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Improving Rumen Fermentation Characteristics and Nutrient Digestibility by Increasing Rumen Degradable Protein in Ruminant Feed using *Tithonia diversifolia* and *Leucaena leucocephala*

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ABSTRACT

The aim of this research was to discover the effect of increasing rumen degradable protein in ruminant diet using *Tithonia diversifolia* and *Leucaena leucocephala* on rumen fermentation characteristics, microbial protein synthesis, protozoa population, methane production, and nutrient digestibility. The *in vitro* method was used in this research in randomized block design with three treatments and five replications. Three diets were formulated containing 50, 55 and 60% rumen degradable proteins (RDP) and were represented as 50, 55 and RDP60. The RDP50 diet contained RDP 50% of crude protein. The 55 and RDP60 diet contained RDP 55 and 60% of crude protein, respectively. The results showed Total Volatile Fatty Acid (100mM vs 98.93mM and 95.60mM) and microbial protein synthesis (88.86mg/100mL vs 84.03mg/100mL and 81.20mg/100mL) were higher in RDP55 diet ($P<0.05$). Decreasing protozoa population and methane production were observed with increasing RDP in diet ($P<0.05$). NH_3 production (15.30-16.15mM) and protein digestibility (62.52-66.12%) increased with the rising of RDP level ($P<0.05$). Beside that, higher dry matter (64.56% vs 61.58% and 58.20%), organic matter (66.67% vs 63.89% and 60.69%), crude fiber (65.37% vs 62.07% and 60.78%), and nitrogen-free extract (66.45% vs 62.87% and 60.57%) digestibility were observed in RDP55 diet ($P<0.05$). Our study revealed that diet containing RDP 55% of crude protein in ruminant feed using *T. diversifolia* and *L. leucocephala* improved rumen fermentation characteristics and nutrient digestibility. Further research is needed to discover the effect of the in vivo feeding trial on animals.

Key words: Legume, Nutrient digestibility, Rumen fermentation characteristics, Rumen degradable protein, Ruminant feed

INTRODUCTION

Inadequate quantity and low quality of feed cause poor ruminant nutrition. Feeding ruminants takes a crucial role in the maintenance and production of ruminants. In case to increase maintenance and production, ruminant feed formulation must pay attention to the nutrient degradability, especially protein and carbohydrate since those are used by both ruminant and rumen microbes. Rumen microbes require rumen degradable protein (RDP) and fermentable carbohydrates in order to effectuate microbial protein synthesis. Meanwhile, ruminant requires *bypass* microbial protein synthesized by rumen microbes and *bypass* protein (Putri et al. 2021). Tedeschi et al. (2015) stated that protein for ruminant has three significant roles: to fulfill the RDP needs of rumen microorganisms for maximum nutrient digestibility and microbial protein synthesis; to fulfill the protein needs of ruminants for maintenance, development, health, and reproduction; and to fulfill the amino acid needs of highly productive ruminants. Feeding leguminous that is higher in protein can supply the protein requirement of ruminant, improve rumen fermentation and increase nutrient digestibility (Camero et al. 2001). A low-protein diet will disrupt microbial protein synthesis, ruminal digestion, existence of nitrogen and carbohydrates by the ruminant (Durango et al. 2021).

Tithonia diversifolia and *Leucaena leucocephala* are leguminous, have been extensively examined and reported to have a high nutritional value, with a high crude protein content, as well as amino acids, vitamins and minerals

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(Ramírez-Rivera et al. 2010; Durango et al. 2021). García et al. (2017) reported that *T. diversifolia* has 24.20% of protein, 40.2% of RDP, 0.32-0.38% of phosphorus, 1.96-2.47% of calcium, and 0.05-0.07% of magnesium. Protein and minerals, especially phosphorus and sulphur, are crucial for microbial protein synthesis. In addition, *T. diversifolia* has low lignin content, leading to high nutrient digestibility (Olabode et al. 2007). *T. diversifolia*'s nutritional value is mainly kept consistent during the dry season (Mauricio et al. 2017). Besides, *T. diversifolia* is a tough weed that proliferates and can help with soil renewal (Olabode et al. 2007; Arief et al. 2020). Not only high nutritional value, *T. diversifolia*'s also has value as a possible methane reducer. Due to their inhibitory effects on rumen ciliate protozoa, anti-nutrients including tannins and saponins have been demonstrated to reduce methane generation in the rumen (Delgado et al. 2012; Ribeiro et al. 2016). The previous study from Holguín et al. (2020) has shown a mixture of *Pennisetum purpureum* and *T. diversifolia* silage (67%:33%) declined methane production until 1.53 mmol/g compared with 100% *P. purpureum* (2.43mmol/g).

