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Macronutrient Digestibilities and Enzyme Activities in Rumen Fluid Supplemented by Protein-Energy Synchronized Index-Based Rations

Afduha Nurus Syamsi[1]*, Harwanto[2], Hermawan Setyo Widodo[1], and Yusuf Subagyo[1]

[1]Department of Livestock Production, Faculty of Animal Science, Jenderal Soedirman University, Purwokerto, 53123, Indonesia
[2]Department of Animal Feedstuff and Nutrition, Faculty of Animal Science, Jenderal Soedirman University, Purwokerto, 53123, Indonesia

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* Corresponding author:
Telp. +62 852 791 29 225
E-mail:
afduha.nurus.syamsi@unsoed.ac.id

ABSTRACT

The protein-energy synchronized (PES) index-based rations can optimize microbial protein synthesis (MPS) and affect enzyme activity and macronutrient digestibility of ration in rumen fluid. This study aimed to examine the in vitro effect of a ration based on PES index on macronutrient digestibility and enzyme activity in rumen fluid. The research was conducted experimentally, consisting of 4 treatment rations with different PES indexes (R1: 0.55; R2: 0.6; R3: 0.65; R4: 0.7). Each treatment was repeated 5 times, so there were 24 experimental units. The material used was the rumen fluid of the Jawa Randu Goat, which was taken at the Sokaraja Slaughterhouse shortly after the goat was slaughtered. Each ration consisted of napier grass, river tamarind, coconut meal, soybean dregs, rice bran, cassava waste, and mineral mix. The results showed that the PES index significantly affected (P<0,01) the digestibility of protein (A), fiber (B), and fat (C), as well as the activity of protease (D) and cellulase (E) enzymes. The orthogonal polynomial test showed that the PES index has a quadratic effect on all test parameters with the following equation: A ($Y= -1229.5X^2 + 1540.6X - 457.57$; $R^2= 0.75$), B ($Y = -800.95X^2 + 955.86X - 264.51$; $R^2 = 0.75$), C ($Y = 868.92X^2 - 1038.2X + 325.1$; $R^2 = 0.81$), D ($Y = -41.4X^2 + 51.697X - 14.982$; $R^2 = 0.77$), and E ($Y = -4.8538X^2 + 5.927X - 1.6241$; $R^2 = 0.84$). The protein-energy synchronized index-based rations increased the in vitro of macronutrient digestibility (protein, fiber, and fat) and enzyme activity on rumen fluid at a medium index level (0.6-0.63).

Keywords: Digestibility, Enzyme activity, Macronutrient, Protein-energy synchronization index

Introduction

The protein-energy synchronized (PES) index-based rations arrangement is a method introduced by Sinclair *et al.* (1993). This ration is prepared by setting a target ration index between 0-1 based on each feedstuff PES index. The principle of preparing this ration is to regulate the use of a feed ingredient in the ration to achieve a balance of ammonia supply from protein degradation and energy supply from carbohydrate degradation. A ration with an index approach to 1 is predicted to have a more simultaneous or synchronous supply of ammonia and energy (Syamsi *et al.*, 2017). The rumen fluid needs to synchronize protein and energy because both are the main factors required to optimize microbial protein synthesis (MPS). Anggraeny *et al.* (2015) stated that energy is a limiting factor for ammonia in the MPS. Therefore, the ammonia supply should not exceed energy. Excess ammonia will be excreted in the urine or can even cause urea toxicity.

Optimization of MPS is an important target to set in the ruminant rations arrangement because it is the leading implementer of feed digestibility in the rumen. The high number of MPS will correlate to the digestibility of nutrients and rumen fermentation products. Rumen microorganisms digest or hydrolyze nutrients by producing certain enzymes depending on the nutrient substrate. Some microbes can produce cellulose enzymes to digest fiber, proteases to digest protein, amylase to digest starch, lipase to digest fats, etc. The activity of digestive enzymes in the rumen can describe the high or low digestive process. The higher the enzyme activity, the higher the digestibility of nutrients in the rumen (Faradha *et al.*, 2019).

