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The effects of energy and protein content in maize forage-based complete diet on *in vitro* ruminal fermentation, gas production, and feed degradability

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ABSTRACT

This research aimed to evaluate the effects of energy and protein contents in a complete diet on *in vitro* gas production, rumen fermentation, and feed degradability. The experiment used a 3x3 factorial arrangement in a randomized block design with two factors and three replications. The first factor was energy content in the complete diets; E_1=12.5MJ/kg DM, E_2=13.5MJ/kg DM, and E_3=14.5 MJ/kg DM. The second factor was protein content in the complete diets; P_1=10.5%, P_2=13.5%, and P_3=16.5%. The complete diet was composed of maize forage silage 37.5 % w/w DM, elephant grass (*Pennisetum purpureum*) 12.5 % w/w DM, and concentrate 50.0 % w/w DM. The concentrate was composed of commercial dairy concentrate feed produced by SAE local dairy cooperative, cassava waste, soybean meal, rice bran, and coffee husk. All of the treatment diets were tested using *in vitro* gas production test. The variables were total, potential, and rate of *in vitro* gas production, NH_3 concentration, efficiency of microbial protein synthesis (EMPS), dry matter degradability (DMD), and organic matter degradability (OMD). Either energy or protein content of the treatment diets had a highly significant effect ($P<0.01$) on the total, potential, and rate of *in vitro* gas production, NH_3 concentration, EMPS, DMD, and OMD, but not for the treatment combination. An increase in energy and protein content in the treatment diets increased the value of all parameters but decreased EMPS.

Keywords: energy, protein, complete diets, degradability, gas production, microbial growth

INTRODUCTION

Protein and energy are the most nutrients required by all organisms after water requirement (McDonald et al., 2010). Upon consumption by ruminants, all of the feed nutrients enter firstly into the reticulo-rumen and are firstly digested or utilized by rumen microbes. The feed consumed by ruminants can directly affect the condition of the reticulo-rumen condition, rumen microbial growth, and feed digestion. Energy in the diet is mostly from carbohydrates, either fiber carbohydrates or mainly non-fiber carbohydrates. The carbohydrates also directly affect rumen condition, especially pH. Fiber carbohydrates increase rumen pH effectively, while non-fiber carbohydrates decrease rumen pH. Carbohydrates also function as a source of carbon skeletons for amino acid synthesis by rumen microbes during their growth. Protein in the diet is in the form of true protein and non-protein nitrogen. Both proteins are also firstly digested and utilized by rumen microbes. Dietary protein is the most nitrogen source for microbial growth in the rumen. All rumen conditions and nutrients affect sensitively rumen microbial growth and population. The rumen microbes are importantly responsible for feed digestion in the rumen, especially for dietary fiber into VFAs which are the main source of energy for host ruminants. Some rumen microbes flow into the abomasum and small intestine and function as the main source of protein for host ruminants.

In addition to the quantity of feed, the balance of the available nutrients, mainly carbohydrates or energy, and protein, is also very important for rumen microbial growth. The availability of protein for rumen microbes in balance with the availability of energy, either in their quantity and time of availability must increase the rumen microbial growth and population as well as their activities in degrading feed, especially fiber carbohydrates. Upon their degradation in the rumen, carbohydrates supply energy and carbon skeletons, and protein supply nitrogen to rumen microbes for synthesis of microbial cells amino acids for

the growth. Leng (1991) stated that balancing energy and protein in the ration will affect the efficiency of nutrient utilization for production.

Feeding a complete diet to ruminants is an important strategy in ruminant feeding management. The complete diet is well formulated and mixed forages and concentrates for ruminants. Feeding a complete diet to ruminants must create better rumen conditions and supply adequate and balanced nutrients for rumen microbial growth than separate feeding of forages and concentrate (Beigh et al., 2017). However, availability of forages is one constraint in ruminant production in the tropics, such as in Indonesia (Hartutik et al., 2022). Most farmers have very limited land, and the focus of its utilization is on growing food or cash crop, not on fodder plants. Farmers mostly collect forages from rangelands. In addition, two different seasons in this area also affect the stability of forage availability, grasses mostly grow better during the rainy season and are scarcely available during the dry season (Achmadi, 2007). Maize (*Zea mays* L.) forage is a common source of forage for ruminants. Heuzé et al. (2017) stated that maize green forage consists of stems, leaves, and ears with high energy for ruminants. Maize forage contains CP 10.9%, fat 2.17%, crude fiber 33.21%, NFE 46.05%, and gross energy 3791 kcal/kg, which is good nutrition for ruminants (Binol et al., 2020). A hectare of maize plantation produces 3 to 7 ton maize forages and can be fed to ruminants as fresh or conserved as silage to maintain nutrition (Zaidi et al., 2013). Wang et al. (2021) stated that ensiling is an important method for keeping the forage nutrient, and it can supply feedstuff throughout the year. Achmadi et al. (2020) mentioned that to achieve feed availability for ruminant production, processing by-product of agroindustry is necessary to be used during the dry season when the availability of roughage feed is low. Maize silage feeding can be in the form of a single feed or mixed with other feed material into a complete diet. Wibisono et al. (2020) reported that formulating a complete diet with 30% maize silage and 70% commercial concentrate showed crude protein content of 12.99%, CF of 14.22%, and NFE of 46.42%. For those based on the review, this study was done to evaluate the effect of energy and protein contents in maize forage silage-based complete diet on *in vitro* ruminal feed fermentation, gas production, and feed degradability.