L. leucocephala is regarded as a high nutritional value forage legume that includes high levels of crude protein, minerals, amino acid content and generates large amounts of biomass (Ningrat et al. 2019; Durango et al. 2021). *L. leucocephala* has a low crude fiber and high tannin content, it prevents excessive protein breakdown in the rumen (bypass protein), allowing small intestine protein absorb true protein (Ningrat et al. 2019). Meena Devi et al. (2013) reported that *L. leucocephala* has 25.9% protein, 2.36% calcium and 0.23% phosphorus. *L. leucocephala* can improve rumen function by speeding up forage breakdown in the rumen. The high protein content will enhance ammonia availability in the rumen, encouraging microbial growth and speeding up forage breakdown. Some studies have shown that 20% of *L. leucocephala* in diet increased nutrient digestibility, rumen fermentability, and reduced methane production (Barros-rodríguez et al. 2013; Ningrat et al. 2019).

This study used an *in vitro* method to examine the impact of novel feeding strategies that increased rumen degradable protein in ruminant feed using *T. diversifolia* and *L. leucocephala* on rumen fermentation characteristics, microbial protein synthesis, protozoa population, methane production and nutrient digestibility.

MATERIALS AND METHODS

Ethical Approval

Ethical approval was not required because this study did not use any live animals.

Study Period and Location

This research was taking place at Ruminant Laboratory in the Faculty of Animal Science, Andalas University, Padang, Indonesia from September to December 2020.

Sample Preparation and Experimental Diets

Fresh native grasses, *Tithonia diversifolia* and *Leucaena leucocephala* were harvested from tropical forages at UPT Teaching Farm, Andalas University, Padang, Indonesia. The samples were dried in a forced-air oven at 60°C for 24 hours before being milled through a 1 mm sieve. Other feeds were obtained from a poultry shop. These samples were formulated into diets with three different amounts of RDP:RUP (50%:50%, 55%:45%, 60%:40%). Proximate analysis was utilized to quantify the amount of dry matter (DM), crude protein (CP), crude fiber (CF), and ether extract (EE) in each diet, according to AOAC (2005) standards. To determine Nitrogen-free extract (NFE) content formula NFE = 100% - (water + ash + CP + EE + CF) was used. Sutardi's formula was used to calculate True Digestible Nutrient (TDN) (Sutardi 1980). The chemical composition of each diet can be seen in Table 1.

Experimental Design

This study used an *in vitro* approach with three treatments and five replications in a randomized block design. The treatments were 50, 55 and RDP60 which contained 50, 55 and 60% rumen degradable proteins (RDP), respectively. The variables observed were pH value of rumen fluid, total VFA concentration, NH_3 concentration, microbial protein synthesis, protozoa population, methane production, and nutrient digestibility.

In vitro Method

Samples of diets were incubated with buffered rumen according to Tilley and Terry method (Tilley and Terry 1963). Rumen liquor was acquired from a slaughterhouse in Padang city from three Kacang goats fed roughage and concentrate *ad libitum* with an average BW±20 kg. The rumen content was filtered with four layers of nylon (100m strainer size) and then placed into thermos flasks after the slaughter (39ºC). As suggested by McDougall (1947), filtered rumen liquor was diluted with buffer solution at a ratio of 1:4 (rumen fluid:buffer solution). An amount of 2.5g sample was mixed with 250mL of mixed solution (rumen liquor and buffer) in each Erlenmeyer flask and incubated anaerobically by injecting CO_2 gas into the Erlenmeyer, which was then immediately sealed with a rubber lid. Each sample was replicated three times. Each Erlenmeyer was kept in an orbital shaking incubator at 39°C and 100rpm for 48h. The pH of the Erlenmeyer was tested using a pH meter after it was immersed in ice water for 48h to cease microbial activity.

