Nutrient digestibility, characteristics of rumen fermentation, and microbial protein synthesis from Pesisir cattle diet containing non-fiber carbohydrate to rumen degradable protein ratio and sulfur supplement

Mardiati Zain[1] , Ujang Hidayat Tanuwiria[2] , Jasmal Ahmari Syamsu[3] , Yunilas Yunilas[4] , Roni Pazla[1] ,
Ezi Masdia Putri[5] , Malik Makmur[5] , Ummi Amanah[1] , Putri Okta Shafura[1] , and Bima Bagaskara[1]

1. Department of Animal Nutrition and Feed Technology, Faculty of Animal Science Andalas University, Kampus Limau
Manis, Padang, West Sumatera, Indonesia; 2. Ruminant and Feed Chemistry Laboratory, Department of Animal
Nutrition and Feed Technology, Faculty of Animal Sciences, Universitas Padjadjaran. Jl. Raya Bandung-Sumedang
Km. 21, Jatinangor, Sumedang 45363, West Java, Indonesia; 3. Department of Animal Nutrition, Faculty of Animal
Sciences, Universitas Hasanuddin, Jl. Perintis Kemerdekaan KM. 10 Kampus UNHAS Tamalanrea, Makassar; 4. Faculty
of Agriculture, Department of Animal Science, Universitas Sumatera Utara, Medan; 5. Research Center for Animal
Husbandry, National Research and Innovation Agency (BRIN), Jl. Raya Jakarta Bogor 11, Cibinong 16915, Indonesia.
Corresponding author: Mardiati Zain, e-mail: mardiati@ansci.unand.ac.id
Co-authors: UHT: ujang.hidayat@unpad.ac.id, JAS: jasmal.syamsu@unhas.ac.id, YY: yunilas@usu.ac.id,
RP: ronipazla@ansci.unand.ac.id, EMP: ezim002@brin.go.id, MM: malikmakmur27@gmail.com,
UA: ummiamanah24@gmail.com, POS: putrioktashafuraa@gmail.com, BB: bimabagaskara0@gmail.com
Received: 24-11-2023, **Accepted:** 26-02-2024, **Published online:** 22-03-2024

Abstract

Background and Aim: To achieve optimal feed efficiency in ruminants, especially Pesisir cattle, it is necessary to maintain a harmonious equilibrium between energy and protein levels within the rumen. Sulfur supplementation can potentially escalate the energy–protein balance in the rumen. The aim of this study was to explore the formulation of ruminant diets by synchronizing rumen degradable protein (RDP) and non-fiber carbohydrate (NFC) while adding sulfur minerals at different levels. Nutrient digestibility, NH_3 concentration, volatile fatty acids (VFA) production, microbial protein synthesis (MPS), and methane gas production were assessed.

Materials and Methods: We employed a randomized block design with a $2 \times 2 \times 3$ factorial arrangement and examined diverse incubation periods of 6, 24, and 48 h. Treatment consisted of RDP (60% and 65%), NFC (35% and 40%), and sulfur (0%, 0.15%, and 0.3%) levels. In this study, the Tilley and Terry *in vitro* technique, which used Pesisir cattle's rumen fluid, was employed to assess the digestibility of dry matter, organic matter, acid detergent fiber, neutral detergent fiber, and RDP-Rumen undegradable protein. In addition, it measures various rumen fluid attributes, including pH, NH_3, VFA, MPS, and methane gas production.

Results: Treatment with a coordinated combination of 65% RDP and 40% NFC combined with 0.15% sulfur supplement yielded significantly improved digestibility and notably reduced methane gas production ($p < 0.05$).

Conclusion: The enhancement in digestibility and reduction in methane gas emissions can be attributed to the interaction of RDP, NFC, and sulfur. Feed digestibility was increased in the 65% RDP treatment with 40% NFC and 0.15% sulfur, along with a decrease in methane gas production.

Keywords: degradable and undegradable protein, digestibility, non-fiber carbohydrate, rumen fermentation, sulfur.

Introduction

Feed formulations based on crude protein requirements are considered less effective in meeting the protein needs of ruminant livestock, especially in high-producing livestock. For ruminant livestock, protein needs come from microbial proteins that lyse into the post-rumen and bypass proteins. Rumen microorganisms synthesize proteins by incorporating nitrogen (N) from rumen degradable protein (RDP), which easily breaks down in the rumen. However, the bypass protein is difficult to degrade by rumen microbes, so it bypasses directly to the post-rumen. This is called rumen undegradable protein (RUP) or undegradable rumen protein. The balance between RDP and RUP in the diet optimizes the protein needs of ruminants. The use of RDP in diet requires an appropriate source of easily degradable carbohydrates in the rumen non-fiber carbohydrate (NFC) so that the availability of protein and energy to synthesize rumen microbial protein is synchronized. The function of rumen microbes is to digest food substances for ruminant livestock, and the lysed microbes will become a source of protein for the host. Therefore, increasing the rumen microbial population is essential for increasing feed digestibility and protein availability for the host animal [1]. We found

that RDP-NFC-based diet still needs further study to improve its potential for ruminant livestock, especially high-production livestock. Advances in RDP-NFC-based diets can be achieved by supplementing ruminants with minerals that play a crucial role.

Minerals are needed in relatively small quantities but play a very important role in animal feed. Various minerals significantly enhance rumen microbial activity [2]. Sulfur often limits the growth of rumen microbes [3]. To optimize the breakdown of feed within the rumen, it is essential to ensure an adequate supply of minerals. Sulfur is a vital mineral that promotes the development and function of rumen microorganisms. Sulfur is the first limiting nutrient for the efficiency of rumen fermentation and has a major effect on the supply of microbial protein to livestock. This mineral content is very low and is often depleted in feed from tropical regions and in feed from agricultural and plant waste. In addition, the bioavailability of minerals in fiber feed is also low [4]. In fact, we need to supplement our diet with minerals. Sulfur is utilized by rumen microbes to form three sulfur-containing amino acids (methionine, cystine, and cysteine). In addition, sulfur is a source of vitamins thiamin and biotin. Therefore, it is necessary to supplement the diet with sulfur to optimize the fermentation process in the rumen.

Pesisir cattle, a local genetic resource found in the West Sumatra province of Indonesia, have the advantage of adapting to harsh environments, making it an excellent candidate for beef cattle that can thrive in the tropics. Compared with other tropical cattle breeds, Pesisir cattle exhibit reduced cooking loss and higher protein content in their meat [5]. To date, research on the nutritional requirements for the development of Pesisir cattle has not been extensive and has been limited to the identification of potential feed sources and their use [6, 7]. On the basis of the above considerations, more in-depth nutritional studies in the form of synchronizing degradable proteins and soluble carbohydrates with sulfur supplementation may optimize rumen function for sustainable growth of Pesisir cattle. We found a lack of data on sulfur supplementation in RDP-NFC-based diet.

Therefore, this study aimed to evaluate the RDP-NFC-based diet supplemented with sulfur by observing nutrient digestibility, NH_3 concentration, volatile fatty acids (VFA) production, microbial protein synthesis (MPS), and methane gas production in Pesisir cattle through in vitro method.