Research on the PES index-based rations has been carried out for a long time, but it has less focused on studying tropical feed ingredients. Researchers generally compile a ration, then

measure the PES index. This study seeks to gather the ration based on the set PES target by the PES index of each feed ingredient inventoried in the previous studies. The previous research has carried out a series of in vitro studies to obtain a protein-energy synchronization index of tropical feed ingredients from both forage and concentrate groups (Syamsi *et al.*, 2018; Syamsi *et al.*, 2020). Seo *et al.* (2012) research showed the highest PES index rations (0.95), the high dry matter, and organic matter digestibility. The increases of dry matter and organic matter digestibilities correlated with increased macronutrient digestibility, such as protein, carbohydrates, and fats. Syamsi *et al.* (2017) added that MPS was higher with an increasing ration PES index. It allows for an increase in the activity of digestive enzymes and increases the digestibility of ration nutrients in the rumen fluid. Cabrita *et al.* (2006) explained a significant effect between the PES index the rumen digestibility and fermented products, even if it had variated impacts in each study. The potential of the preparation of rations based on the PES index and its application to tropical feed ingredients needs to be studied well. This study aimed to examine the in vitro effect of a ration based on the PES index on macronutrient digestibility and enzyme activity in rumen fluid.

Materials and Methods

Experimental design

The experiment was carried out at the Nutrition and Animal Feeding Laboratory of the Faculty of Animal Science, Jenderal Sudirman University, from August to October 2021. The material used was the mixed rumen fluids of the three different Java Randu goats taken at the Sokaraja abattoir shortly after the goats were slaughtered. The rumen fluid was collected using a stabilized tube at 39^0C. The research experiment used a completely randomized design (CRD) consisting of 4 treatments, and each was 5 times repeated, so there were 20 experimental units. The rations arrangement was on Syamsi *et al.* (2020). The ration is prepared based on the target index determined through the index of each feed ingredient used. The method of preparing the feed used is the trial-error method. The list of feed ingredients and nutrient content is in Table 1.

In vitro digestibility measurement

The ration samples were incubated with rumen fluid according to the in vitro methods by Tilley and Terry (1969). Samples were weighed 2 g and put in a fermenter tube, then 24 mL of Mcdougall's (Buffer solution) was added and incubated in a shaker water bath with a temperature of 39ºC. The sample solution was then added with 16 mL of rumen fluid and flowed with CO_2 gas for 30 seconds. The sample was then closed using a ventilated rubber cover and 4 hours incubation for enzyme activity and 48 hours for digestibility measurement. Then it was separated from the residue and supernatant using a 4,000 rpm centrifuge for 15 minutes. The residue was used to measure digestibility, while the supernatant measured enzyme activity. The following formula measured the digestibility of protein, fiber, and fat content.

$$\text{Digestibility (\%)} = \frac{\text{nutrient of sample (g)} - \text{nutrient of residue(g)}}{\text{nutrient of sample (g)}} \text{ x } 100\%$$

Enzyme activity measurement

Cellulase activity (Solahuddin *et al.*, 2021). The first step is the isolation of the cellulase enzyme. Rumen fluid in cellophane bags was immersed in 0.05 M acetate buffer solution for 10 hours, then centrifuged at 3,400 rpm for 30 minutes. The supernatant was added to 40% ammonium sulfate little by little and stirred with a magnetic stirrer at a cold temperature (40ºC). The solution was allowed to stand at the same temperature, then centrifuged to obtain sediment. The sediment was dissolved in 0.05 M acetate buffer and dialyzed again to get a purer cellulase enzyme.

The second stage is making a standard glucose curve by dissolving 0.05 g of glucose with 50 mL of aquabidest, then making several concentrations, namely 0.1; 0.2; 0.3; 0.4; 0.5; and 0.6 mg/mL. Each solution was taken of 1.5 mL glucose standard and added by 1.5 mL of distilled water into the test tube. Each test tube was added with 1.5 mL of 3,5-di nitrosalicylic acid (DNS) reagent and homogenized. The test tube was heated for 5 minutes (100ºC) and cooled for 50 minutes (4ºC), then the absorbance was measured using a spectrophotometer at a wavelength (λ) of 540 nm UV-Vis.

