Rumen Fermentation Profiles of Protein-Energy Synchronization Index-Based Ration: an *In Vitro* Study

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ABSTRAK

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Penelitian bertujuan untuk mengkaji pengaruh ransum berbasis indeks sinkronisasi protein-energi (SPE) terhadap profil fermentasi rumen. Materi yang digunakan yaitu cairan rumen Kambing Jawa Randu yang diambil sesaat setelah kambing dipotong. Komposisi ransum perlakuan terdiri dari rumput gajah, lamtoro, bungkil kelapa, ampas tahu, dedak, onggok, dan mineral mix yang disusun berdasarkan indeks SPE masing-masing bahan pakan. Pengujian dilakukan secara in vitro dan mengunakan rancangan acak lengkap (RAL). Perlakuan terdiri dari 4 indeks SPE yaitu 0,55 (R1); 0,6 (R2); 0,65 (R3); dan 0,7 (R4) dan masing-masing terdapat 5 ulangan. Data dianalisis dengan analisis variansi dan diuji menggunakan orthogonal polynomial (OP). Hasil analisis variansi menunjukkan bahwa indeks SPE tidak berpengaruh terhadap produksi asetat (C2) dan butirat (C4), tetapi berpengaruh sangat nyata terhadap kecernaan bahan kering (KBK), kecernaan bahan organik (KBO), pH, volatile fatty acids (VFA) total, propionate (C3), C2:C3 rasio, metan (CH4), amonia (NH3), dan sintesis protein mikroba (SPM). Hasil uji OP menunjukkan pengaruh kuadrater pada semua variable dengan persamaan yaitu $Y= - 81{,}601X^2+375{,}04X-310{,}78$ (KBK), $Y= -433{,}69X^2+ 522{,}69X–128{,}75$ (KBO), $Y=-44X^2+54{,}04X–9{,}9$ (pH), $Y= -2160X^2+2576{,}8X–604{,}2$ (VFA Total), $Y= -481{,}8X^2+585{,}01X–143{,}45$ (C3), $Y = 50{,}93X^2 – 60{,}177X + 21.067$ (C2:C3), $Y = 202{,}45X^2 – 223{,}18X + 103{,}41$ (CH_4), $Y= 436X^2 - 552{,}28X + 181{,}08$ (NH_3), dan $Y= -1012X^2+1260X–311{,}64$ (SPM). Ransum dengan indeks SPE 0,6 memiliki profil fermentasi rumen terbaik. Formulasi ransum dengan indeks 0,6 lebih efektif dibandingkan lainnya berdasarkan pada hasil propionate yang tinggi, metan yang rendah, NH_3 yang rendah, dan SPM yang tinggi. Ransum dengan indeks 0,6 tersusun atas 30% rumpt gajah, 30% lamtoro, 10% bungkil kelapa, 10% ampas tahu, 10% dedak, 9% onggok, dan 1 % mineral mix.

Kata Kunci: Ransum, Indeks Sinkronisasi Protein-Energi, Fermentasi Rumen, *In Vitro*

ABSTRACT

Syamsi AN, Ifani M. 2023. Rumen fermentation profiles of protein-energy synchronization index-based ration: an *in vitro* study. JITV 28(1):22-33. DOI:http://dx.doi.org/10/14334/jitv.v28.i1.3091.

The study examined the effect of protein-energy synchronization (PES) index-based rations on the rumen fermentation profile. The material used was the Jawa Randu goat's rumen fluid, collected soon after the goat was slaughtered. The treatment ration consisted of elephant grass, lamtoro (*Leucaena leucocephala*), coconut meal, tofu waste, bran, cassava waste, and mineral mix arranged based on the PES index of each feed ingredient. The research was conducted in vitro using a completely randomized design (CRD). The treatment consisted of 4 PES indexes, namely 0.55 (R1); 0.6 (R2); 0.65 (R3); 0.7 (R4), and each had 5 replications. Data were analyzed by ANOVA and orthogonal polynomials (OP). The results of the ANOVA showed that the PES index did not affect the production of acetate (C2) and butyrate (C4) but had a highly significant effect on dry matter digestibility (DMD), organic matter digestibility (OMD), pH, a total of volatile fatty acids (VFA), propionate (C3), C2:C3 ratio, methane (CH_4), ammonia (NH_3), and microbial protein synthesis (MPS). The analysis of OP showed a quadratic effect on all variables with the equation $Y= - 81.601X^2+375.04X–310.78$ (DMD), $Y= -433.69X^2+522.69X–128.75$ (OMD), $Y=-44X^2+54.04X– .9$ (pH), $Y= -2160X^2+2576.8X–604.2$ (VFA Total), $Y= -481.8X^2+585.01X–143.45$ (C3), $Y = 50.93X^2–60.177X+21.067$ (C2:C3), $Y = 202.45X^2–223.18X+103.41$ (CH_4), $Y= 436X^2 – 552.28X+181.08$ (NH_3), dan $Y= -1012X^2+1260X–311.64$ (MPS). Protein-energy synchronization (PES) index of 0.6 achieved the best rumen fermentability. The formulation of the PES index-based ration of 0.6 had the most effective compared to other indexes based on the high of propionate, the lowest methane, the lowest NH_3, and the best microbial protein synthesis. The ration with an index of 0.6 is composed of 30% elephant grass, 30% Leucaena leucocephala, 10% coconut meal, 10% tofu waste, 10% rice brand, 9% cassava waste, and 1% mineral mix.

Key Words: Ration, Protein-Energy Synchronization Index, Rumen Fermentation, *In Vitro*

INTRODUCTION

The principle of preparing rations for ruminants is to stimulate the optimal development of rumen microorganisms. The role of rumen microorganisms is crucial for the feed fermentation process and fulfills more than 60% of the protein requirement for the animal (Liu et al. 2021). The main growth factor for all types of rumen microbes is the availability of ammonia and energy, while the need for cofactors differs for each species. The two compounds have a positive associative relationship, so the availability of one compound affects the use of other compounds in microbial protein synthesis (MPS). Therefore, synchronization of the availability of the two compounds is essential to achieve optimizing MPS and can be done through the preparation of rations based on the protein-energy synchronization index (Zhang et al. 2020).