Materials and Methods

Ethical approval

Because no animals were used in this study, there is no need for ethical approval. Rumen fluid was obtained from the slaughtered cattle of a slaughterhouse.

Study period and location

The study was conducted from June to August 2023 at the Ruminant Nutrition Laboratory of the Faculty of Animal Husbandry at Andalas University.

Experimental diets

The experimental diets consisted of elephant grass, *Indigofera zollingeriana*, *Gliricidia sepium*, corn, bran, tofu dregs, top mix, and sulfur minerals according to treatment. In this study, a 2 × 2 × 3 treatment factorial design was implemented using a randomized block design, which consisted of two levels of RDP (60% and 65%), two levels of NFC (35% and 40%), and three levels of sulfur (0%, 0.15%, and 0.3%). The experimental diet consisted of 50:50% forage and concentrate (based on dry matter [DM]). Nutrients in the experimental diet are presented in Table-1.

In vitro procedure and sample measurement

We performed the *in vitro* investigation using the method described by Tilley and Terry [8]. This method is widely used, and Tilley and Terry confirmed a high correlation between *in vitro* and *in vivo* digestibility. Rumen fluid was obtained from Pesisir cattle slaughtered at a slaughterhouse which was fed with forage and concentrate. Rumen fluid was filtered through a nylon mesh with a pore size of 100 μm and bubbled with CO_2 in a water bath shaker at 39°C. Filtered rumen fluid was diluted 1:4 with a buffer solution (McDonald, 1947). A 2.5-g sample was placed into an Erlenmeyer tube, and 250 mL of rumen fluid and buffer solution were added. *In vitro* feed fermentation in this experiment was performed in an incubator shaker with three types of incubation times: 6 h, 24 h, and 48 h. The fermentation process shall be terminated by immersing the Erlenmeyer tube in a block of ice. Subsequently, the pH of the rumen was measured using a pH meter, and the supernatant was separated from the residue using centrifugation. The rumen fermentation characteristics were evaluated in the supernatant, while the residue was used for feed digestibility analysis. The resulting supernatant was stored in the freezer until NH_3, volatile fatty acids (VFA), and Microbial Protein Synthesis (MPS) analysis was required. The NH_3 concentration was computed following the procedure outlined by Conway and O'Malley [9]. Conway and O'Malley techniques are widely used, and it is simple to perform. Partial VFA concentration was quantified by gas chromatography. Methane gas production (CH_4) was calculated from partial VFA data using the following formula [10]: $CH_4 = 0.45 \times$ acetate $– 0.275 \times$ propionate $+ 0.40 \times$ butyrate. The MPS assessment followed the procedure outlined by Lowry et al. [11].

Statistical analysis

Data collected in this study were analyzed using the Statistical Package for the Social Sciences version 25 software (IBM SPSS Statistics, NY, USA). Duncan's multiple range test was employed to examine the variations among the treatment groups following this analysis. A quadratic regression model using Minitab version 20.3 (Solutions Analytics, Pennsylvania, USA) was applied to process the data for estimating the rumen methane production.

Table-1: Chemical composition of experimental diets (%).

Treatments											
RDP			60						65		
NFC		35			40			35			40	
Sulfur	0	0.15	0.3	0	0.15	0.3	0	0.15	0.3	0	0.15	0.3
Feed compositions												
Elephant grass	40	40	40	40	40	40	30	30	30	37	37	37
Indigofera zollingeriana	10	10	10	7	7	7	3	3	3	7	7	7
Gliricidia sepium	10	10	10	13	13	13	27	27	27	16	16	16
Coconut cake	9	9	9	7	7	7	4	4	4	3	3	3
Corn	16	16	16	5	5	5	20	20	20	12	12	12
Rice bran	12	12	12	14	14	14	4	4	4	3	3	3
Tofu waste	2	2	2	13	13	13	11	11	11	21	21	21
Mineral	1	1	1	1	1	1	1	1	1	1	1	1
Chemical compositions												
CP	15.34	15.34	15.34	15.04	15.04	15.04	16.75	16.75	16.75	16.48	16.48	16.48
TDN	65.09	65.09	65.09	65.02	65.02	65.02	66.23	66.23	66.23	66.46	66.46	66.46
Crude fiber	21.52	21.52	21.52	21.97	21.97	21.97	20.39	20.39	20.39	20.10	20.10	20.10
Dry matter	88.60	88.60	88.60	89.15	89.15	89.15	89.74	89.74	89.74	89.64	89.64	89.64
OM	89.60	89.60	89.60	90.15	90.15	90.15	90.74	90.74	90.74	90.64	90.64	90.64
Ash	10.40	10.40	10.40	9.85	9.85	9.85	9.26	9.26	9.26	9.36	9.36	9.36
EE	4.03	4.03	4.03	3.69	3.69	3.69	3.51	3.51	3.51	3.04	3.04	3.04
NFE	48.71	48.71	48.71	49.46	49.46	49.46	50.09	50.09	50.09	51.01	51.01	51.01
NDF	33.92	33.92	33.92	29.91	29.91	29.91	33.62	33.62	33.62	29.32	29.32	29.32
ADF	20.36	20.36	20.36	18.17	18.17	18.17	18.70	18.70	18.70	17.64	17.64	17.64
CEL*	16.74	16.74	16.74	15.26	15.26	15.26	14.68	14.68	14.68	14.67	14.67	14.67
HEMI*	13.56	13.56	13.56	11.75	11.75	11.75	14.92	14.92	14.92	11.68	11.68	11.68
LIG*	2.90	2.90	2.90	2.23	2.23	2.23	3.49	3.49	3.49	2.34	2.34	2.34
SIL*	0.71	0.71	0.71	0.67	0.67	0.67	0.53	0.53	0.53	0.63	0.63	0.63

RDP=Rumen degradable protein, NFC=Non-fiber carbohydrate, CP=crude protein, TDN=True digestibility Nutrient , OM=organic matter, EE=extract ether, NFE=nitrogen-free extract, ADF=Acid detergent fiber, NDF=Neutral detergent fiber, *CEL=Cellulose, HEMI=Hemicellulose, LIG=Lignin, SIL=Silica

Results

Nutrient digestibility

Diet digestibility increased significantly ($p < 0.01$) with increasing RDP levels. The NFC content and the ratio of RDP, NFC, and sulfur also had a significant ($p < 0.05$) effect on digestibility. *In vitro* of dry matter digestibility (IVDMD) and *In vitro* of organic matter digestibility (IVOMD) with an incubation time of 48 h increased from 54.27% to 62.77% and 55.72% to 63.89%, respectively. *In vitro* of acid detergent fiber digestibility (IVADFD) varied between 59.20% and 69.83%. There was an increase in *In vitro* of Neutral detergent fiber digestibility (IVNDFD), RDP increased from 56.54% to 66.69%, RUP varied between 68.46% and 80.67%, and RUP ranged from 19.33% to 31.54%. Table-2 lists IVDMD, IVOMD, IVADFD, IVNDFD, RDP, and RUP.