The supernatant and residue were then separated using a centrifuge at 3000rpm for 5min at 4°C. The NH_3, total VFA, microbial protein synthesis and number of protozoa analyses supernatant was stored in bottles in a -18ºC freezer. The Conway and O'Malley (Conway and O'Malley 1942) method was used to determine the NH_3 concentration. Steam distillation method was used according to Abdurachman and Askar (2000) to determine total VFA concentration. Lowry's technique (Lowry et al. 1951) was used to determine microbial protein production. The number of protozoa was determined by way of Ogimoto and Imai (1981). Methane production was determined by using partial VFA components followed by Abdurachman and Askar (2000) procedures. Moss et al. (2000) equation was used to calculate *in vitro* methane (CH_4) emissions based on partial VFA component concentrations. Meanwhile, the residue was filtered through Whatman paper No.41 and dried in an oven at 60ºC in 48h for nutrient digestibility. Nutrient digestibility was

determined by subtracting nutrient residue from their initial amounts before the incubations, respectively. The *in vitro* was conducted in five replications, and each replication was served by two bottles containing only a mixed solution of rumen liquor and buffer. However, no sample was incubated as blanks or as a correction factor in the calculation of nutrient digestibility. These formulas were used to compute *in vitro* nutrient digestibility:

$$\text{IVDMD} = \frac{DM\ samples - (DM\ residual - DM\ blanks)}{DM\ samples}\ x\ 100\%$$

$$\text{IVOMD} = \frac{OM\ samples - (OM\ residual - OM\ blanks)}{OM\ samples}\ x\ 100\%$$

$$\text{IVCPD} = \frac{CP\ samples - CP\ residual}{CP\ samples}\ x\ 100\%$$

$$\text{IVEED} = \frac{EE\ samples - EE\ residual}{EE\ samples}\ x\ 100\%$$

$$\text{IVCFD} = \frac{CF\ samples - CF\ residual}{CF\ samples}\ x\ 100\%$$

$$\text{IVNFED} = \frac{NFE\ samples - NFE\ residual}{NFE\ samples}\ x\ 100\%$$

Where:

IVDMD: *In vitro* dry matter digestibility, IVOMD: *In vitro* organic matter digestibility, IVCPD: *In vitro* crude protein digestibility, IVEED: *In vitro* extract ether digestibility, IVCFD: *In vitro* crude fiber digestibility, IVNFED: *In vitro* nitrogen-free extract digestibility, DM: dry matter, OM: organic matter, CP: crude protein, EE: extract ether, CF: crude fiber, NFE: Nitrogen-Free Extract.

Statistical Analysis

Statistical Package for the Social Sciences (SPSS) software was used to analyze the collected data using analysis of variances (IBM SPSS Statistics, USA) version 21.0.

RESULTS

Rumen Fermentation Characteristics

The increase of RDP in ruminant feed using *T. diversifolia* and *L. leucocephala* as legumes did not influence pH value of rumen fluid (P>0.05), despite there was an increase in pH value. In this study, the high RDP levels tended to reduce the total VFA production (P<0.05). Diet with RDP50 has shown the highest total VFA production compared to 55 and RDP60 diet. It indicated that RDP50 has a high content of fermentable carbohydrate. Meanwhile, NH_3 production was increased in line with increasing of RDP levels (P<0.05). It was revealed that these diets are high in soluble protein. Ruminant diets containing 50% RDP can improve rumen fermentation properties. Rumen fermentation characteristics of this study can be seen in Table 2.

Microbial Protein Synthesis (MPS), Total Protozoa and Methane Production

The increase of RDP in ruminant feed caused microbial protein production to decline in this study, which used *T. diversifolia* and *L. leucocephala* as legumes (P<0.05). Furthermore, the RDP50 diet reduced the quantity of protozoa in the rumen fluid (P<0.05) compared to 55 and RDP60 diets, otherwise RDP50 tend to have high methane production in the rumen (P<0.05) compared to 55 and RDP60. Including 50% of RDP in ruminant diet can enhance microbial protein production in the rumen while reducing protozoa numbers. Microbial protein synthesis, total protozoa and methane production of this study can be seen in Table 3.