The cellulase activity was tested by adding 1.5 mL of supernatant (enzyme), 1.5 mL of 1% CMC solution, and 1.5 mL of phosphate buffer pH 7. Then it homogenized with a vortex and then incubated for 15 minutes at 55°C. A total of 1.5 mL of DNS reagent was added to the solution, then boiled for 5 minutes in a water bath at 100ºC, then cooled for 50 minutes (4ºC), then the absorbance was measured using a spectrophotometer at a wavelength (λ) of 540 nm UV-vis.

Protease activity (Bergmeyer, 1983). Rumen fluid is taken from the incubation supernatant by filtration under cold conditions. The filtered liquid was centrifuged at 10,000 *g* for 10 minutes at 4ºC. The supernatant was taken as a crude enzyme source, then reacted with 60% ammonium sulfate. It is then stirred using a magnetic stirrer for 1 hour and allowed to stand for 24 hours at 4ºC. The supernatant was then centrifuged at 10,000 g for 15 minutes at 4ºC.

The enzyme obtained was taken and then dissolved in phosphate buffer pH 7.0 with a ratio of 10:1 (precipitate of 100 mL rumen fluid supernatant dissolved in 10 mL phosphate buffer pH 7.0). A total of 1 mL of 2% casein solution was mixed with 1 mL of boric buffer (0.01 M) pH 8.0, 0.20 mL of 0.05 M hydrochloric acid, and 0.20 mL

of protease enzyme extract to determine its activity. Then it was incubated in a water bath with a set temperature variation, namely: 30ºC, 35ºC, 40ºC, 45ºC, and 50ºC for 10 minutes, then added 2 mL of 0.1 M trichloroacetic acid (TCA), then incubated for 10 minutes and then centrifuged. The 1.5 mL filtrate was mixed with 5 mL of 0.4 M disodium carbonate and 1 mL of Folin Ciocalteu's reagent and allowed to stand for 20 minutes then read the absorbance at a wavelength of 578 nm. For blank determination, the enzyme solution was replaced with distilled water and for standard determination, the enzyme solution was replaced with tyrosine standard (5 mmol/mL).

Enzyme activity formula

$$EA = \frac{SpA - BA}{StA - BA}\ x\ F\ x\ \frac{1}{T}$$

Notes:
EA : Enzyme activity (U/mL)
SpA : Sample absorbance
StA : Standard absorbance
BA : Blank absorbance
F : Diluent factor
T : Incubation time

Data analysis

The data obtained were analyzed using analysis of variance to determine its significance. Orthogonal polynomials were used to further tested the significant parameters.

Results and Discussion

Macronutrient digestibility

A protein-energy synchronization (PES) index-based rations were prepared to optimize microbial protein synthesis (MPS), but this is not the main objective of this method. The increase in MPS through the simultaneous availability of energy and ammonia is expected to impact increasing nutrient digestibility and synthesis of fermentation products in the rumen fluid. Table 2 shows that the average digestibilities of crude protein, crude fiber, and crude fat in rations based on the PES index are between 17.41%-25.59%, 12.69%-22.34%, and 15.98%-24.48%. This result is much lower than that of Antisa *et al.* (2020), which explains that the digestibility of the three macronutrients is above 50%. The low TDN of each treatment (Table 1) caused the low degradability of macronutrients. Setiyawan *et al.* (2019) state that the TDN ration for goats is at least 65%.

The analysis of variance showed that the PES index had a very significant effect (P<0.01) on the digestibility of crude protein, crude fiber, and crude fat. Henning *et al.* (1991) stated that differences in the level of synchronization of protein and energy in the ration significantly affect in vitro nutrient digestibility. The performance of rumen microorganisms on macronutrient digestibility is highly dependent on the simultaneous availability of energy and ammonia. Syamsi *et al.* (2017) prove that MPS increases commensurate with the increase in the PES index ration. The setting of the PES index causes differences in the percentage of feed ingredients in the ration. It impacts different levels of different nutrients, different supplies of energy and ammonia, and different digestibility of each ration.