Characteristics of rumen fermentation

The synchronization treatment between RDP: NFC:Sulfur did not influence the pH value, total and partial VFA, and methane gas production ($p > 0.05$). Several factors, including total VFA, iso-butyrate, acetate, and valerate, exhibited noteworthy significance ($p < 0.05$) due to variations in RDP content. NH_3 and MPS levels were noticeably ($p < 0.05$) influenced by the RDP: NFC:Sulfur synchronization. NH_3 levels ranged from 13.61 mg/100 mL to 25.63 mg/100 mL of rumen fluid. MPS concentrations ranged from 197.43 to 249.09 mg/100 mL. The total VFA concentration ranged from 113.43 to 118.77 mM. The pH value ranges from 6.59 to 6.90. Tables-3 and 4 present variations in the characteristic values of rumen fermentation. In Figure-1, a relationship is evident between the experimental diet and the extent of ruminal methane gas production, where the incubation periods of 6, 24, and 48 h showed a downward trend in methane gas emissions. The estimated ruminal methane production ranged from 11.58 to 15.77 mM.

Discussion

Digestibility of dry matter and organic matter

As the incubation time for feed fermentation increases, the digestibility of both IVDMD and IVOMD becomes higher. The optimal incubation time is 48 h. The RDP:NFC 65 and 40 treatment and 1.5% sulfur supplementation resulted in the highest digestibility As a mineral source, sulfur triggers an increase in the rumen microbial population, thereby optimizing the digestibility [12]. Supplementation with 0.15% sulfur minerals in the diet at an RDP: NFC ratio of 65:40 produced the highest digestibility. This is consistent with the findings of Pazla *et al*. [12], in which the highest digestibility was observed with 0.17% sulfur mineral supplements. Putri *et al*. [13] reported that feed containing high crude fiber (CF) will thicken plant cell walls to reduce digestibility. On the other hand, feed ingredients are easier to digest if they contain a low amount of CF because the cell walls of

Table-2: IVDMD, IVOMD, IVADFD, IVNDFD, RDP, and RUP (%) of experimental diets.

Time	IVDMD											
	111	112	113	121	122	123	211	212	213	221	222	223
6 h	24.38ab	24.57ab	19.82bc	24.90ab	28.67a	27.60a	27.37a	27.51a	25.42ab	20.82bc	17.82c	17.31c
24 h	41.00c	40.97c	42.26bc	53.87a	48.68bc	51.01abc	51.87ab	51.64ab	51.94ab	54.08a	55.84a	57.30a
48 h	55.65ab	56.07ab	54.27b	55.07ab	56.65ab	56.40ab	61.22ab	58.90ab	60.12ab	60.91ab	62.77a	61.45ab

Time	IVOMD											
	111	112	113	121	122	123	211	212	213	221	222	223
6 h	24.02bc	23.82bc	19.67cd	25.02ab	22.56c	27.56a	27.20a	27.84a	23.39c	18.26cd	17.72d	17.87d
24 h	39.75e	46.92de	43.71de	55.30ab	49.90cd	51.24ab	50.13ab	50.83ab	52.85b	53.79b	56.97ab	58.66a
48 h	57.03ab	57.50ab	55.72b	56.48ab	58.17ab	58.07ab	62.07ab	58.68ab	57.32ab	60.79ab	63.89a	62.33ab

Time	IVADFD											
	111	112	113	121	122	123	211	212	213	221	222	223
6 h	34.25bc	34.31bc	33.31bc	32.22bc	34.75bc	38.12ab	38.93ab	42.52ab	43.90a	43.64a	40.99ab	24.49c
24 h	58.46ab	62.83ab	60.61ab	58.01ab	57.70ab	54.26b	60.96ab	60.17ab	60.92ab	61.68ab	62.68ab	65.75a
48 h	62.21a	67.10bc	59.20de	62.71a	57.12e	60.31de	64.23cd	62.55a	65.11abc	69.79ab	69.83ab	68.11bc

Time	IVNDFD											
	111	112	113	121	122	123	211	212	213	221	222	223
6 h	29.84b	32.48b	27.63b	34.48ab	34.68ab	36.02ab	32.82b	31.16b	37.35a	28.71b	29.97b	25.06c
24 h	45.93e	47.08e	46.36e	56.28ab	49.34ef	52.59de	51.61d	51.84d	54.00cd	54.89cd	57.05ab	58.27ab
48 h	57.22cd	57.38cd	56.73d	56.54d	60.07cd	58.06cd	64.92bc	62.60bc	63.73bc	66.69ab	66.16ab	63.11bc

Time	RDP											
	111	112	113	121	122	123	211	212	213	221	222	223
6 h	51.52e	53.36ef	57.44d	56.86de	56.70de	56.83de	66.90ab	66.79ab	64.84b	61.33c	66.22ab	68.55a
24 h	60.55e	60.86e	62.00de	55.58f	64.85cd	65.56c	62.01de	68.91b	64.61cd	66.61bc	73.96a	74.57a
48 h	68.46e	77.17bc	73.80cd	74.01d	76.95bc	74.48d	79.21bc	77.96bc	73.83d	80.41ab	80.67ab	72.05a

Time	RUP											
	111	112	113	121	122	123	211	212	213	221	222	223
6 h	48.48a	46.65ab	42.57c	43.18bc	43.30bc	43.17bc	33.10ef	33.21ef	35.16e	38.67d	33.79ef	31.45f
24 h	39.46b	39.14b	38.01bc	44.42a	35.15cd	34.44d	37.99bc	31.10e	35.39cd	33.40de	26.04f	25.43f
48 h	31.54a	22.84bc	26.20b	25.99b	23.05bc	25.52b	20.79cd	22.04cd	26.17b	19.59cd	19.33cd	27.95b

111=RDP 60%, NFC 35%, Sulfur 0%; 112=RDP 60%, NFC 35%, Sulfur 0.15%; 113=RDP 60%, NFC 35%, Sulfur 0.3%; 121=RDP 60%, NFC 40%, Sulfur 0%; 122=RDP 60%, NFC 40%, Sulfur 0.15%; 123=RDP 60%, NFC 40%, Sulfur 0.3%; 211=RDP 65%, NFC 35%, Sulfur 0%; 212=RDP 65%, NFC 35%, Sulfur 0.15%; 213=RDP 65%, NFC 35%, Sulfur 0.3%; 221=RDP 65%, NFC 40%, Sulfur 0%; 222=RDP 65%, NFC 40%, Sulfur 0.15%; 223=RDP 65%, NFC 40%, Sulfur 0.3%, IVDMD=*In vitro* of dry matter digestibility, IVOMD=*In vitro* of organic matter digestibility, IVADFD=*In vitro* of Acid detergent fiber digestibility, IVNDFD=*In vitro* of neutral detergent fiber digestibility, RDP=Rumen degradable protein, RUP=Rumen undegradable protein, NFC=Non-fiber carbohydrate
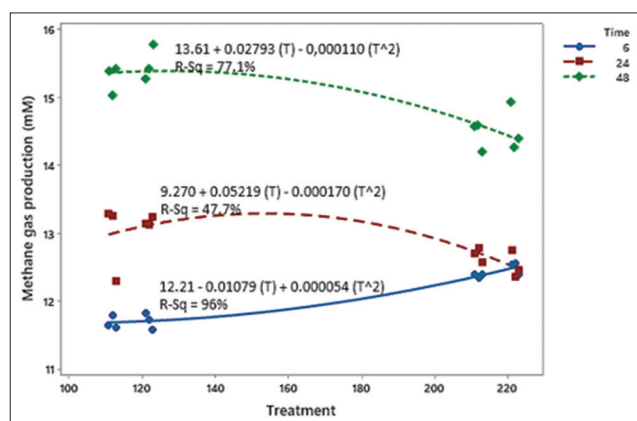Superscript a,b,c,d,e,f in different parameter and observation time means significantly different (p > 0.05)

Figure-1: Correlation between RDP:NFC:Sulfur ration and methane gas production of experimental diets. NFC=Non-fiber carbohydrate, RDP=Rumen degradable protein.

the ingredients are thin, so rumen microbes easily degrade them.