Table 1: Ingredients and chemical composition of experimental diets

Component	Diets		
	RDP 50	RDP 55	RDP 60
Ingredient composition (%)			
Native grass	20	30	38
Tithonia diversifolia	27	15	2
Leucaena leucocephala	23	25	30
Tofu waste	8	13	17
Cassava (*Manihot esculenta*)	12	8	2
Rice bran	9	8	10
Mineral	1	1	1
Total	100	100	100
Chemical Composition (%)			
Dry matter (DM)	89.17	89.72	90.28
Crude protein (CP)	16.85	16.23	16.03
RDP (%CP)	50.00	55.00	60.00
RUP (%CP)	50.00	45.00	40.00
Crude fiber (CF)	18.43	19.99	21.81
Ether extract (EE)	4.12	4.13	4.35
Nitrogen-free extract (NFE)	46.05	45.70	44.56
True Digestible Nutrient (TDN)	68.43	67.68	66.96

Table 2: Rumen fermentation characteristics of dietary treatments

Variables	Treatments			SEM
	RDP 50	RDP 55	RDP 60	
pH	6.90	6.94	6.97	0.10
Total VFA (mM)	100^a	98.93^a	95.60^b	4.88
NH_3 (mM)	15.30^b	15.45^b	16.15^a	0.87

Superscript means significantly different in a row (P<0.05). SEM: standard error of the mean.

Table 3: Microbial protein synthesis, total protozoa, and methane production of dietary treatments

Variables	Treatments			SEM
	RDP 50	RDP 55	RDP 60	
MPS (mg/100mL)	88.86^a	84.03^a	81.20^b	0.67
Number of protozoa ($x10^5$)	4.77^a	5.57^a	3.93^b	0.05
Methane production (mM)	13.67^a	11.77^b	11.58^b	0.43

Superscript means significantly different in a row (P<0.05). SEM: standard error of the mean.

The Digestibility of Nutrient

The increase of RDP in ruminant feed using *T. diversifolia* and *L. leucocephala* as legumes tended to decrease the nutrient digestibility (P<0.05). The highest nutrient digestibility was observed in RDP50 diet and tend to decrease in 55 and RDP60 diets. Providing 50% RDP in ruminant ration can increase nutrient digesitibiliy in the rumen. Nutrient digestibility of this study can be seen in Table 4.

DISCUSSION

Rumen Fermentation Characteristics

There are no significant differences except in the total VFA and NH_3 production. Ruminal pH value is a crucial parameter that affects microbial growth and fermentation.

Table 4: Nutrient digestibility of dietary treatments

Digestibility (%)	Treatments			SEM
	RDP 50	RDP 55	RDP 60	
IVDMD	64.56[a]	61.58[a]	58.20[b]	0.61
IVOMD	66.67[a]	63.89[a]	60.69[b]	0.61
IVCPD	62.52[b]	63.43[b]	66.12[a]	0.51
IVEED	61.37[b]	64.05[a]	60.91[b]	0.59
IVCFD	65.37[a]	62.07[a]	60.78[b]	0.98
IVNFED	66.45[a]	62.87[b]	60.57[b]	0.61

Superscript means significantly different in a row (P<0.05). SEM: standard error of the mean: *IVDMD, *in vitro* dry matter digestibility; IVOMD, *in vitro* organic matter digestibility; IVCPD, *in vitro* crude protein digestibility; IVEED, *in vitro* ether extract digestibility, IVCFD, *in vitro* crude fiber digestibility; IVNFE, *in vitro* nitrogen-free extract digestibility.

The diet treatments demonstrated significant rumen fermentation characteristics after 48h of incubation (Table 2). This study's pH levels ranged from 6.90 to 6.97, which is still within the normal range of 5.5 to 7.0 (Puniya et al. 2015). Also, according to Putri et al. (2021), an increase in RDP in the diet had no effect on pH. The alteration of ruminal pH value interfered the activity and growth of rumen microbes. The decrease of pH value caused ruminal acidosis which leads to negative effect on ruminal fermentation (Li et al. 2014). The VFA energy was diverted to keep bacterial cells at a neutral pH, which disrupted microbial protein production. Beside that, the level of pH value above 7.5 caused alkalosis that can destroy microbial activity (Kumbhar et al. 2018).