Preparing rations based on the protein-energy synchronization (PES) index differs from the Cornell net carbohydrate and protein system (CNCSP) and practical synchronization methods. This ration is compiled with a synchronization value measured quantitatively through indexation with a scale of 0-1. The higher the PES index of the ration, the more harmonious the supply of ammonia and energy in the rumen (Sinclair et al. 1993; Waldi et al. 2017). Although MPS is the main target, the final result of preparing this ration is feed use efficiency through increased rumen fermentation activity. Suhada et al. (2016) proved that the higher PES index of the ration resulted in higher efficiency of microbial protein synthesis and higher total volatile fatty acids (VFA) production. The best energy efficiency is also obtained from rations with the highest index based on the ratio of acetate: propionate and methane (CH_4) production, which decreases with increasing PES index.

Preliminary research has been carried out to inventory the protein-energy synchronization index of various types of local feed ingredients from grass, legumes, energy source concentrates, and protein source concentrates. The PES index of each feed ingredient is used as the basis for compiling the ration by setting the scale planned by the researcher. However, this method has not been widely used, especially on local feed ingredients with different characteristics than sub-tropical regions. Therefore, this study aimed to examine the effect of preparing rations based on the protein-energy synchronization index on the rumen fermentation profile.

MATERIALS AND METHODS

Materials

The material used in this study was the rumen fluid of the Jawa Randu Goat, which was fed with 14% protein content and 65% TDN. The composition of the treatment ration consisted of elephant grass, lamtoro (*Leucaena leucocephala*), coconut meal, tofu waste, rice bran, cassava waste, and mineral mix.

Research variables

The variables measured in this study were dry matter and organic matter digestibility, pH, microbial protein synthesis (MPS), ammonia (NH_3), total volatile fatty acids (VFA), acetate (C2), propionate (C3), butyrate (C4), C2:C3 ratio, and methane (CH_4).

Methods

The research was conducted experimentally at the Laboratory of Nutrition and Animal Feed Science, Faculty of Animal Science, Jenderal Soedirman University, Purwokerto, Indonesia, from April to September 2021.

The in vitro method of Tilley & Terry (1963) was used in this research.

The ration is formulated based on the PES index of each feedstuff used. First, it is obtained by measuring the hourly degradation of organic matter (OM) and protein. The times used to measure the degradation were 2, 4, 6, 8, 12, 24, 48, and 72 hours for forages and 2, 4, 6, 8, 12, and 48 hours for concentrate. Degradability at all time observations is then entered into a linear equation to obtain degradation of g protein or Kg OM hourly. Then the degradation of g protein and Kg OM hourly is used to calculate the PES index of each feedstuff with the following equation.

$$PES\ index = 20 - \frac{\sum_{1-24}^{n} \sqrt{(20 - \frac{N}{OM}\ hourly)^2}}{24}$$
$$20$$

where n is observation time; N/OM hourly is the degradation rate of protein, compared to the organic matter hourly; 20 is 20 g N-protein/kg OM degradability on the rumen; 24 is 24 hours (Hermon et al. 2008).

The PES index of feedstuff used results from preliminary research. After the PES index of each feed ingredient is obtained, the ration formulation is compiled based on the PES index. First, it is determined by multiplying the grams used for each feed ingredient in the ration by each PES index. Then multiplication is performed for each synchronization. index is added up and divided by the total gram of ration. The PES index used in the study were 0.55, 0.6, 0.65, and 0.7.

Experiment design and data analysis

The completely randomized design (CRD) was used with four types of rations with different protein-energy

Table 1. Feed composition and nutrient content used as treatments

Feedstuff	R1	R2	R3	R4
PES indexes	*0.55*	*0.6*	*0.65*	*0.7*
Elephant grass (%)	20	30	40	50
Leucaena leucocephala (%)	40	30	20	8
Coconut meal (%)	5	10	11	11
Tofu waste (%)	15	10	7	8
Rice bran (%)	15	10	7	8
Cassava waste (%)	4	9	14	14
Mineral mix (%)	1	1	1	1
Total (%)	100	100	100	100
Nutritional content *				
Dry matter (%)	82.65	83.34	84.03	82.91
Moisture (%)	17.35	16.66	15.97	17.09
Organic matter (%/DM)	87.27	84.62	83.86	83.47
Ash (%/DM)	12.73	15.38	16.14	16.53
Crude protein(%/DM)	15.55	14.80	13.91	12.40
Crude fiber (%/DM)	33.39	33.65	29.44	29.30
Extract ether (%/DM)	6.32	4.30	3.60	3.24
NFE (%/DM)	32.01	31.87	36.90	38.53
TDN (%/DM)	52.46	50.05	51.81	50.99

R1= ration with PES index 0.55; R2= ration with PES index of 0.6; R3= ration with PES index of 0.65; R4= ration with PES index 0.7; NFE= nitrogen free extract; TDN= total digestible nutrients; TDN= (70.60 + 0.259 CP + 1.01 EE) – (0.76 CF + 0.0991 NFE)

synchronization indexes, each treatment consisting of 5 replications; thus, there were 20 experimental units. The data obtained were tabulated and then analyzed using ANOVA. The treatments that significantly affect the variable is tested using orthogonal polynomials. The treatments are described in Table 1.

Measuring nutrition ration treatment

The nutrition of the treatment ration was measured by proximate analysis (AOAC 2005) to determine the nutrient content of each ration. The calculated nutrient content was dry matter (DM), moisture, organic matter (OM), ash, fat (extract ether), crude fiber, crude protein, nitrogen-free extract (NFE), and total digestible nutrient (TDN).