According to Antonius *et al.* [14], an elevated breakdown of organic matter in the diet corresponds to increased nutrient sufficiency for livestock. The digestibility pattern of organic matter is similar to that of DM digestibility. The reason is that most of the dry material is organic. High digestibility increases livestock productivity because nutrients are optimally used [15]. In addition, the nutrient content of the feed plays an important role to play in influencing increased nutrient digestibility. Lv *et al.* [16] and Hao *et al.* [17] have similarly documented that a well-balanced nutrient supply leads to heightened microbial growth and improved nutrient digestibility, leading to enhanced microbial growth.

Degradable and undegradable proteins

To meet the protein requirements of ruminant animals, several factors must be considered. These factors include the provision of N to support microbial

Table-3: Total-VFA and Individual VFA (mM) of experimental diets.

Time	Total-VFA											
	111	**112**	**113**	**121**	**122**	**123**	**211**	**212**	**213**	**221**	**222**	**223**
6 h	101.68	101.7	100.41	101.28	101.26	100.88	102.12	102.36	102.83	102.93	102.27	102.21
24 h	107.59	108.16	105.96	108.55	108.3	107.85	106.81	105.9	106.34	106.21	105.95	106.2
48 h	117.31	116.88	116.53	116.42	116.52	118.77	114.82	114.73	114.77	114.41	113.43	113.72

Time	Acetate											
	111	**112**	**113**	**121**	**122**	**123**	**211**	**212**	**213**	**221**	**222**	**223**
6 h	23.03	23.19	22.52	23.11	23.1	22.73	24.24	24.32	24.56	24.69	24.45	24.14
24 h	27.07	27.35	25.35	27.35	27.31	27.21	26.07	25.83	25.72	25.72	25.26	25.49
48 h	31.8	31.16	31.56	31.28	31.48	32.37	29.22	29.62	28.36	29.76	28.51	28.84

Time	Propionate											
	111	**112**	**113**	**121**	**122**	**123**	**211**	**212**	**213**	**221**	**222**	**223**
6 h	17.58	17.33	16.88	17.13	17.35	17.27	17.07	17.16	17.38	17.23	16.8	16.83
24 h	18.87	19.31	19.28	19.53	19.51	18.95	19.06	18.39	18.95	18.23	18.85	18.85
48 h	20.92	21.19	20.43	20.5	20.38	21.35	20.5	20.68	20.91	19.95	20.23	20.23

Time	Butyrate											
	111	**112**	**113**	**121**	**122**	**123**	**211**	**212**	**213**	**221**	**222**	**223**
6 h	15.27	15.3	15.29	15.3	15.24	15.26	15.23	15.25	15.26	15.41	15.41	15.39
24 h	15.71[a]	15.63[ab]	15.46[bc]	15.50[bc]	15.47[bc]	15.50[bc]	15.53[bc]	15.52[bc]	15.51[bc]	15.45[bc]	15.42[c]	15.41[c]
48 h	17.07	17.09	17.06	17.08	17.11	17.69	17.62	17.38	17.94	17.53	17.46	17.43

Time	Iso-butyrate											
	111	**112**	**113**	**121**	**122**	**123**	**211**	**212**	**213**	**221**	**222**	**223**
6 h	15.26	15.28	15.26	15.26	15.20	15.23	15.20	15.23	15.22	15.22	15.22	15.23
24 h	15.47	15.39	15.34	15.40	15.36	15.40	15.40	15.40	15.40	15.46	15.41	15.45
48 h	15.81	15.78	15.79	15.84	15.84	15.76	15.78	15.66	15.82	15.72	15.77	15.76

Time	Iso-valerate											
	111	**112**	**113**	**121**	**122**	**123**	**211**	**212**	**213**	**221**	**222**	**223**
6 h	15.30	15.35	15.26	15.28	15.21	15.21	15.22	15.22	15.23	15.22	15.22	15.23
24 h	15.27f	15.27f	15.33[ef]	15.46[cd]	15.41[de]	15.47[cd]	15.43[de]	15.46[cd]	15.44[cde]	15.77[a]	15.56[bc]	15.59[b]
48 h	15.97[ab]	15.95[ab]	15.96[ab]	15.98[ab]	15.97[ab]	15.92[ab]	15.99[ab]	15.81[b]	16.02[a]	15.84[ab]	15.85[ab]	15.85[ab]

Time	Valerate											
	111	**112**	**113**	**121**	**122**	**123**	**211**	**212**	**213**	**221**	**222**	**223**
6 h	15.24[ab]	15.25[ab]	15.20[ab]	15.20[ab]	15.16[b]	15.18[ab]	15.16[b]	15.18[ab]	15.18[ab]	15.16[b]	15.17[ab]	15.39[a]
24 h	15.20[c]	15.21[c]	15.20[c]	15.31[bc]	15.24[bc]	15.32[bc]	15.32[abc]	15.30[abc]	15.32[abc]	15.58[a]	15.45[a]	15.41[a]
48 h	15.74	15.71	15.73	15.74	15.74	15.68	15.71	15.58	15.72	15.61	15.61	15.61

111=RDP 60%, NFC 35%, Sulfur 0%; 112=RDP 60%, NFC 35%, Sulfur 0.15%; 113=RDP 60%, NFC 35%, Sulfur 0.3%; 121=RDP 60%, NFC 40%, Sulfur 0%; 122=RDP 60%, NFC 40%, Sulfur 0.15%; 123=RDP 60%, NFC 40%, Sulfur 0.3%; 211=RDP 65%, NFC 35%, Sulfur 0%; 212=RDP 65%, NFC 35%, Sulfur 0.15%; 213=RDP 65%, NFC 35%, Sulfur 0.3%; 221=RDP 65%, NFC 40%, Sulfur 0%; 222=RDP 65%, NFC 40%, Sulfur 0.15%; 223=RDP 65%, NFC 40%, Sulfur 0.3%, VFA=Volatile fatty acids, NFC=Non-fiber carbohydrate, RDP=Rumen degradable protein, Superscript [a,b,c,d,e,f] in different parameter and observation time means significantly different ($p > 0.05$)

growth, resistance to degradation, the ability to supply high bypass protein, and a high biological value [18]. Ruminants rely on degradable proteins and bypass proteins for productivity. In addition, protein solubility in the feed affects protein degradation within the rumen. Feed with a high level of solubility will undergo rapid degradation in the rumen. However, feed with high protein solubility but disulfide bonds tends to undergo slow degradation and may even penetrate the post-rumen. In this study, rumen protein digestibility (RDP) increased with increasing RDP and NFC content, indicating that the synchronization of RDP and NFC positively affected rumen microbial activity. In line with the previous research by Putri *et al.* [13],

increasing RDP positively influences rumen microbes in providing N for protein synthesis. These results are also in line with other research reports that a diet with an RDP: NFC ratio of 60:35 produces the most optimal digestibility [19]. High rumen microbial activity increases nutrient digestibility due to increased RDP [20]. Increasing RUP content can reduce rumen microbial activity due to the low N supply for rumen MPS, thereby reducing digestibility [13, 21, 22].