Total VFA is the product from energy feed digested by rumen microbial in the rumen (Yang et al. 2016). The carbohydrate content of the diet and its degradability determine the amount of VFA produced in the rumen (Durango et al. 2021). High fermentable carbohydrate in the diet results in the accumulation of VFA in the rumen. Diet with RDP50 has shown the highest total VFA production compared to 55 and RDP60 diet. It indicated that RDP50 has a high content of fermentable carbohydrate, namely cassava as much as 12% of the total diet compared to 55 and RDP60 which was 8 and 2%. The availability of fermentable energy is usually the first limiting factor for microbial growth in the rumen. The supply of carbohydrate and nitrogen in the rumen has a critical impact on microbial growth (Uddin et al. 2015). Low fermentable carbohydrate in 55 and RDP60 caused unsynchronization of protein-energy. It lessens the efficiency of microbial protein synthesis in the rumen. Rumen metabolites such as VFA and NH_3 are reduced due to inadequate microbial protein synthesis in the rumen.

Furthermore, the higher content of legumes, protein, and TDN on RDP50 compared to 55 and RDP60 diet is helpful for microbes in the microbial protein synthesis. *T. diversifolia* and *L. leucocephala* as legumes supplied nitrogen for microbial growth and improved nutrient digestibility. This study is in line with Barros-rodríguez et al. (2013) that feeding 20-40% *L. leucocephala* into the diet can improve rumen fermentation and forage breakdown. Also, Ramírez-Rivera et al. (2010) claimed that combination of 20% *T. diversifolia* in the diet with Taiwan grass improved rumen fermentation, intake, and digestibility.

Moreover, as predicted, the major effect of increasing dietary crude protein and RDP levels on ruminal fermentation patterns was mirrored in the change in ruminal NH_3 levels. Protein solubility had a crucial impact on NH_3 concentration. The simpler protein to break down by rumen bacteria, the more soluble it is (Zain et al. 2020; Pazla et al. 2021). The amount of protein, the rate of degradation, and the time of feeding all influence the concentration of NH_3 (Ningrat et al. 2019; Makmur et al. 2020). Table 2 has shown that the production of NH_3 was increased in line with the increase in RDP (P<0.05). It indicated that these diets contain high soluble protein, especially from *T. diversifolia* which contains 40.2% of RDP (García et al. 2017) and *L. leucocephala* with 50.77% of RDP (Putri et al. 2019). As the quantity of dietary protein increased, the total of NH_3 also rising that enable the bacteria to use the protein in the form of NH_3. Furthermore, the degree of NH_3 in the rumen indicates a high soluble protein level and dry matter digestibility in the meal. Previous research has shown that an increase in RDP leads to an increase in NH_3, allowing protein to be utilized by bacteria in the form of NH_3 (Zhao et al. 2015; Yang et al. 2016; Paula et al. 2017). Putri et al. (2021) reported that diet with 12-14% protein and 55-65% RDP content improved the accumulation of NH_3 in the rumen.

RDP is important in the rumen's NH_3 regulation. RDP is used by rumen microbes as a nitrogen source for protein synthesis. When protein is digested, NH_3 is the metabolite of rumen microbial activity. Rumen microorganisms, particularly proteolytic bacteria, use RDP to transform protein into peptides by secreting protease enzymes. Proteolytic bacteria produce peptidase, an enzyme that converts peptides to amino acids. Furthermore, proteolytic bacteria release deaminase enzymes that convert amino acids to NH_3, which is used in microbial protein synthesis. Microbial protein synthesis boosts NH_3 production by 6-21mMol (McDonald et al. 2010). The efficient usage of NH_3 for microbial production, however, is determined by energy availability (Camero et al. 2001). As a result, NH_3 generation is expected to help microbial protein synthesis, implying that for rumen microbial protein production, legumes (*T. diversifolia* and *L. leucocephala*) provide a lot of protein and organic matter.