MATERIALS AND METHODS

Location and Time

This study was conducted from August 2020 to March 2021 in the Faculty of Animal Science, Brawijaya University Sumber Sekar Field Laboratory for making the maize forage silage and in Animal Feed and Nutrition Laboratory to evaluate the *in vitro* rumen fermentation, gas production, and degradability and samples analysis.

Method

This experiment used a 3x3 factorial arrangement in a randomized block design with two factors. The first factor was energy contents in the complete diets as the second priority nutrients required by the body after water, i.e. E_1=12.5MJ/kg DM, E_2=13.5MJ/kg DM, and E_3=14.5 MJ/kg DM. The second factor was protein contents in the complete diets as the third priority nutrients required by the body, i.e. P_1=10.5% DM, P_2=13.5% DM, and P_3=16.5% DM. Thus, in total, there were nine treatment combinations. Each treatment ration was evaluated using *in vitro* gas production test according to the procedure of Makkar et al. (1995). The evaluation was done three times using rumen fluids collected at three different times as replication.

The complete diet was composed of maize forage silage 37.5 % w/w DM, elephant grass (*Pennisetum purpureum*) 12.5 % w/w DM, and concentrate 50.0 % w/w DM. The concentrate was composed of commercial dairy concentrate feed produced by SAE local dairy cooperative, cassava waste, soybean meal, rice bran, and coffee husk. The silage was made of maize forage harvested 65 days after planting, molasses, and *Lactobacillus plantarum* 1 x 10^6 CFU/mg. The maize forage was wilted for a day and chopped into 2-5cm particle size. The forage was then properly mixed with a mixture of molasses and *Lactobacillus plantarum* 1x10^6 cfu/g (10: 1 ratio) as much as 6% of maize forage weight, put in airtight plastic bag silo and incubated for 14 days.

The nutrient content of each feed ingredient as analyzed using proximate analysis (AOAC, 2005) and the energy content as estimated using the procedure of Menke et al. (1979) is presented in Table 1, and feed composition in each treatment diet is presented in Table 2.

Table 1. Nutrient content of feedstuffs used in each treatment diet

Feedstuff	DM (%)	OM (% DM)	Ash (% DM)	CF (% DM)	Fat (% DM)	CP (% DM)	ME** (MJ/kg DM)
SAE dairy concentrate feed	97.60	90.06	9.94	14.97	4.70	18.38	18.39
Cassava waste	92.59	82.87	17.13	25.39	0.44	1.76	20.57
Soybean meal (SBM)	93.53	91.62	8.38	4.04	2.57	47.53	15.14
Rice bran	90.63	87.40	12.60	16.20	13.00	10.15	12.49
Coffee husk	94.14	89.42	10.58	34.00	1.49	10.11	9.74
Maize forage silage	94.54	89.36	10.64	22.45	0.94	7.80	12.39
Elephant grass	96.12	85.95	14.05	31.99	2.35	12.08	11.81

** Metabolizable Energy content as estimated using the procedure of Menke et al. (1979).

Table 2. Composition of ingredients in each treatment diet on DM basis

Treatment	ME content (MJ/kg DM)	Protein Content (%)	Maize silage (%)	E. grass (%)	Concentrate (%)	Rice bran (%)	Cassava waste (%)	SBM (%)	Coffee husk (%)
E_1P_1	12.5	10.5	37.5	12.5	18	12	4.5	-	15.5
E_1P_2	12.5	13.5	37.5	12.5	23	7	-	5.5	14.5
E_1P_3	12.5	16.5	37.5	12.5	18	-	-	14.5	17.5
E_2P_1	13.5	10.5	37.5	12.5	24	6	11.5	-	8.5
E_2P_2	13.5	13.5	37.5	12.5	50	-	-	-	-
E_2P_3	13.5	16.5	37.5	12.5	36.5	-	2.3	11.2	-
E_3P_1	14.5	10.5	37.5	12.5	30	-	19.5	0.5	-
E_3P_2	14.5	13.5	37.5	12.5	23	-	17.6	9.4	-
E_3P_3	14.5	16.5	37.5	12.5	13.5	-	17	19.5	-

All of the treatment diets were evaluated using *in vitro* gas production test according to the procedure of Makkar et al. (1995) three times as replication using rumen liquid collected from a rumen fistulated cow feed on fresh elephant grass and concentrate at 60%:40% DM weight ratio in three different days.