The orthogonal polynomial test showed that the PES index had a quadratic effect on crude protein and crude fiber digestibility. The impact of the PES index on crude protein digestibility

Table 1. The ration composition based on protein-energy synchronization (PES) index

Feedstuffs	R1	R2	R3	R4
PES Index	0.55	0.6	0.65	0.7
Napier grass (%)	20	30	40	50
River tamarind (%)	40	30	20	8
Coconut meal (%)	5	10	11	11
Soybean dregs (%)	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
Nutrition	R1	R2	R3	R4
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

Source: Syamsi *et al.* (2021); DM: Dry matter; NFE: Nitrogen free extract; TDN: Total digestible nutrient.

Table 2. Macronutrient digestibility in rations based on in vitro protein-energy synchronization index

Digestibility	Treatments				Sig.
	R1	R2	R3	R4	
Crude protein (%)	17.41±0.96	25.59±0.82	23.02±0.78	18.90±0.33	**
Crude Fiber (%)	18.38±0.93	22.34±0.76	16.74±0.91	12.69±0.45	**
Crude Fat (%)	16.65±0.98	15.98±0.89	16.47±0.91	24.48±0.34	**

Sig: Significance; **Highly significant.

resulted in the equation Y= -1229.5X^2 + 1540.6X - 457.57 with a coefficient of determination (R^2) = 0.75. The effect of the PES index on the digestibility of crude fiber resulted in the equation Y = -800.95X^2 + 955.86X - 264.51 with a coefficient of determination (R^2) = 0.75. Figure 1a showed that protein digestibility increased from index 0.55 (R1) to a peak (XP) at index 0.63, then declined after that. Figure 1b showed that crude fiber digestibility increased from index 0.55 (R1) to a peak (XP) at index 0.6 (R2), then continued to decrease after that. The highest percentage of protein digestibility (YP) was 25.03%, and the highest crude fiber digestibility (YP) was 20.67%.

Figures 1 (a and b) showed a contradictory result with the hypothesis that claimed nutrient digestibility increases with increasing PES index (Sinclair *et al.*, 1993). This difference could be due to the rhythms between protein content and ration nitrogen free extract (NFE) (Table 1). The protein content of the ration decreased along with the increase in the PES index, while the NFE level increased from an index of 0.55 (R1) to 0.6 (R2), then increased again after that. Lee *et al.* (2003) stated that the performance of rumen microorganisms would be strongly influenced by the balance of fermentable carbohydrates (NFE) and protein degradation in the ration. Conceptually, the setting of the PES index should have an effect on the rhythmic rhythm between NFE and protein in the ration. Syamsi *et al.* (2018) further explained that energy is a limiting factor for ammonia in MPS, so the availability oga adaf energy from digestible fermentable carbohydrates must be adjusted to ammonia from protein digestibility.

Syamsi *et al.* (2020) and Syamsi *et al.* (2021) explained that local feed ingredients have different characters than sub-tropical feed ingredients. Some feed ingredients, such as river tamarind, have certain antinutrients that can affect overall digestibility. Utomo (2012) further explained that river tamarind is a proteinaceous roughage (a source of fiber with high protein content). River tamarind protein is bound in a strong matrix and tannins, so it has lower degradation in the rumen. The presence of antinutrients and the slow degradation of protein from river tamarind are other reasons that may cause the digestibility of protein and fiber to be non-linear with the increase in the PES index of the ration.

The PES index also has a quadratic effect on crude fat digestibility through the orthogonal polynomial test. The effect of the PES index on crude fat digestibility resulted in the equation Y = 868.92X^2 - 1038.2X + 325.1 with a coefficient of determination (R^2) = 0.81. Figure 2 showed a different crude fat digestibility chart than the crude protein and crude fiber digestibility chart. Crude fat digestibility decreased from PES index 0.55 (R1) to the bottom point (XP) at PES index 0.6 (R2), then increased steadily after that. The lowest average crude fat digestibility is at index 0.6, which is 14.99%, while the highest average digestibility is at index 0.7, 24.48%.