Microbial Protein Synthesis, Total Protozoa and Methane Production

The most essential process in ruminant nitrogen metabolism is ruminal microbial protein synthesis. Protein from rumen bacteria accounts for more than half of the amino acids absorbed in the small intestine. It also has the same amino acid composition as the proteins needed for milk production and meat production. In this study, microbial protein synthesis declined due to the rise of RDP (P<0.05). This study contradicts with Javaid et al. (2011), Brooks et al. (2012) and Akhtar et al. (2017) who claimed that increasing of RDP in the diet tends to raise microbial protein synthesis, because rumen microbes utilize nitrogen from RDP. The highest MPS was in RDP50 diet (88.86mg/100mL) compared to 55 and RDP60 which was 84.03 and 81.20mg/100mL, respectively. It's possible because the RDP50 diet contains a nutrient that allows rumen bacteria to manufacture protein. *T. diversifolia* and *L. leucocephala* contain a large amount of crude protein and TDN (True Digestible Nutrient). This results in a high rate of aminogenesis, which allows rumen bacteria to

proliferate faster and produce more metabolites. Beside that, higher sulfur content from *T. diversifolia* and *L. leucocephala* are essential for microbial protein synthesis. Similarly, Zain et al. (2019) reported that due to the presence of significant microbial growth factors; sulfur and calcium minerals in *L. leucocephala*, especially for cellulolytic microbial growth and fungi; and the availability of nutrients for optimal microbial growth, the inclusion of legumes, specifically *L. leucocephala*, increases microbial protein synthesis. In addition, legumes supplied enough organic matter for microbial protein synthesis (Makmur et al. 2020). Furthermore, the amount of *adenosine triphosphate* (ATP) available for rumen microbial development of microbial dry matter produced per unit of carbohydrate fermented is influenced by the sources and amount of carbohydrate (Lu et al. 2019).

As the rumen microbial population grows, so does ammonia usage, fiber digestibility, and microbial protein production. As a result, the feed can be digested more quickly, increasing the total digestibility of the diet. By enhancing nitrogen availability in the rumen, it increases the synthesis of rumen microbial proteins. Although the rumen accumulates a lot of nitrogen, it can reduce protein production if it isn't supported by a lot of fermentable carbohydrate. The composition of *T. diversifolia* in RDP50 diet (27%) was higher than 55 and RDP60 diet which was 15 and 2%. It increased the availability of nutrients for rumen microbes. Beside that, Pazla et al. (2018) reported that 16% addition of *T. diversifolia* to fermented oil palm fronds and elephant grass increased microbial protein synthesis (79.21mg/100mL) compared to 12, 8 and 4% inclusion of *T. diversifolia* which was 63.25, 55.36 and 53.56mg/100mL, respectively. It was supported with *T. diversifolia* leaves which are high in complex amino acids including lysine, arginine, aspartate, glutamate, methionine, cystine, isoleucine, tyrosine, and phenylethyl, as well as enough P and Mg to support rumen microbial development (Fasuyi et al. 2010; Pazla et al. 2018).

Due to secondary metabolites such as anti nutrients, legumes are well-known as methane reducers. Anti-nutrients, like as tannins and saponins, have been reported to lower CH_4 emissions by inhibiting rumen ciliate protozoa (Delgado et al. 2012; Ribeiro et al. 2016). In this study, 27% *T. diversifolia* (RDP50) supplementation can compress number of protozoa in the rumen fluid compared to 8 and 2% supplementation of *T. diversifolia*, otherwise RDP50 tend to have high methane production in the rumen. This is indicated that RDP50 contain high fermentable carbohydrate and acknowledged that feedstuffs with higher gas generation and IVDMD produce more CH_4 per gram of incubated dry matter (Meale et al. 2012; Terry et al. 2016). González et al. (2020) reported that diet with 67% *Cenchrus purpureus*: 33% *T. diversifolia* tend to decrease methane production compared to 100% *C. purpureus*, 33% *C. purpureus*: 67% *T. diversifolia*, and 100% *T. diversifolia.* Likewise, 30% *T. diversifolia* added to a diet based on star grass (*Cynodon nlemfuensis*) reduced methane emission. The presence of secondary metabolites as condensed tannins and saponin in *T. diversifolia* caused it (Delgado et al. 2012). Ribeiro et al. (2016) reported that PCR-DGGE profiling revealed considerable variations in archaeal communities depending on the quantity of *T. diversifolia* in the diet. According to their findings, including up to 15.4% *T. diversifolia* in the mix had no influence on methane generation. Despite the genetic diversity of the archaeal populations, no variations in CH_4 production were observed. These data support the idea that changes in community diversity are not always linked to changes in activity due to functional redundancy and relative abundances of the microorganisms involved. Methane production can be influenced by dietary components, feed additives that have a direct effect on methanogenic population concentrations, as well as alterations in the bacterial ecology that affect the availability of methanogens's substrates (e.g. formate and hydrogen).