Proteins degraded in the rumen (RDP) are converted into NH_3, which is later used for MPS. The walls of the rumen absorb ammonia, which is quickly released, and the microbes use some of it. The activity of rumen microbes in synthesizing their body

Table-4: pH, NH3 and MPS of experimental diets.

Time	pH											
	111	**112**	**113**	**121**	**122**	**123**	**211**	**212**	**213**	**221**	**222**	**223**
6 h	6.94	6.91	6.88	6.91	6.81	6.98	6.99	6.98	6.96	6.92	6.96	6.97
24 h	6.97	6.99	6.82	6.87	6.87	6.63	6.63	6.67	6.81	6.92	6.85	6.84
48 h	6.69	6.68	6.89	6.59	6.65	6.78	6.73	6.88	6.82	6.77	6.90	6.85
Time	**NH$_3$ (mg/100mL)**											
	111	**112**	**113**	**121**	**122**	**123**	**211**	**212**	**213**	**221**	**222**	**223**
6 h	4.00[bc]	4.47[bc]	3.12[ef]	2.77[f]	3.83[de]	5.53[b]	5.38[b]	6.17[a]	5.59[ab]	5.53[b]	5.51[b]	5.81[ab]
24 h	8.08[b]	8.93[ab]	10.42[ab]	8.51[ab]	8.93[ab]	10.63[ab]	12.12[a]	10.84[ab]	9.99[ab]	9.35[ab]	9.72[ab]	9.14[ab]
48 h	17.43[bc]	19.34[b]	16.13[bc]	13.61[c]	17.64[bc]	18.28[bc]	20.16[ab]	19.95[b]	22.32[ab]	21.75[ab]	21.79[ab]	25.63[a]
Time	**MPS (mg/100mL)**											
	111	**112**	**113**	**121**	**122**	**123**	**211**	**212**	**213**	**221**	**222**	**223**
6 h	93.18[e]	95.00[e]	122.19[c]	92.28[e]	108.59[d]	87.74[e]	177.48[ab]	182.92[a]	178.39[ab]	171.14[b]	178.39[ab]	171.14[b]
24 h	191.99[d]	191.99[d]	191.99[d]	183.83[e]	194.71[d]	184.74[e]	238.22[b]	234.59[c]	250.00[a]	234.59[b]	227.34[c]	220.99[c]
48 h	200.14[d]	211.02[c]	200.61[d]	197.43[d]	198.33[d]	199.24[d]	243.65[ab]	243.65[ab]	249.09[a]	240.03[ab]	247.28[a]	236.37[b]

111=RDP 60%, NFC 35%, Sulfur 0%; 112=RDP 60%, NFC 35%, Sulfur 0.15%; 113=RDP 60%, NFC 35%, Sulfur 0.3%; 121=RDP 60%, NFC 40%, Sulfur 0%; 122=RDP 60%, NFC 40%, Sulfur 0.15%; 123=RDP 60%, NFC 40%, Sulfur 0.3%; 211=RDP 65%, NFC 35%, Sulfur 0%; 212=RDP 65%, NFC 35%, Sulfur 0.15%; 213=RDP 65%, NFC 35%, Sulfur 0.3%; 221=RDP 65%, NFC 40%, Sulfur 0%; 222=RDP 65%, NFC 40%, Sulfur 0.15%; 223=RDP 65%, NFC 40%, Sulfur 0.3%, MPS=Microbial protein synthesis, NFC=Non-fiber carbohydrate, RDP=Rumen degradable protein, Superscript a,b,c,d,e,f in different parameter and observation time means significantly different (p > 0.05)

protein depends on the pH value, rumen microbes, organic matter, and DM of the feed, feed sources of easily fermentable energy, N compounds, balance of feed sources of energy and protein, ratio of forage and concentrate, and feed rate in the rumen [23]. Crude protein is a nutrient in feed that determines the process of MPS because protein degradation shows that N compounds for rumen microbes can be available, but the N concentration must be sufficient and the energy source does not come from protein [24]. When N availability is in harmony with the energy in the rumen, it promotes an increase in MPS, resulting in increased microbial activity and feed digestion ability. This resulted in augmented *in vitro* MPS in diets with elevated RDP levels [25].

Fiber fraction digestibility (Acid detergent fiber [ADF] and Neutral detergent fiber [NDF])

The highest digestibility of ADF and NDF was observed in the 65% RDP, 40% NFC, and 1.5% sulfur supplementation treatments. A smaller fiber content may increase digestibility because rumen microbes will digest it more easily. According to Wahyono *et al.* [26], a reduced fiber fraction component makes cellulose and hemicellulose digestion more accessible to microorganisms and increases digestibility. In this study, the fiber fraction content (ADF and NDF) decreased with increasing RDP and NFC content. The highest ADF and NDF digestibility in the RDP: NFC:Sulfur 65:40:1.5 treatment resulted in the lowest ADF and NDF content among all treatments (Table-1). In addition, the level of lignin (LIG) in the feed is one of the factors that affect digestibility. LIG binds to the fiber fraction, making it difficult for rumen microbes to break down [27]. Therefore, it is important to balance RDP and NFC in high-fiber diets

to optimize rumen microbial activity. Hao *et al.* [17] emphasized that a well-balanced nutrient supply provides ample substrate for rumen microbes, which promotes their growth and improves nutrient digestibility.

Characteristics of rumen fermentation

In this study, the rumen pH value was found to fall within the standard range, which typically ranges from 5 to 7 [27]. The pH value of the rumen, a factor that determines the rumen fermentation process [19], describes the good or bad condition of the rumen. In addition to its nutritional content, a normal pH value in accordance with rumen conditions will optimize the performance of microbes in digesting feed. This is because the pH of the rumen is suitable so that microbes are able to increase their activity in digesting the feed. Our findings suggest that environmental factors do not affect rumen pH. Rumen microbes thrive in a pH range of 6.0–7.0 and produce ammonia (NH$_3$) and VFAs as byproducts of fermentation. It should be noted that this pH range does not influence the activity or composition of the rumen microbes [28]. In addition, it has cellulolytic microbial activity [29].

Ammonia (NH3) is a very important fermentation product and shows how much feed protein is degraded by rumen microbes [30]. In addition, protein quality in ruminant diet can be determined by measuring NH$_3$ concentration [31]. The NH$_3$ product in the rumen is used by rumen microbes for body synthesis. According to McDonald *et al.* [32], the NH$_3$ concentration required for the growth of rumen microbes ranges between 85 and 300 mg/L. Leng [33] reported that the NH$_3$ concentration in the rumen fluid varies between 1 and 34 mg/100 mL. The concentration of NH$_3$ in this study was within the normal range (Table-4). Rumen NH$_3$ products from protein degradation are

absorbed in the rumen walls and some will be recycled again (N recycling); therefore, it is important in the metabolic processes of ruminant animals [34].