Variable

The variables measured in this study were total gas production, gas production potential, and gas production rate based on the difference in gas volume in the syringe after 48 hours of sample incubation and the initial volume, pH, temperature, NH_3 concentration of supernatant, dry matter and organic matter degradability, the efficiency of microbial protein synthesis (EMPS) measured after 48 hours sample incubation according to the procedure of Blümmel et al. (1997).

Data Analysis

The data were analyzed using ANOVA of a 3x3 factorial arrangement in a randomized block design with two factors. If the treatment showed a significant effect ($P<0.5$), a mean comparison was continued with Duncan Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

Nutrient contents of the treatment diets

The nutrient contents of all treatment diets are presented in Table 3. DM content ranged from 94.59 (E_3P_3) to 96.25% (E_2P_2), OM content from 87.89 (E_3P_1) to 89.40% DM (E_1P_3), ash from 10.60 (E_1P_3) to 12.11% DM (E_3P_1), CF from 18.92 (E_2P_3) to 23.47% DM (E_1P_1), fat from 1,86 (E_3P_3) to 3.3% DM (E_1P_1, CP content from 10.52% (E_2P_1) to 16.51% DM (E_2P_3) and ME from 12.52 (E_1P_2) to 14.50 MJ/kg DM (E_3P_3).

To adjust crude protein and energy content of the treatment diets that were as treatments in this experiment was used mainly SBM as a protein source and cassava waste as an energy source for major adjustment, and rice bran and coffee pulp for minor adjustment (Table 2). The energy content of the treatment diets increased using cassava waste, while the protein content of the treatment diets increased by using soybean meal replacing concentrate feed.

Cassava waste contains high energy in respect of its protein content (20.57% DM vs 1.76 MJ/kg DM), while SBM contains high protein in respect of its energy content (47.53% DM vs 15.14 MJ/kg DM). MLA (2015) stated that the major nutrient components of feed that contribute to energy content are carbohydrates, fat, and protein. The different components of feed provide different amounts of energy to the animal and will be used in different ways by the animal. Lukuyu et al. (2014) mentioned that cassava waste provides great energy

for ruminants. Hartutik et al. (2020) stated that SBM could increase the value of CP content and total digestible nutrient (TDN).

Table 3. Nutrient content of treatment diets

Treatment diets	DM* (%)	OM* (% DM)	Ash* (% DM)	CF* (% DM)	Fat* (% DM)	CP* (% DM)	ME** (MJ/Kg DM)
E_1P_1	94.62	88.54	11.46	23.47	3.30	10.61	12.62
E_1P_2	95.01	89.09	10.91	22.15	3.00	13.45	12.52
E_1P_3	95.04	89.40	10.60	21.65	2.13	16.41	12.59
E_2P_1	94.94	88.24	11.76	22.79	2.73	10.52	13.49
E_2P_2	96.25	89.28	10.72	19.90	3.00	13.63	13.25
E_2P_3	95.67	89.29	10.71	18.92	2.66	16.51	13.50
E_3P_1	95.24	87.89	12.11	21.88	2.16	10.54	14.49
E_3P_2	94.97	88.17	11.83	20.71	2.05	13.44	14.45
E_3P_3	94.59	88.37	11.63	19.54	1.86	16.48	14.50

*Proximate analysis in Animal Feed and Nutrition Lab. Faculty of Animal Science, Brawijaya University (2021).
**Calculated according to Menke et al. (1979).

The effect of treatment diets on the parameters of in vitro gas production test

In vitro gas production tests for all of the treatment diets were done according to the procedure of Makkar et al. (1995). Based on the average pH and temperature of the substrate in the syringes after 48 hours of incubation time were 6.87±0.03 and 37.59±0.29° C (Table 4), respectively, were not significantly different between the treatments and volume of gas production in the syringes that increased steadily during the tests (Figure 1), so that it convinced that feed fermentation processes by microbes during the tests took place properly. The results of the tests are presented in Table 4 and are discussed below. Owen and Goetsch (1988) stated that to achieve maximum microbial growth, rumen conditions must have a pH in the range of 5.5-7.2 and a temperature between 38-41^{0}C.