Measuring digestibility

The in vitro method used to measure supernatant and treatment residues is the in vitro method from Tilley & Terry (1963). The first stage is taking rumen fluid using a thermos filled with hot water at 39^0C. The rumen fluid was taken immediately after the goats were slaughtered at the abattoir. The rumen fluid is squeezed and filtered using gauze and put in a warm thermos drained of water. Next, in vitro digestion was carried out using a 250 ml Erlenmeyer which had been filled with 2 g of sample, added 16 ml of rumen fluid and 24 ml of McDougall's solution, then put in a shaker bath with a temperature of 39^0C, the Erlenmeyer was shaken with CO_2 flowing for 30 seconds, with a pH of 6.5 -6.9 and then covered with ventilated rubber, and fermented for 2 x 24 hours. After 24 hours, the fermenter tube was centrifuged at 4,000 rpm for 15 minutes. The substrate will separate into a precipitate at the bottom and a supernatant (liquid) at the top. The supernatant was taken for subsequent analyses (pH, NH_3, partial VFA, and CH_4). At the same time, the residue or precipitate was then incubated for 24 hours for hydrolytic incubation to measure the dry matter digestibility (DMD) and organic matter digestibility (OMD). Both were calculated based on the equation made by Tilley & Terry (1963) followed.

$$Digestibility: \frac{DM/OM\ sample - (DM/OM\ residue - DM/OM\ blank)}{DM/OM\ sample}$$

Measuring pH

The rumen fluid's pH was measured using a pH meter. First, the pH meter is calibrated sequentially with an alkaline solution (buffer) and an acidic solution. Second, the tip of the pH meter flowed with distilled water to keep the pH neutral. Then the pH meter is inserted into the supernatant. The pH meter display will show the read pH number. The pH measurement activities were carried out individually for each sample, and at each sample change, the pH meter was rinsed with distilled water.

Measuring volatile fatty acids

The first step is boiling the water in a boiling flask and washing the sample holder with distilled water. Next, put 5 ml of rumen fluid into the sample holder, and then add 1 ml of 15% H_2SO_4. Next, the distillate was accommodated in a 250 ml Erlenmeyer flask filled with 0.5 N NaOH until the distillate volume reached 100 ml. Next, the distillate was added with two drops of phenolphthalein and titrated with 0.5 N HCl until a color change occurred. Finally, as a control, a blank was made with 5 ml of 0.5 N NaOH with 0.5 N HCl. After the sample data and the blank are obtained, enter them into the equation below.

$$VFA\ level = \left((Y - Z)x\ NHCL\ x\ \left(\frac{1000}{5}\right)\right) nM$$

where Y is ml of HCl required for blank titration, and Z is ml of HCl required for distillate titration.

The rumen fluid's partial VFA levels (acetate, propionate, butyrate) were measured using the gas chromatography technique described by Matsui et al. (1992). The standard solutions contained the following VFA: acetate 52.54% molar, propionate 13.42% molar, isobutyrate 5.40% molar, n-butyrate 10.89% molar, isovalerate 4.23% molar, n- valerate 4.61% molar. Calculations other than the previous explanation can also be carried out using this technique: VFA (% molar) = VFA area of the sample x standard VFA content divided by standard VFA area. Partial VFA was measured by using the gas chromatography technique by taking 4 ml of rumen fluid using a pipette and then centrifuging it at 10,000 rpm for 15 minutes. Next, the supernatant was added to as much as 2 ml into a 5 ml plastic tube. Next, add 30 mg of 5-sulphosalicylic acid. Then the solution is shaken, centrifuged at 3,000 rpm for 10 minutes at 4^0C, then filtered with a millipore until a clear liquid is obtained. A total of 1 l of clear liquid was injected into the gas chromatography, which was previously injected with a standard solution of VFA. The concentration in the sample is calculated using the VFA formula = sample height divided by the standard height times the standard concentration, according to the individual solution to be measured.

Estimation of methane level

Methane gas (CH_4) levels were not measured directly through laboratory tests but were calculated based on the proportion of partial VFA obtained. The gas content of CH_4 is computed using the mM formula developed by Orskov & Ryle (1996), namely CH_4= 0.5 a – 0.25 p+0.5 b (a= acetate, p= propionate, and b= butyrate).

Measuring ammonia

NH_3 levels were measured by using the Conway micro diffusion technique. The first step is to prepare the Conway cup by smearing vaseline on the cup and lid. Next, 1 ml of boric acid was taken and dropped in the middle of the cup. Then on the skating to the right of the cup, 1 ml of rumen fluid was added. Next, the cup is tilted, then 1 ml of saturated Na_2CO_3 is added to the slide to the left of the cup. Next, the cup is closed and moved slowly so that all the liquid is mixed and left for 24 hours at room temperature. After 24 hours, the solution was titrated with 0.01 N H_2SO_4 until the color changed to pink. After that, it is calculated by the following equation, NH_3= (ml titrant x N H_2SO_4 x (1000/1)) mM (National Bureau of Standards 1967).

Measuring MPS

According to Zinn & Owens (1986), measuring MPS has several stages. In the first stage, 0.5 g of dry residue sample (residue in the oven at 60^0C for two days) was pulverized with mortar, then put into a closed 25 ml culture tube. The second stage involved adding 2.5 ml of $HClO_4$ (70%) into the culture tube. After that, the culture tubes were tightly closed and incubated in a water bath at 90-95^0C for one hour. After completion of incubation (marked by a change in the color of the solution to black-like charcoal), the sample was crushed into a small powder with a glass rod so that the sample was easily extracted. Then, 17.5 ml of 0.0285M $NH_4H_2PO_4$ pH 2 buffer solution was added to the tube and stirred until homogeneous with a vortex stirrer. The solution was heated again in a water bath at a temperature of 90-95^0C for 15 minutes. The heating results were filtered with Whatman paper no 4. The resulting filtrate was taken at 0.5 ml and put into a centrifuge tube. The 0.5 ml of 0.4 M $AgNO_3$ and 9 ml of 0.2 M $NH_4H_2PO_4$ buffer solution were added and allowed to stand for 30 minutes in the darkroom (Better precision is shown by incubating overnight in a refrigerator at 5^oC. After that, the solution was centrifuged for at least 15 minutes at 5,000 rpm and decadent the supernatant. The precipitate formed was rinsed with distilled water pH 2 and centrifuged again. The results of the second centrifuge were added 10 ml of

0.5 N HCl, stirred evenly using a vortex, tightly closed the culture tube, then heated again in a water bath with a temperature of 90-95ºC for 30 minutes. Light absorption (absorbance) was read at a wavelength of 260 on an ultraviolet spectrophotometer (Walker & Nader 1986). The standard is 100, 200, 300, and 400 mg yeast RNA. The procedure was the same as for the sample and diluted at 1:20 after seven steps.

$$Y = a + bx$$

where Y is Absorption spectrophotometer ultraviolet, a is Intercept, b is regression coefficient, and x is MPS.

