do not have a negative impact on microbial fermentation. Previous studies reported that changes in RDP and NFC balances equivalent to a ratio of 0.21-0.31 do not affect the pH of incubation liquid containing rumen fluid inoculants from dairy cows (Rosmalia *et al.*, 2022) and Balinese cattle (Syamsu *et al.*, 2024).

Although the increasing NFC level generally tends to lower ruminal pH due to enhanced fermentation and production of VFAs, the specific impact can vary depending on the type and characteristic of NFC (Yan *et al.*, 2019; de Melo *et al.*, 2021). It was suspected the high NFC level

Table 3. Effect of Different RDP and NFC Ratios of Diets on In vitro Fermentation Characteristic and Microbial Protein Synthesis

Parameters	Treatments <sup>1</sup>				SEM	P-value
	T1	T2	T3	T4		
Ruminal pH	6.54	6.55	6.52	6.55	0.005	0.294
Ruminal N-NH <sub>3</sub> (mg/dl)	7.67	8.42	8.42	7.29	0.321	0.551
Individual volatile fatty acid						
Acetate (mM)	18.96	17.71	18.23	18.45	0.272	0.466
Propionate (mM)	9.35	8.73	8.84	8.97	0.139	0.439
A/P <sup>2</sup>	2.03	2.03	2.07	2.06	0.015	0.722
Butyrate (mM)	7.19	6.74	7.08	7.21	0.13	0.591
Iso butyrate (mM)	0.05a	0.03b	0.06a	0.06a	0.129	0.039
Iso valerate (mM)	0.32	0.31	0.3	0.3	0.053	0.963
n-valerate (mM)	0.44	0.41	0.44	0.45	0.544	0.846
MPS (mg/dl)	115.6 <sup>b</sup>	303.41 <sup>a</sup>	311.62 <sup>a</sup>	289.41 <sup>a</sup>	26.228	0.009
Efficiency of MPS (per l)	0.46 <sup>b</sup>	1.23 <sup>a</sup>	1.23 <sup>a</sup>	1.19 <sup>a</sup>	0.144	0.007

MPS = Microbial protein synthesis. A/P = Acetate to propionate ratio.

<sup>1</sup>Treatments of different RDP and NFC ratio in complete feeds: T1 = 0.32 (10.35:32.70); T2 = 0.29 (9.89:34.49); T3 = 0.26 (9.61:37.45) and T4 = 0.23 (9.36:39.78); <sup>2</sup>A/P = acetate to propionate ratio;

<sup>a,b,c</sup> Means with different superscripts in the row indicate significant difference (P < 0.05).

in this study (T4) is dominated by resistant starches which ferment more slowly, leading to a more gradual production of VFAs and a more stable ruminal pH value. In addition, it is also possible because the interaction of lignin and tannins with NFC enables buffering of pH changes (Jayanegara *et al.*, 2019).

The stability of pH value in neutral was in line with the production of the main short-chain fatty acids, namely acetate, propionate and butyrate, which was not affected by treatments (P > 0.05). The decrease in the ratio of RDP-NFC from 0.32 to 0.23 did not affect the molarity of short-chain VFA production (P > 0.05) except for isobutyrate, which showed a significant difference (P < 0.05). The incubation liquid of T2 had the lowest isobutyrate molarity (0.03 mM) compared to T1 (0.05 mM), T3 (0.06 mM) and T4 (0.06 mM), which were equal to each other (P > 0.05).

Besides isobutyrate acid, the different RDP-NFC ratios also had an impact on the variation of concentration and efficiency of MPS (P < 0.05), but N-NH<sub>3</sub> concentration was not impacted by treatments (P > 0.05). Liu *et al.* (2020) stated that the increasing BCVFAs utilisation for the MPS process reduces N-NH<sub>3</sub> concentration. The concentration of N-NH<sub>3</sub> from 7.29 to 8.42 mg/dl (Table 3) is within a normal range to provide

adequate nitrogen for the MPS process. Chen *et al.* (2022) stated that the minimum concentration of N-NH<sub>3</sub> to support an optimal MPS process is 5 mg/dl. It indicates that the RDP at the lowest level in this study is 93.6 g/kg, or 9.36% of DM (T4), sufficient to meet the nitrogen requirement for MPS. Thus, the variation in MPS (P < 0.05) is suspected to be due to the effect of differences in the proportion of NFC in feed. The availability of NFC in animal feed plays a crucial role in providing energy and carbon skeletons necessary for microbial protein synthesis in the rumen.