Bauman and Lock (2006) explained that the ration fat content would undergo hydrolysis of ester bonds in the fat-derived, followed by the biohydrogenation process of unsaturated fatty acids after the fat is hydrolyzed into free fatty acids. Wina and Susana (2013) added that 85% of ration fat content would undergo biohydrogenation into free fatty acids (saturated), sugar, phosphate, and glycerol. Unsaturated fatty acids from feed cannot be utilized directly by rumen metabolism, so they must go through this process. The ration fat content showed a decrease in line with the increase in the PES index, but the highest digestibility was found at the index of 0.7. It could be due to the increased use of coconut meals in R3 and R4. Syamsi *et al.* (2017) revealed that coconut meals have a relatively high short-chain fatty acid, so its use in rumen metabolism is much faster.

Enzyme activity

Microorganisms are the main actors in fermentation or digestion in the rumen. Microorganisms work by producing specific enzymes to digest certain nutrients. Lipase enzymes of microbe regulate fatty acid metabolism in the rumen and limit the biohydrogenation of polyunsaturated fatty acids. Xylanase breaks down the polysaccharide β-1,4-xylan, the main hemicellulose chain, while cellulase breaks down crystalline cellulose in cellulose fibers. Dehydrogenases, ureases, and proteases can react with proteins and urea, which supply ideal protein nutrients to the host (Hao *et al.*, 2021). Table 3 shows the activity of two main enzymes in the rumen, namely proteases and cellulases.

Table 3 showed that the protease enzyme activity in the study was between 0.917-1.16 U/mL, higher than the results by Moharrerya and Das (2001), which found the average activity of the protease enzyme was between 0.152-0.201 U/mL. The cellulase enzyme activity in the study was between 0.151-0.198 U/mL, higher than Solahuddin *et al.* (2021), which is between 0.145-0.147 U/mL. Hao *et al.* (2021) explained that differences in rumen microbial enzyme activity could be caused by genetics, type of livestock, feed substrate, number of microbes, rumen pH, and rumen fluid temperature. The variance analysis showed that the PES index had a very significant effect ($P<0.01$) on the activity of protease and cellulase enzymes. These results are in line with crude protein and crude fiber (Table 2). The different percentage of feed ingredients in each index causes differences in the nutrient content of the ration. Protein and NFE differences in each ration caused microbial protein synthesis and metabolic activity differences.

The orthogonal polynomial analysis showed that the PES index had a quadratic effect on the activity of protease and cellulase enzymes.

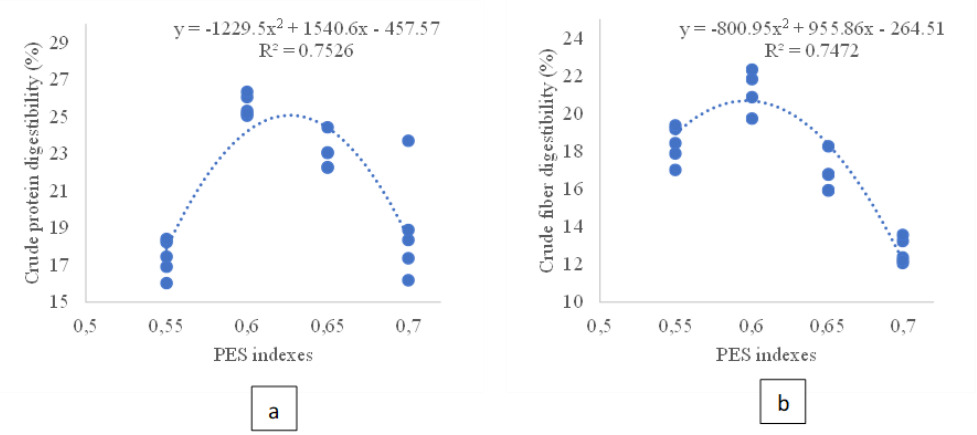

Figure 1. The PES index effect on in vitro crude protein and crude fiber digestibility Graphs.