RESULTS AND DISCUSSION

Digestibility value

The protein-energy synchronization (PES) index-based ration preparation aims to increase the quantity of rumen microbial synthesis and its ability to carry out optimal ration fermentation. Dietary nutrient digestibility is an essential indicator of rumen fermentability. High digestibility values indicate optimal microbial activity in utilizing and converting nutrients into fermented products needed by the animal. The results showed that dry matter digestibility (DMD) and organic matter digestibility (OMD) is relatively smaller than the standard digestibility, which can reach 50-80%, even more (Yanuartono et al. 2019). Mahyuddin (2008) explained that in vitro digestibility measurements resulted in lower digestibility compared to the actual conditions in the rumen. The results of in vitro digestibility are also very diverse, depending on the level of control of the factors that affect in vitro digestibility. Factors influencing in vitro digestibility are temperature stability, rumen fluid source, rumen fluid handling, artificial saliva, CO_2 supply, valve ventilation control, and others. However, the digestibility rhythm produced is a vital overview that can be used as a reference in determining the digestibility of the ration in the rumen.

The analysis of variance showed that the PES index-based ration had a highly significant effect ($P<0.01$) on DMD and OMD. The Tukey analysis showed that the DMD in R1, R2, and R3 is not different and is higher than in R4. Meanwhile, the OMD on R2 and R3 were not different and higher than R1, then R1 was higher than R4. The orthogonal polynomial (OP) analysis showed a quadratic response to the DMD ($Y= -81.601X^2+ 375.04X-310.78$; ($R^2$) = 0.71) and OMD ($Y= -433.69X^2 + 522.69X–128.75$; ($R^2$) = 0.92). Figure 1a showed that DMD initially increased in rations from a PES index of 0.55 (R1) to a PES index of 0.6 (R2) and then continued to decrease in rations with a PES index of 0.65 (R3) and 0.7 (R4). The peak was in the ration with a PES index of 0.6 and a DMD of 51.55%. Similar graphic dynamics are shown in the OMD results for all treatments. The quadratic graph (Figure 1b) is generated with the peak point at the PES index of 0.6 with an OMD of 48.74%.

Based on Tukey and OP analysis, it is known that R4 has the lowest DMD and OMD, which may be due to the ratio between protein and NFE rations being quite large; even the PES concept is also closely related to the balance in the availability of protein and NFE rations. R4 has the highest NFE but the lowest protein, with a ratio of 3.11. Waldi et al. (2017) and Indah et al. (2020) explained that a high proportion of NFE and protein could affect the availability of ammonia and energy, which needs to be balanced. This high proportion of NFE and protein will correlate with the slowdown in microbial protein synthesis (MPS) and ultimately affect the slowness of the DMD and OMD processes. NFE and protein ratios in R1, R2, and R3, respectively, were 2.06, 2.15, and 2.65, which makes it possible that there is no difference in the Tukey analysis. Although the Tukey analysis showed no difference, the OP analysis showed that the highest DMD was obtained in R2. Besides that, Tukey analysis showed that the OMD at R1 is lower than R2 and R3. Franco et al. (2016) stated that factors affecting the digestibility value are the amount and content of nutrients consumed. The high value of DMD on R2 is due to the relatively evenly distributed feedstuff compared to others in fiber, protein, and energy sources. Protein and carbohydrates (NFE) are nutrients that majorly affect digestibility. The composition of this ration resulted in crude protein content and NFE, respectively, 14.80% and 31.87%. Although R1 had a lower protein and NFE ratio than R2, the use of Leucaena in this treatment was the highest. Montoya-Flores et al. (2020) stated that adding leucaena to the treatment will affect digestibility. Leucaena is a legume that contains high protein but contains anti-nutrients, namely tannin, and mimosine, which can interfere with the performance of rumen microorganisms. According to Ariani et al. (2015), leucaena leaves should be no more than 40 percent to reduce the risk of poor performance of rumen microorganisms. The decrease in DMD in the R3 and R4 treatments on OP analysis was due to the higher ratio of protein and NFE. The increase in this ratio is in line with the lower crude protein content and higher NFE levels, so the synchronization of protein and energy becomes increasingly unbalanced, which is in line with the opinion of Syamsi et al. (2018) that the synchronization index is primarily determined by comparing protein levels and the NFE of the ration. Protein is the primary source of ammonia supply, and NFE is a fermentable carbohydrate that provides available energy in the rumen to increase the use of ammonia in MPS.

The digestibility of organic matter is closely related to the degradation of dry matter because most of the dry matter is a component of organic matter. Hartono et al. (2016) stated that DMD and OMD have a positive correlation, so OMD will follow the dynamics of decreasing or increasing DMD. The DMD and OMD

Table 2. Dietary digestibility profile based on protein-energy synchronization index *in vitro*

Digestibility	Treatment				
	R1	R2	R3	R4	Sig.
Dry matter (%)	50.76±0.52^{a}	51.24±0.82^{a}	51.18±1.00^{a}	48.55±0.42^{b}	**
Organic matter (%)	47.68±0.33^{b}	48.32±0.25^{a}	48.18±0.51^{a}	44.49±0.32^{c}	**

R1= index 0.55, R2= index 0.6, R3= index 0.65, R4= index 0.7. **highly significant effect