The Digestibility of Nutrient

In this research, nutrient digestibility declined as RDP increased ($P<0.05$), proving that in this study microbial protein production is unaffected by protein-energy synchronization and RDP levels. The rumen microbial activity was linked to nutrient digestibility. The higher the RDP level, the more nitrogen was available for microbial protein synthesis, which improved microbe activity and feed digestibility. Meanwhile, in this study the highest nutrient digestibility was in RDP50 compared to 55 and RDP60. However, these results contradict to the study by Costa (2017) who stated that RDP can improve nutrient digestibility and microbial protein synthesis when added to feed at specific amounts. Decreasing of protein dan TDN content on 55 and RDP60 diets caused a decresing of microbial protein synthesis. In addition, the composition of *T. diversifolia* and *L. leucocephala* as protein source and cassava as fermentable carbohydrate were decreased in 55 and RDP60. We hypothesized that these diets were unable to achieve optimal protein-energy synchronization, resulting in microbial protein production and nutrient digestibility declines.

The highest nutrient digestibility was shown in RDP50 diet which contains of 27% *T. diversifolia* and 23% *L. leucocephala*. Likewise, Jamarun et al. (2017) highlighted that 20% of *T. diversifolia* combined with 80% Napier grass showed the highest nutriet digestibility. The authors stated that the highest nutrient digestiblity of supplemented 20% *T. diversifolia* was due to the greatest phytic acid antinutrition degradation in the rumen. Rumen microbes produce fitase, an enzyme that breaks down the phosphor-phytate bond, and phosphor can be used as a mineral supply for microbial protein synthesis by rumen microbes. Fasuyi et al. (2010) reported that *T. diversifolia* contained high phytic acid amount of 79.1mg/100mg compared to other anti-nutrients namely tannin, oxalate, saponin, alkaloid, flavanoid which was 0.39, 1.76, 2.36, 1.23 and 0.87mg/100g respectively. Lamid et al. (2014) reported that rumen bacteria of ruminant origin (*Actinobacillus sp.* and *Bacillus pumilus*) produced the enzyme phytase, which allowed phosphor to be released and used for microbial protein synthesis. Odedire and Oloidi (2014) also mentioned that inclusion of 10-30% of *T. diversifolia* supports the growth of West African Dwarf goats, especially during the period of drought without any deleterious effects by increasing nutrient digestibiliy.

In addition, the high protein level in the RDP50 diet causes an increase in nutritional digestibility. Microbial protein production was increased with an adequate amount

of protein. The optimal activity of rumen microbes increased nutrient digestibility, implying that the rumen was in better shape, allowing for improved fermentation. Improved rumen fermentation and microbial activity resulted in increased enzyme synthesis, improved dry matter degradation, and reduced nutrient loss. High digestibility enhanced ruminant productivity as the nutrients could be optimally utilized. It's possible that the improvement is due to nutritional abundance rather than the requirement to improve digestion. The availability of a synchronized nutrition supply provided bacteria with sufficient metabolic substrates, promoting growth and nutrient digestibility. Similarly, Ramírez-Rivera et al. (2010) stated that diet with a high protein content and then a 20% protein inclusion of *T. diversifolia* increases the digestibility of dry matter, organic matter, and neutral detergent fiber.

Conclusion

We concluded that diet with RDP 50% which contains of 27% *T. diversifolia* and 23% *L. leucocephala* can improve rumen fermentation characteristics and nutrient digestibility. It can be seen from the content of pH value (6.90), Total VFA (100mM), NH_3 (15.30mM), microbial protein synthesis (88.86mg/100mL), number of protozoa ($4.77x10^5$), methan production (13.67mM), IVDMD (64.56%), IVOMD (66.67%), IVCPD (62.52%), IVEED (61.37%), IVCFD (65.37%), and IVNFED (66.45%). These information need further study to discover the effect on livestock with *in vivo* method.

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Autrhor's Contribution

M. Zain and R.M. Sari supervised the experiment and wrote original manuscript. R.M. Sari, N. Jamarun, R.W.S Ningrat and Elihasridas conducted the experiment and data analysis. E.M. Putri prepared tables and finalize draft. The final version of the manuscript was read and approved by all authors.

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