Ruminants are animals whose main energy source is rumen fermentation products, namely, VFAs. There were no differences in the total VFA concentrations between the treatments. The VFA values obtained in this study are consistent with those reported by Savari *et al.* [35] and Rosmalia *et al.* [19], who reported that RDP and NFC levels do not affect total VFA concentrations. High or low concentrations of VFA are influenced by several factors such as the speed at which the feed is fermented, the amount of substrate that is digested, the rate at which the feed is consumed, and the amount of VFA absorbed [36]. However, in this study, the synchronization of RDP: NFC:Sulfur did not affect the total VFA concentration. This result arises from the equilibrium between RDP and NFC release, which facilitates the optimization of protein synthesis within rumen microbes and the generation of elevated levels of fermentation products. VFA production occurs in the rumen, and these VFAs are then taken up by epithelial cells lining the rumen wall. Both VFA production and the equilibrium of absorption within the rumen wall influence the absorption process [37]. The type of feed, depolymerization speed and type of rumen microbes available in the rumen influence VFA production [19]. Acetate contains the highest proportion of all types of VFA. Glucose, the primary source of energy in ruminants, stems primarily from VFA propionate, which serves as a substrate for gluconeogenesis. In addition, acetate and butyrate are precursors of long-chain fatty acid formation [38]. Notably, the individual proportions of VFA remained unaffected by the synchronization of RDP, NFC, and sulfur supplementation in this study. However, the previous study has indicated that an increase in NFC leads to a heightened proportion of propionate [39]. It is assumed that this is due to the different NFC sources. However, further studies by Broderick *et al.* [40] and Paula *et al.* [41] underscored that dietary NFC has a limited impact on the rumen fermentation process, particularly in relation to propionate production. In addition, the molar concentration of n-butyrate increased with increasing NFC levels. Butyrate stimulates growth and expansion in rumen papillae [42]. Metabolic processes that entail the oxidative removal of amino groups and carboxyl groups from branched-chain amino acids (isoleucine, valine, and leucine) result in the formation of branched-chain VFAs, such as iso-valerate, iso-butyrate, and valerate. In addition, carbohydrates and carboxylic acids produce n-valerate [43]. Branched-chain amino acids (leucine, isoleucine, proline, and valine) are synthesized from branched-chain VFAs (such as iso-butyrate, iso-valerate, and n-valerate) produced by cellulolytic bacteria [43]. The balance between RDP and NFC in the diet significantly affected the amount of iso-valerate in the rumen fluid. These results differ from those of previous studies, which indicated that balancing RDP and NFC in the feed did not significantly affect iso-butyrate, n-valerate, and iso-valerate content [39].

Microbial protein synthesis

Rumen microbial growth can be observed through MPS. The greater the number of microbes in the rumen, the more protein they produce [44]. The synthesis of microbial proteins results from the harmonious coordination of energy sources in the feed with proteins that are readily broken down in the rumen [16]. Ruminants obtain most of their protein from micro-organisms in their rumen. Ruminant animals require 80%–90% of their amino acids from microbial proteins [45]. Enhancing MPS requires an increase in the dietary RDP. Conversely, if there is more RUP in the diet, microbes will produce less protein, and the animal will be able to digest less of its food. This study shows that increasing the feed RDP and NFC can provide sufficient N from NH_3 and carbon (C) atoms from VFA. High NFC in the diet can increase MPS because it provides carbohydrates and energy that can be quickly fermented [46]. Proteins and carbohydrates are the primary nutrients required for MPS. A balanced supply of N and C can be determined using the synchronization index formula [47], which calculates the amount of organic matter and N degraded per hour. MPS can also be enhanced by increasing the synchronization index [48]. High MPS levels are also influenced by several mineral and vitamin factors [23]. In this study, sulfur supplementation was able to increase MPS, so the MPS concentration was higher (Table-4) compared with prior research by Putri *et al.* [13] and Rosmalia *et al.* [19]. Sulfur supplementation increases the efficiency of MPS [49].

Methane gas production

Rapid gas production at 24–48 h intervals can be attributed to the exponential growth phase of rumen microbes, which have adapted to their new environment and access to abundant substrates. These data are in accordance with research by Karabulut *et al.* [50], where gas production data almost doubled when the observation time interval was 12 and 24 h. In addition to producing VFA, the fermentation of feed in the rumen also produces gases such as CO_2, CH_4, NO_2, and NO. These gases originate from the fermentation of organic substances in the feed consumed by animals. The quantity of gas produced depends on the nutritional content of the feed. Increased carbohydrate content in the feed leads to increased gas production in the rumen from feed fermentation. Increased activity of cellulolytic bacteria increases acetate production, leading to an increase in the acetate-to-propionate ratio [34]. Methane gas

production originating from rumen fermentation positively correlates with the acetate and propionate ratio and is also an indicator of CH_4 production [51]. Acetate and butyrate are VFAs that produce hydrogen (H) during its formation process, and propionate uses H atoms during its formation process. Methanogenic bacteria use the H atom to synthesize CH_4, so increasing acetate and butyrate will increase methane production [52].

Conclusion

Effective enhancement of rumen fermentation and MPS is achieved by supplementing rumens with a dietary RDP to NFC ratio. The research findings conclude that the interaction between RDP, NFC, and sulfur significantly enhances digestibility and reduces methane gas production. Feed digestibility was increased in the 65% RDP treatment with 40% NFC and 0.15% sulfur, along with a decrease in methane gas production.

Authors' Contributions

MZ and RP: Designed the study and drafted and reviewed the manuscript. EMP: Supervised field and laboratory works. UA, UHT, JAS, and MM: Performed field and laboratory works and tabulated data. YY, POS, and BB: Collected and prepared samples and performed data analysis. All authors have read, reviewed, and approved the final manuscript.

Acknowledgments

Competing Interests

The authors declare that they have no competing interests.

Publisher's Note

Veterinary World remains neutral with regard to jurisdictional claims in published institutional affiliation.