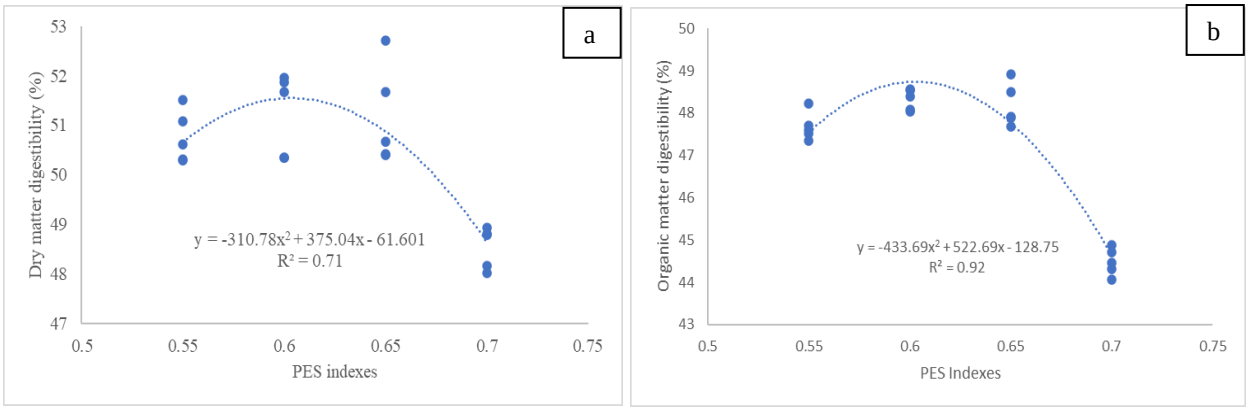

Figure 1. Digestibility graph of dry matter (a) and organic matter (b)

Table 3. Profile of rumen fermentation products in rations based on in vitro protein-energy synchronization index

Fermentation products	Treatment				
	R1	R2	R3	R4	Sig.
pH	6.5±0.07^{b}	6.72±0.11^{a}	6.6±0.10^{ab}	6.38±0.08^{c}	**
VFA Total (mM)	158.80±2.05^{b}	166.80±1.30^{a}	155.60±1.14^{c}	142.00±1.87^{d}	**
C2 (mM)	110.68±8.0	109.34±3.88	117.74±5.26	116.19±2.02	ns
C3 (mM)	32.33±2.46^{b}	34.78±0.99^{a}	32.56±1.30^{b}	30.19±0.60^{c}	**
C4 (mM)	9.71±0.45	10.15±0.75	10.72±1.16	9.30±1.05	ns
C2:C3 Ratio	3.43±0.90^{c}	3.15±0.18^{d}	3.62±0.16^{b}	3.85±0.11^{a}	**
CH_4 (mM)	42.40±3.27^{bc}	40.90±1.82^{c}	45.37±2.05^{ab}	45.89±1.23^{a}	**
NH_3 (mM)	9.32±2.89^{a}	6.16±2.03^{c}	6.72±0.43^{bc}	7.92±0.35^{ab}	**
MPS (g/ml)	74.49±0.48^{c}	82.08±4.27^{a}	77.65±3.87^{b}	75.12±1.53^{c}	**

R1= index 0.55, R2= index 0.6, R3= index 0.65, R4= index 0.7, ns= no significant effect, VFA= volatile fatty acids, C2= acetate, C3= propionate, C4= butyrate, CH_4= methane, NH_3= ammonia; MPS= microbial protein synthesis. **highly significant effe

values are also influenced by rumen environmental conditions, affecting rumen microbial activity. One of the main ones is the degree of acidity or rumen pH. Kitkas et al. (2022) stated that the rumen's pH standard was 5.5-6.8; the closer to 6.8, the better. Table 3 shows that the pH in R1, R2, and R3 is above 6.5, and R4 is below. It also makes it possible that there is no DMD difference in the Tukey analysis. Besides that, the pH of R2 was the highest, so R2 was the highest DMD and OMD on OP analysis, in line with the opinion of Li et al. (2021) that the lower the pH, the lower the digestibility because microorganisms generally have optimum activity at a pH close to neutral.

A PES index-based-ration is based on the potential of a simultaneous ammonia and energy supply through an indexed overview. The PES ration index is set between 0.0 to 1.0, where the index closer to 1.0 indicates the most simultaneous potential in providing the two nutrients. This result is different from the research by Chumpawadee et al. (2005), which shows that the synchronization index has a linear effect on DMD and OMD, where the DMD and OMD will increase with

increasing PES index. Based on Table 1, the type of feed ingredient also needs to be considered. The preparation of the rations in Table 1 only focuses on achieving the ration index, which impacts nutrient levels that are different from what is expected. For example, the ratio between NFE proteins does not decrease with increasing index numbers. In addition, the use of feed ingredients such as legumes has an impact on the digestibility value, which is due to the anti-nutrients they carry. Then it gives non-linear results increasing in DMD or OMD.

Rumen fermentation products

The feed eaten by ruminants will undergo a fermentation process in the rumen. Most sources of protein and carbohydrates will degrade their fermentation into various fermentation products. The type of feed, temperature, and degree of acidity (pH) strongly influence rumen fermentation products. The optimal pH for rumen metabolism is in the range of 6.2-7. Therefore, a decrease in pH below 6.2 will cause a slowdown in the performance of fiber-digesting bacteria.

In contrast, a reduction in pH below 5.6 will cause the death of fiber-digesting bacteria, an increase in lactate-producing bacteria, and the appearance of early symptoms of acidosis (Kim et al. 2018; Li et al. 2021). The results showed that the average rumen pH of all treatments ranged from 6.38-6.72 (Table 3). This condition indicates the rumen fluid is at an ideal pH for rumen microbe activity, suggesting that the PES index-based-ration can optimize the rumen pH conditions to be within the normal range.