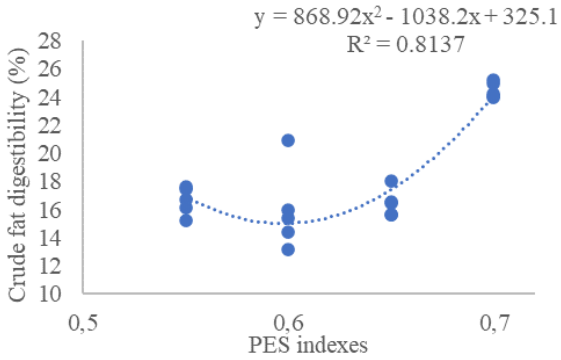

Figure 2. The PES index effect on in vitro crude fat digestibility Graphs.

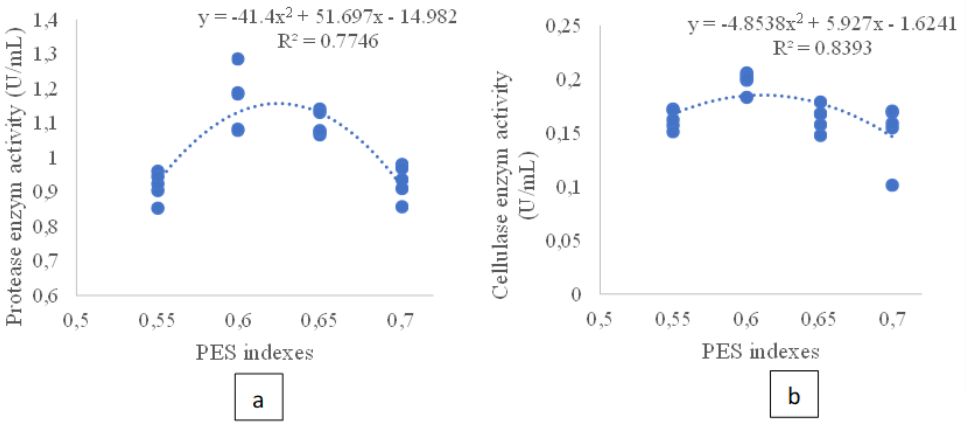

Figure 3. The effect of the PES index on the in vitro activity of protease and cellulase enzymes Graphs.

Table 3. In vitro enzyme activity in ration based on protein-energy synchronization index

Enzym activity	Treatments				Sig.
	R1	R2	R3	R4	
Protease (U/mL)	0.917±0.001	1.164±0.007	1.098±0.001	0.930±0.002	**
Cellulase (U/mL)	0.163±0.008	0.198±0.007	0.164±0.001	0.151±0.008	**

Sig: Significance; **Highly significant.

The effect of the PES index on the protease enzyme activity got the equation Y = -41.4X^2 + 51.697X – 14.982 with a coefficient of determination (R^2) = 0.77, while the cellulase enzyme activity got the equation Y = -4.8538X^2 + 5.927X – 1.6241 with a coefficient of determination (R^2) = 0.84. The peak points obtained from Figure 3a are XP = 0.62 and YP = 1.16, while in Figure 3b are XP = 0.61 and YP = 0.19. Supporting results were shown in previous research by Kaswari *et al.* (2007) and Achmadi *et al.* (2016) that the medium PES index (0.5-0.52)

has a higher microbial protein synthesis than the lower or higher-level PES index. Hao *et al.* (2021) confirmed that enzyme activity is strongly influenced by the number of microbes in the rumen. The higher a certain microorganism, the higher the enzyme activity.

The difference between the rhythm of protein and NFE levels in each ration and the use of river tamarind in the ration was thought to be the primary influence on these results. Table 1 showed decreased protein and fiber content with an increasing PES index in the ration. However, the results show that R2 tends to have higher enzyme activity. The results of previous research from Waldi *et al.* (2017), Syamsi *et al.* (2018), and Syamsi *et al.* (2021) regarding rumen fermentation products in rations with different PES indexes also showed similar results. Although the index has been widened or narrowed, the results show a quadratic effect. More in-depth studies through in sacco and in vivo techniques are needed to clarify the results better.

Conclusions

The protein-energy synchronized index-based rations increased the in vitro of macronutrient digestibilities (protein, fiber, and fat) and enzyme activity on rumen fluid at a medium index level (0.6-0.63).

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