References

1. Zain, M., Despal, Tanuwiria, U.H., Pazla, R., Putri, E.M. and Amanah, U. (2023) Evaluation of legumes, roughages, and concentrates based on chemical composition, rumen degradable and undegradable proteins by *in vitro* method. *Int. J. Vet. Sci*., 12(4): 528–538.
2. Vigh, A., Criste, A., Gragni, K., Moquet, L. and Gerard, C. (2023) Ruminal solubility and bioavailability of inorganic trace mineral sources and effects on fermentation activity measured *in vitro*. *Agriculture*, 13(4): 879.
3. Rosmalia, A., Astriani, A Sahroni, W.P., Permana, I.G. and Despal, D. (2022) Effect of rumen degradable protein and sulfur supplementation on *in vitro* digestibility and ruminal fermentation. *IOP Conf. Ser. Earth Environ. Sci.*, 951: 012013.
4. Philp, J.N.M., Cornish, P.S., Te, K.S.H., Bell, R.W., Vance, W., Lim, V., Li, X., Kamphayae, S. and Denton, M. (2021) Insufficient potassium and sulfur supply threaten the productivity of perennial forage grasses in smallholder farms on tropical sandy soils. *Plant Soil*, 461(1): 617–630.
5. Khasrad, K, Sarbaini, S, Arfa`I., A and Rusdimansyah, R. (2017) Effect of cattle breeds on the meat quality of longissimus dorsi muscles. *Pak. J. Nutr.*, 16: 164–167.
6. Putri, E.M., Zain, M., Warly, L. and Hermon, H. (2019) *In vitro* evaluation of ruminant feed from West Sumatera based on chemical composition and content of rumen degradable and rumen undegradable proteins. *Vet. World*, 12(9): 1478–1483.
7. Pazla, R., Adrizal., A and Sriagtula, R. (2021) Intake, nutrient digestibility and production performance of Pesisir cattle fed *Tithonia diversifolia* and *Calliandra calothyrsus*-based rations with different protein and energy ratios. *Adv. Anim. Vet. Sci.*, 9(10): 1608–1615.
8. Tilley, J.M.A. and Terry, R.A. (1963) A two-stage technique for *in vitro* digestion of forage crops. *J. Br. Grassland Soc.*, 18(2): 104–111.
9. Conway, E.J. and O'Malley, E. (1942) Microdiffusion methods. Ammonia and urea using buffered absorbents (revised methods for ranges greater than 10μg. N). *Biochem. J.*, 36(7–9): 655–661.
10. Moss, A.R., Jouany, J.P. and Newbold, J. (2000) Methane production by ruminants: Its contribution to global warming. *Anim. Res.*, 49(3): 231–253.
11. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the Folin reagent. *J. Biol. Chem.*, 193(1): 265–275.
12. Pazla, R., Zain, M., Ryanto, H.I. and Dona, A. (2018) Supplementation of minerals (phosphorus and sulfur) and *Saccharomyces cerevisiae* in a sheep diet based on a cocoa by-product. *Pak. J. Nutr.*, 17(7): 329–335.
13. Putri, E.M., Zain, M., Warly, L. and Hermon, H. (2021) Effects of rumen-degradable-to-undegradable protein ratio in ruminant diet on *in vitro* digestibility, rumen fermentation, and microbial protein synthesis. *Vet. World*, 14(3): 640–648.
14. Antonius, A., Pazla, R., Putri, E.M., Negara, W., Laia, N., Ridla, M., Suharti, S., Jayanegara, A., Asmairicen, S., Marlina, L. and Marta, Y. (2023) Effectiveness of herbal plants on rumen fermentation, methane gas emissions, *in vitro* nutrient digestibility, and population of protozoa. *Vet. World*, 16(7): 1477–1488.
15. Sharif, M., Qamar, H. and Wahid, A.A. (2019) Effect of rumen degradable protein concentrations on nutrient digestibility, growth performance and blood metabolites in Beetal kids. *Concepts Dairy Vet. Sci.*, 2(5): 249–253.
16. Lv, X., Cui, K., Qi, M., Wang, S., Diao, Q. and Zhang, N. (2020) Ruminal microbiota and fermentation in response to dietary protein and energy levels in weaned lambs. *Animals (Basel)*, 10(1): 109.
17. Hao, X., Diaoa, X., Yu, S., Ding, N., Mu, C., Zhao, J. and Zhang, J. (2018) Nutrient digestibility, rumen microbial protein synthesis, and growth performance in sheep consuming rations containing sea buckthorn pomace. *J. Anim. Sci.*, 96(8): 3412–3419.
18. Tedeschi, L.O., Fox, D.G., Fonseca, M.A. and Cavalcanti, L.F.L. (2015) Models of protein and amino acid requirements for cattle. *Rev. Bras. Zootec.*, 44(3): 109–132.
19. Rosmalia, A., Permana, I.G. and Despal, D. (2022) Synchronization of rumen degradable protein with non-fiber carbohydrate on microbial protein synthesis and dairy ration digestibility. *Vet. World*, 15(2): 252–261.

20. Javaid, A., Shahzad, M.A., Nisa, M. and Sarwar, M. (2011) Ruminal dynamics of ad libitum feeding in buffalo bulls receiving different level of degradable protein. *Livest. Sci.*, 135(1): 89–102.

21. Valizadeh, A., Kazemi-Bonchenari, M., Khodaei-Motlagh, M., Moradi, M.H. and Salem, A.Z.M. (2021) Effects of different rumen undegradable to rumen degradable protein ratios on performance, ruminal fermentation, urinary purine derivatives, and carcass characteristics of growing lambs fed a high wheat straw-based diet. *Small Rum. Res.*, 197: 106330.

22. Akhtar, M., Nisa, M. and Javais, J. (2017) Effect of varying levels of dietary rumen undegradable protein on dry matter intake, nutrient digestibility and growth performance of crossbred cattle heifers. *Gomal Univ. J. Res.*, 33(2): 58–67.

23. Uddin, M.J., Haque, K.Z., Jasimuddin, K.M. and Nurul, I.M. (2015) Nutritional evaluation and chemical compositions of feedstuffs for ruminant using *in vitro* gas production technique. *Ann. Vet. Anim. Sci.*, 2(2): 2312–9123.

24. Dineen, M., McCarthy, B., Dillon, P., LaPierre, P.A., Fessenden, S., Matthews, C., Galvin, N. and Van Amburgh, M.E. (2020) Rumen metabolism, omasal flow of nutrients, and microbial dynamics in lactating dairy cows fed fresh perennial ryegrass (*Lolium perenne* L.) not supplemented or supplemented with rolled barley grain. *J. Dairy Sci.*, 103(12): 11332–11348.

25. Kelln, B.M., Penner, G.B., Acharya, S.N., McAllister T.A., McKinnon, J.J., Saleem, A.M., Biligetu, B. and Lardne, H.A. (2023) Effect of mixtures of legume species on ruminal fermentation, methane, and microbial nitrogen production in batch and continuous culture (RUSITEC) systems. *Can. J. Anim. Sci.*, 103(4): 326–337.

26. Wahyono, T., Sugoro, I., Jayanegara, A., Wiryawan, K.G. and Astuti, D.A. (2019) Nutrient profile and *in vitro* degradability of new promising mutant lines sorghum as forage in Indonesia. *Adv. Anim. Vet. Sci.*, 7(9): 810–818.

27. Puniya, A.K., Singh, R. and Kamra, D.N. (2015) Rumen Microbiology: From Evolution to Revolution. Springer, Berlin, India.

28. Poulsen, M., Jensen, B.B. and Engberg, R.M. (2012) The effect of pectin, corn and wheat starch, inulin, and pH on *in vitro* production of methane, short-chain fatty acids and on the microbial community composition in rumen fluid. *Anaerobe*, 18(1): 83–90.

29. Pazla, R., Jamarun, N., Zain, M., Arief, A, Yanti, G., Putri, E.M. and Candra, R.H. (2022) Impact of *Tithonia diversifolia* and *Pennisetum purpureum*-based ration on nutrient intake, nutrient digestibility and milk yield of Etawa crossbreed dairy goat. *Int. J. Vet. Sci.*, 11(3): 327–335.