The analysis of variance showed that the diet based on the PES index had a highly significant effect ($P<0.01$) on rumen pH. Tukey's analysis showed that R2 and R3 had the highest pH, but R3 was not different from R1, and R4 was the lowest. The OP analysis showed a quadratic response of the PES index to pH (Figure 2a) with the equation $Y= -44X^2+54.04X–9.9$ and a coefficient of determination (R^2) = 0.67. Figure 2a shows that the pH increased from R1 to R2, then decreased in R3 and R4. The peak reached P (0.61:6.69), meaning it obtained the highest pH at the index of 0.61 with a pH of 6.69. The degree of pH of the rumen fluid is a balance between the buffer capacity and the alkaline or acidic properties of the fermentation product. Cahyaningtyas et al. (2019) explained that carbohydrates are one of many factors influencing rumen pH. High fiber content can help improve the pH balance of the rumen. The R2 treatment in Table 1 has the highest fiber content (33.65%) but the lowest NFE content (31.87%). Jasin and Sugiyono (2014) stated that the high content of NFE in the ration causes the pH to be quiet because it is a fermentable carbohydrate quickly used by lactate-producing microorganisms. Tables 1 and 3 prove that

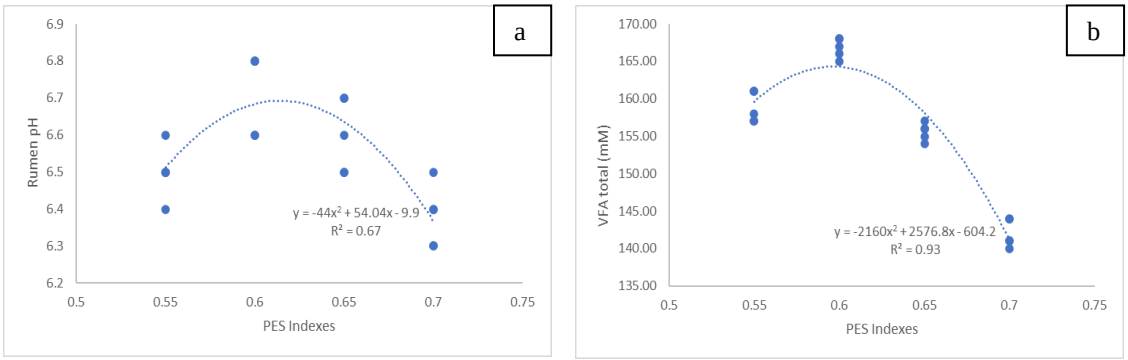

Figure 2. Graph of the pH (a) and a total of volatile fatty acids (b) on rumen fluid

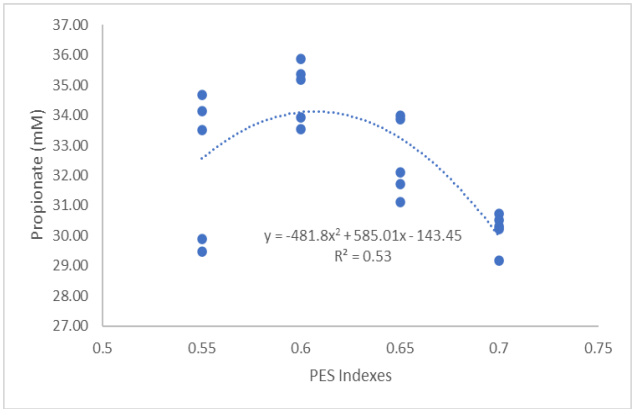

Figure 3. Graph of propionate production on rumen fluid

rations with decreasing fiber content and increasing NFE levels resulted in a lower pH. The crude fiber content of R1, R3, and R4 were 33.39%, 29.44%, and 29.30%; the NFE levels were 32.01%, 36.90%, and 38.53%, respectively.

VFA production describes the conversion rate of carbohydrates or organic matter into energy sources for ruminants and indicates the quality of feed fermentability in the rumen (Muchlas et al. 2014). The average concentration of the total VFA product in the study ranged from 142.00-166.80 mM (Table 3). These results indicate that the PES index-based ration can produce a reasonably high VFA because the standard VFA in rumen fluid ranges from 80-180 mM (Rahayu et al. 2018). The analysis of variance showed that the ration based on the PES index had a highly significant effect ($P<0.01$) on the total VFA production. The Tukey'sanalysis showed that the higest VFA was R2, then followed by R1, R3, and R4. The orthogonal polynomial analysis indicates that the PES index has a quadratic impact with the equation Y= -2160 X2+2576.8 X–604.2 (Figure 2b) and a coefficient of determination (R^2)= 0.93. The peak reached the PES index of 0.6 with a VFA of 164.31 mM. Tukey's and OP's analysis showed that the R2 was the highest on VFA production. The R2 ration with an index of 0.6 (Table 1) was prepared with a balanced proportion of feedstuff and causes the nutritional content produced is also quite balanced, especially in NFE and protein. The balance of these two nutrients in R2 (with a low ratio) supports faster microbial development than other treatments. In addition, the R2 content of crude fiber is quite high, which has an impact on controlling rumen pH. It supports high DMD and OMD at an index of 0.6. Conversely, Li et al. (2021) explained that carbohydrates are the primary substrate in rumen fermentation. Therefore, VFA production is strongly influenced by the fermentability of this substrate. The optimum pH level allows amylolytic microorganisms and cellulose and hemicellulose digesters to work optimally. Therefore, the production of VFA in this study strongly correlates with both digestibility and the degree of rumen pH. VFA composition is acetate (C2), propionate (C3), and butyrate (C4), while the other small portion is formic acid, isocaproic acid, isobutyrate, isovalerate, and valerate. Concentrations of C2, C3, and C4 from the fermentation of feed ingredients describe the solubility of carbohydrates and proteins during the fermentation process (Wahyuni et al. 2014; Syamsi & Waldi, 2021). C2 levels were between 109.34-117.74 mM, C3 levels were 30.19-34.78 mM, and C4 levels were 9.30-10.72 mM. When calculated based on the average value of all treatments, the proportion of C2 is 72.76%, C3 is 20.86%, and C4 is 6.38%. Syamsi et al. (2018) stated that the expected proportion of partial VFA in the rumen consists of 65% C2, 20% C3, and 12% C4.

The analysis variance of the PES index on partial VFA showed no significant effect ($P>0.05$) on the levels of C2 and C4 but a highly significant effect on the levels of C3 ($P<0.05$). Syamsi et al. (2018) also prove that the PES index did not affect C2 and C4. These two compounds have different formation pathways with propionate. The production of C2 and C4 is closely related to fiber degradability. Table 1 shows that the crude fiber content of the treatment tends to decrease with increasing PES index. Nevertheless, it does not affect C2 and C3 production. Aling et al. (2020) stated that cellulolytic microorganisms digest fiber. These microorganisms require quite diverse metabolite compounds (cofactors). The availability of these cofactors dramatically affects its performance in producing C2 and C3. The composition of the rations in Table 1 only focuses on achieving the PES index, but there is no addition of specific metabolites to improve cellulolytic microbial performance.