Microbial protein synthesis in T2 (303.41 mg/l), T3 (311.62 mg/l), and T4 (289.41 mg/l) was significantly higher (P < 0.05) than in T1 (115.6 mg/l). They are in line with the MPS efficiency; T2 (1.23), T3 (1.23) and T4 (1.19) were significantly higher (P < 0.05) than T1 (0.46) in the efficiency of MPS. It means the microbial synthesis of T1 is less efficient because of unsynchronous energy and nitrogen release or unavailability of enough carbon skeleton (Bezerra *et al.*, 2026). The low proportion of NFC at T1 exacerbates the deficiency of a carbon skeleton because the digested carbohydrates are mainly utilised for ATP energy production. This causes a diminished synthesis of its microbial proteins.

High isobutyrate concentrations without an increase in MPS by the batch culture technique

of *in vitro* experiment strengthen the suspicion that MPS inefficiency occurred in T1. The decrease in the ratio of RDP and NFC from 0.32 (T1) to 0.29 (T2) with an increase of NFC from 327.0 to 344.9 g/kg of DM diet increases the availability of carbon skeletons from the glycolysis pathway for the MPS process, although the VFAs concentration of T2 decreased.

The MPS of T2 (303.41 mg/l) is as high as T3 (311.62 mg/l); however, the molarity of VFAs in T3 is relatively higher than in T2. The RDP and NFC balance of 0.26 with 96.1 and 374.5 g/kg of DM proportion of RDP and NFC (T3) provides a synchronous release of energy and N-NH<sub>3</sub>, as well as increases VFA production. In addition, T3 is observed to provide enough energy for microbial protein synthesis and for the host animals. This phenomenon is suspected because of the differences in energy production pathways. Ruminal bacteria of T2 use acetogenic pathways to produce the energy, while T3 employs the glucogenic pathways. A result of energy production through acetogenic pathways is an increased gas production (Zhang *et al.*, 2022). The majority of hemicellulose and cellulose were degraded by cellulolytic bacteria through acetogenic pathways with a high potential for gas production (CO<sub>2</sub> and CH<sub>4</sub>), resulting in less efficient fermentation due to the considerable loss of fermented carbohydrates as gases (Schut, 2022).

## CONCLUSION

The decrease of the RDP-NFC ratio from 0.32 to 0.23 in complete feed comes with no disruption of *in vitro* ruminal fermentation, whereas pH values are consistently neutral and the N-NH<sub>3</sub> is at normal thresholds. An RDP-NFC ratio of 0.26 (9.61:37.45) in complete feed is recommended because it runs efficiently in nutrient utilisation, as indicated by the high levels of MPS and VFA concentration, with less gas production. This RDP-NFC balance requires validation through *in vivo* trials to ascertain authentic animal responses. The findings of this study may serve as a reference, particularly for establishing optimal RDP-NFC ratio intervals in *in vivo* evaluations of complete feeds for lamb fattening in tropical regions.

## ACKNOWLEDGEMENTS

This study was supported by the Agency for Agricultural Extension and Human Resources Development (BPPSDMP) of the Ministry of Agriculture of the Republic of Indonesia. The author would like to thank the head of Politeknik Pembangunan Pertanian Yogyakarta Magelang (Polbangtan Yoma), Central Java. A special thank is also expressed to Albert Martinus Hardjananta, Rosa Devi Aprilia, and Tarisa Rizky Bahtiyar Putri for their assistance in sampling and collecting data.

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