30. Noersidiq, A., Marlida, Y., Zain, M., Kasim, A., Agustin, F. and Huda, N. (2020) The Effect of urea levels on *in-vitro* digestibility and rumen fermentation characteristic of ammoniated oil palm trunk. *Int. J. Adv. Sci. Eng. Inf. Technol.*, 10(3): 1258–1262.

31. Zain, M., Putri, E.M., Rusmana, W.S.N., Erpomena., E and Makmur, M. (2020) Effects of supplementing *Gliricidia sepium* on ration based ammoniated rice straw in ruminant feed to decrease methane gas production and to improve nutrient digestibility (*in-vitro*). *Int. J. Adv. Sci. Eng. Inf. Technol.*, 10(2): 724–729.

32. McDonald, P., Edwards, R.A., Greenhalgh, J.F.D., Morgan, C.A., Sinclair, L. and Wilkinson, R.G. (2010) Animal Nutrition. 7th ed. Pearson, London, UK.

33. Leng, R.A. (1980) Principle and Practice of Feeding Tropical Crop and by Products to Ruminant. Department of Biochemistry and Nutrition University of New England. Armidale, Australia.

34. Zhang, G.W., Wang, C., Du, H.S., Wu, Z.Z., Liu, Q., Guo, G., Huo, W.J., Zhang, J., Zhang, Y.L., Pei, C.X. and Zhang, L. (2020) Effects of folic acid and sodium selenite on growth performance, nutrient digestion, ruminal fermentation and urinary excretion of purine derivatives in Holstein dairy calves. *Livest. Sci.,* 231: 1–7.

35. Savari, M., Khorvash, M., Amanlou, H., Ghorbani, G.R., Ghasemi, E. and Mirzaei, M. (2018) Effects of rumen-degradable protein: Rumen-undegradable protein ratio and corn processing on production performance, nitrogen efficiency, and feeding behavior of Holstein dairy cows. *J. Dairy Sci.*, 101(2): 1111–1122.

36. Beckett, L., Gleason, C.B., Bedford, A., Liebe, D., Yohe, T.T., Hall, M.B., Daniels, K.M. and White, R.R. (2021) Rumen volatile fatty acid molar proportions, rumen epithelial gene expression, and blood metabolite concentration responses to ruminally degradable starch and fiber supplies. *J. Dairy Sci.*, 104(8): 3988–3999.

37. Gleason, C.B., Beckett, L.M. and White, R.R. (2022) Rumen fermentation and epithelial gene expression responses to diet ingredients designed to differ in ruminally degradable protein and fiber supplies. *Sci. Rep.*, 12: 2933.

38. Morales, A.G., Vibart, R.E., Li, M.M., Jonker, A., Pacheco, D. and Hanigan, M.D. (2021) Evaluation of Molly model predictions of ruminal fermentation, nutrient digestion, and performance by dairy cows consuming rye-grass-based diets. *J. Dairy Sci.*, 104(9): 9676–9702.

39. Ma, T., Tu, Y., Zhang, N.F., Deng, K.D. and Diao, Q.Y. (2015) Effect of the ratio of non-fibrous carbohydrates to neutral detergent fiber and protein structure on intake, digestibility, rumen fermentation, and nitrogen metabolism in lambs. *Asian Australas. J. Anim. Sci.*, 28(10): 1419–1426.

40. Broderick, G.A., Faciola, A.P. and Armentano, L.E. (2015) Replacing dietary soybean meal with canola meal improves production and efficiency of lactating dairy cows. *J. Dairy Sci.*, 98(8): 5672–5687.

41. Paula, E.M., Monteiro, H.F., Silva, L.G., Benedeti, P.D.B., Daniel, J.L.P., Shenkoru, T. and Faciola, A.P. (2016) Effects of replacing soybean meal with canola meal differing in rumen-undegradable protein content on ruminal fermentation and gas production kinetics using 2 *in vitro* systems. *J. Dairy Sci.*, 100(7): 5281–5292.

42. Zhong, H., Yu, W., Wang, M., Lin, B., Sun, X., Zheng, N., Wang, J. and Zhao, S. (2023) Sodium butyrate promotes gastrointestinal development of preweaning bull calves via inhibiting inflammation, balancing nutrient metabolism, and optimizing microbial community functions. *Anim. Nutr.*, 14: 88–100.

43. Tresia, G.E., Tiesnamurti, B., Alwiyah, A. and Anwar, A. (2023) Effects on *in vitro* digestibility and rumen fermentation of maize straw silage as a partial dietary replacement for Napier grass. *J. Anim. Feed Sci.*, 33(1): 1–9.

44. Zain, M., Rusmana, W.S.N., Erpomen, E, Putri, E.M. and Makmur, M. (2019) The effects of leguminous supplementation on ammoniated rice straw based completed feed on nutrient digestibility on *in vitro* microbial protein synthesis. *IOP Conf. Ser. Earth Enviorn. Sci.*, 287(1): 012018.

45. Russell, J.B., Muck, R.E. and Weimer, P.J. (2009) Quantitative analysis of cellulose degradation and growth of cellulolytic bacteria in the rumen. *FEMS Microbiol. Ecol.*, 67(2): 183–197.

46. Wei, Z., Xie, X., Xue, M., Valencak, T.G., Liu, J. and Sun, H. (2021) The effects of non-fiber carbohydrate content and forage type on rumen microbiome of dairy cows. *Animals* (*Basel*), 11(12): 3519.

47. Sinclair, L.A., Garnsworthy, P., Newbold, J.R. and Buttery, P.J. (1993) Effect of synchronizing the rate of dietary energy and nitrogen release on rumen fermentation and microbial protein synthesis in sheep. *J. Agric. Sci.*, 120(2): 251–263.

48. Zhang, J., Zheng, N., Shen, W., Zhao, S. and Wang, J. (2020) Synchrony degree of dietary energy and nitrogen release influences microbial community, fermentation, and protein synthesis in a rumen simulation system. *Microorganism*, 8(2): 231.

49. Supapong, C. and Cherthong, A. (2020) Effect of sulfur concentrations in fermented total mixed rations containing fresh cassava root on rumen fermentation. *Anim. Product.*

Sci., 60(11): 1429–1434.

50. Karabulut, A., Canbolat, O., Kalkan, H., Gurbuzol, F., Sucu, E. and Filya, I. (2007) Comparison of *in vitro* gas production, metabolizable energy, organic matter digestibility and microbial protein production of some legume hays. *Asian Australas. J. Anim. Sci.*, 20(4): 517–522.

51. Ábrego-Gacía, A., Poggi-Varaldo, H.M., Mendoza-Vargas, A., Mercado-Valle, F.G., Ríos-Leal, E., Ponce-Noyola, T. and Calva-Calva. G. (2021) Effects of fermented oat straw as a lovastatin carrier on *in vitro* methane production and rumen microbiota. *Front. Energy Res.*, 9: 630701.

52. Sun, X., Cheng, L., Jonker, A., Munidas, S. and Pacheco, D. (2022) A review: Plant carbohydrate types-the potential impact on ruminant methane emissions. *Front. Vet. Sci.*, 9: 880115.

****