In contrast to acetic and butyric acids, the PES index-based ration significantly affected propionate. Tukey's analysis showed that R2 was the highest C3 production, followed by R1 and R3 (not different), and the lowest was R4. The orthogonal polynomial analysis on propionate shows a quadratic effect with the equation Y= -481.8 X2+585.01 X–143.45 and the coefficient of determination (R^2)= 0.53. Propionate is the primary glucogenic fatty acid produced in the digestion of carbohydrates by ruminants. Propionate production has the same dynamics as total VFA production, namely an increase in R2 and a decrease in R3 and R4 (Figure 3), with the peak at R2 (0.61; 34.13%). The different formation pathways compared to C2 and C4 lead to significant C3 production. The NFE in R4 was the highest, followed by R3, R1, and R2. Although the NFE level of R2 is the lowest, DMD and OMD are the highest and achieve the same index of 0.61. In addition, propionate formation is closely related to the degradation of carbohydrates from the starch or NFE that occurs in 2 pathways, succinate, and acrylate (Syamsi et al. 2018; Isipato et al. 2020). The formation of C3 through the acrylic pathway utilizes lactic acid in the rumen fluid. This theory is very much in line with the measurement of pH degrees (Figure 2a) which shows a similar graphic rhythm. The higher the pH conditions obtained, the higher C3, namely the ration with an index of 0.61. The high pH indicates that the utilization of lactate in the production of C3 through the acrylate pathway also increases.

The study shows that the ratio of C2:C3 is between 3.15-3.85. it was high compared to the research result by Syamsi & Waldi (2021), which was between 1.77-1.94. The average C2 production is relatively high, with a calculation reaching 72.76%, and an increase does not follow it in the average propionate production. Therefore there is a high gap between C2 and C3. Ifani et al. (2021)

stated that in the stoichiometry of carbohydrate conversion to VFA, acetate production had a higher tendency, among others. This condition is referred to as acetogenic, where the activity of cellulolytic bacteria is more dominant than amylolytic. The analysis of variance on the PES index-based ration showed a highly significant effect ($P<0.01$) on the C2:C3 ratio and methane production. Tukey's analysis showed the order of C2:C3 from low to high, i.e., R2, R1, R3, and R4. The orthogonal polynomial analysis on the C2:C3 ratio indicates a quadratic effect with the equation $Y = 50.93X2–60.177X+21.067$ and the coefficient of determination (R^2)= 0.66 (Figure 4a). The lowest point of the graph is obtained at the ratio of 3.29 and the index of 0.59. Although the PES index had no significant effect on C2, Table 1 shows that the production of C2 in R2 had a reasonably low production compared to other treatments. In addition, the production of C3 in R2 was the highest. Therefore, the lowest C2:C3 ratio is achieved in this index range.

The research got methane production ranging from 40.90-45.89 mM. Tukey's analysis showed that R3 and R4 had the highest CH_4 production, but R3 was not different from R1, then R1 and R2 were the lowest. The quadratic effect influenced methane with the equation $Y= 202.45X2–223.18X+103.41$ and a coefficient of determination $R^2= 0.38$. The lowest point was at an index of 0.55, with a methane production of 41.90 mM (Figure 4b). Mitsumori and Sun (2008) stated that methane production highly correlates with the C2:C3 ratio. It is evidenced in Figures 4a and 4b, which show the same graphic trend. The higher C2:C3 gap ratio indicates the low use of hydrogen (H_2) in the propionate formation pathway, so it is more widely used in methanogenesis. For every 1 mole of methane, it takes 1 mole of CO_2 and 4 moles of H_2. These compounds are commonly produced in the C2 and C3 formation pathways from Ac-CoA. Each production of 1 mole of C2 will make 2 moles of CO_2 and 4 moles of H_2, while each output of 1 mole of butyrate will have 2 moles of CO_2 and 4 moles of H_2. On the other hand, the production of C3 makes more use of these two compounds, where 1 mole of C3 requires 3 moles of CO_2 and 5 moles of H_2. Therefore, increased C3 output will limit the availability of CO_2 and H_2, reducing the methanogenesis process (Syamsi et al. 2018; Yanuartono et al. 2019). Therefore, the lowest methane production was in the R2 ration index range because it had the highest C3 production with a low C2 average output compared to the others. Ammonia (NH_3) concentration is one of the indicators to determine feed protein degradability, microbial activity, and rumen microbial population (Susilo et al. 2019). The analysis of variance showed that the PES index-based ration had a highly significant effect on the concentration of NH_3. Tukey's analysis showed that R1 was the highest NH_3 production, but R1 was not different from R4, R4 was not different from R3, then R3 and R2 were the lowest. Analysis of the orthogonal polynomial shows a quadratic effect with the equation $Y= 436 X2-552.28 X+181.08$ with a coefficient of determination (R^2)= 0.52 (Figure 5a). The NH_3 graph decreases in the diet with a PES index of 0.6 (R2) at 6.16 mM to 0.55 (R1) at 9.32±2.89 mM. Then the concentration of NH_3 increased again to 0.65 (R3) and 0.7 (R4). According to Arias et al. (2020), the concentration of NH_3 in rumen fluid is influenced by protein degradation, rumen villi absorption, and microbial protein synthesis (MPS). This research was carried out using the in vitro method so that the observed concentration of NH_3 could only be associated with protein degradability and MPS. Figures 1a and 1b show that DMD and OMD produce closed quadratic graphs with the highest digestibility at an index of 0.61.

In contrast, the concentration of NH_3 produces an open quadratic graph with the lowest attendance at the index of 0.6. These results are contradictory, but high feed degradability does not determine the concentration of NH_3 because most of these compounds are also used in MPS. If MPS activity is high, then the use of NH_3 will also be increased so that its concentration is observed to be low in the rumen. The analysis of variance showed that the ratio based on the PES index had a highly significant effect on the MPS. Tukey's analysis showed that the highest MPS was R2, followed by R3, and the lowest was R1 and R4. MPS had a quadratic effect with the equation $Y= -1012 X2+1260X–311.64$ with a coefficient of determination (R2) = 0.59 (Figure 5b). The MPS increased from the index of 0.5 (R1) to 0.6 (R2). They then decreased with the lowest production in the index of 0.7 (R4). These results indicate an association with low NH_3 production in the same index range. It means that the activity of MPS is high, so many utilize NH_3 as a source of the N chain. High MPS in the rumen can describe the high colony and rumen microbial activity. Ifani et al. (2021) stated that factors affecting microbial protein synthesis were dry matter consumption, nitrogen compounds, fermented energy supply, the ratio of forage to concentrate in the ration, synchronization of nitrogen and energy, rumen environment, rate of food, vitamins, and minerals.

The complete results of the research show that there is an interrelated relationship between each variable. However, the results of this study are conceptually different from the basic concept developed by the inventor of this synchronization method, namely Sinclair et al. (1993). The basic idea of the synchronization index states that the digestibility and fermentability of the rumen will increase along with the increase in the PES index of the ration. Syamsi and Waldi (2021) said that studies on preparing the PES index-based ration showed varied results. Some studies show a positive effect with a linear effect, some have a quadratic effect, and some even have no significance. This research results from a

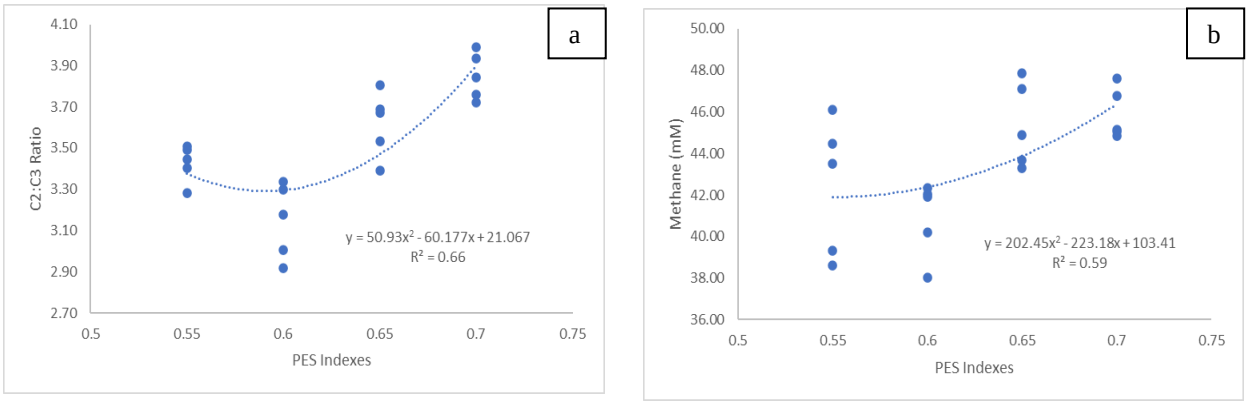

Figure 4. Graph of C2:C3 ratio (a) and methane production (b) of rumen fluid

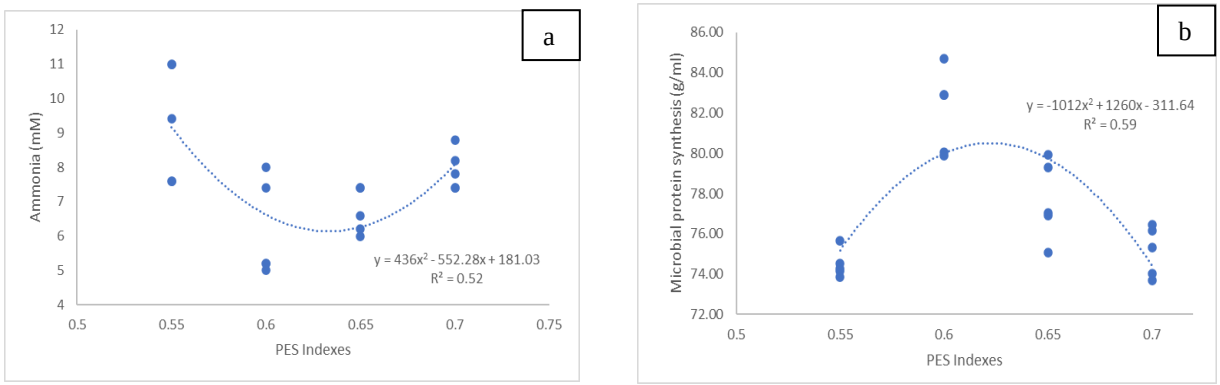

Figure 5. Graph of NH_3 (a) and microbial protein synthesis (b) of rumen fluid

follow-up study from a series of previous studies, namely the inventory of the PES index on various ruminant feedstuff carried out in vitro. Therefore, developing a list of feed ingredients PES index research needs to be done in Sacco. Then, the results can be tested again to be compared with the results of this study. Some critical notes observed from previous studies and other research results are that the preparation of rations based on the PES index must still pay attention to the nutrient composition of the resulting rations. The balance between protein, fiber, and NFE levels must still be considered to achieve a harmonious supply of ammonia and energy in the rumen fluid. On the other side, the rations in this study were prepared by calculating the individual index of each feedstuff. Therefore, designing rations must focus on achieving a predetermined index, which is also an important note; different formulating mechanisms must be compared. It means that the ration is prepared with a specific ratio of protein and NFE, then the PES index of the ration is determined through the in-sacco process. The development of this research is still very possible and produces more valid data.

CONCLUSION

Protein-energy synchronization (PES) index of 0.6 achieved the best rumen fermentability. Furthermore, the provision of the PES index-based ration of 0.6 was the most effective compared to other indexes based on the high propionate, the lowest methane, the lowest NH_3, and the best microbial protein synthesis. The ration with an index of 0.6 is composed of 30% elephant grass, 30% *Leucaena leucocephala*, 10% coconut meal, 10% tofu waste, 10% rice brand, 9% cassava waste, and 1% mineral mix.

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