According to Guo et al. (2022), rumen pH generally ranges from 6 to 7 and can be used to judge the ruminal environment and health. Rumen pH is affected by VFA interaction in chyme with buffer salt in saliva, and the absorption of VFAs by the rumen epithelium and outflow with chyme. A diet with high non-fiber carbohydrates decreased pH value along with decreased acetate: propionate ratio.

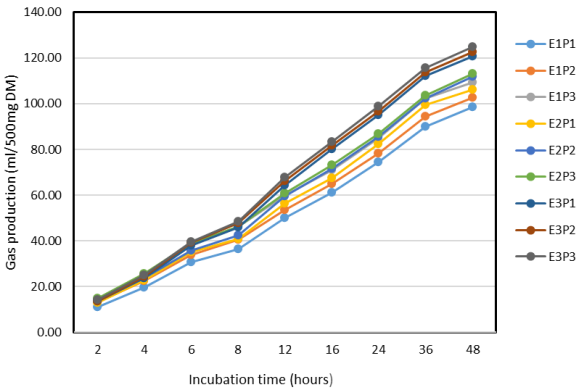

Figure 1. *In vitro* gas production curve of each treatment diet

Data in Table 4 show that the content of energy and protein in the diet, each gave a very significant effect (P<0.01) on all parameters, including the total and the potential of *in vitro* gas production, degradability of DM (DMD) and OM (OMD) as well as NH_3 concentration and EMPS. Protein content in the diets gave also significant effect (P<0.5) on the rate of *in vitro* gas production, but not for energy content in the diets. The value of all parameters increased due to the increase of energy as well as protein content in the diet, except the value of EMPS decreased due to the increase of energy as well as protein content in the diet. However, the treatment combination between energy and protein contents in the treatment diets did not give a significant effect (P>0.05) on all parameters, although all of the data increased consistently due to the increase of energy and protein contents in the diet.

As shown in Table 4, DMD and OMD, the total gas production and its rate of production as one of the products of DM and OM digestion increased as the energy and protein content in the treatment diets

increased where the highest values were in the treatment diets with highest energy (E3) and protein (P3) contents. The data were in line with the research of Sultan et al. (2010), who did an evaluation on nutrient digestibility and feedlot performance of lambs fed diets varying protein and energy contents. Zheng et al. (2020) mentioned that the larger the amount of gas produced, the better the feed fermentation.

Ammonia or NH_3 is the final product of crude protein degradation in the rumen, which is mostly from feed protein and, to a lesser extent, from lysis rumen microbes that release their cell protein. Ammonia is the main nitrogen source for rumen microbial growth, especially for rumen bacteria and fungi. With the availability of energy and other nutrients, ammonia is incorporated with carbon skeletons to synthesize cell protein during rumen microbial growth. Hence in *in vitro* digestibility test, the ammonia pool in the samples is directly affected by protein content and quantity of feed sample, as well as its degradation in the rumen liquid minus ammonia utilization for microbial growth. McDonald et al. (2002) stated that the concentration of NH_3 in the rumen is influenced by the protein content of the feed, rumen pH, the solubility of protein feed ingredients, and the time after feeding. Data in Table 4 show that the concentration of NH_3 in the treatment diets ranges from 4.29-7.75 mMol. Some of the NH_3 concentrations in this research were less than ideal as the optimal NH_3 concentration in the rumen, according to McDonald et al. (2010), ranges from 6 to 21 mMol. The concentration increased as the protein and energy content, as well as DM and OM degradability increased, and microbial biomass, as well as the efficiency of microbial protein synthesis decreased.

EMPS data in Table 4 ranged from 34.06-53.77 g microbial N/kg FOM. The optimal EMPS ranges from 30-40 g N/kg FOM but normally ranges from 10-70 g N/kg FOM (Karsli and Russell, 2001). The EMPS values decreased as the energy and protein content in the treatment diets increased, which were in contrast with the ammonia concentration, gas production as well as DMD and OMD that increased as the energy and protein content in the treatment diets increased. Karsli and Russell (2001) reported that microbial protein synthesis is highly dependent on the adequacy of nutrients available for their growth, especially energy in the form of ATP as a result of degradation of organic matter and N as a result of degradation of protein in the rumen.

In a closed cell or microbial cultures such as *in vitro* digestibility test, the growth of cell or microbe is usually divided into lag, exponential, stationary, and death phases (Prescott et al., 2002; Peleg and Corradini, 2011). During the lag phase, cells or microbes undergo intracellular changes to adjust to a new environment, and little or no cell reproduction takes place. During the exponential phase, cells reproduce at a rate proportional to the number of cells leading to an exponential increase in the number of cells. The stationary phase follows when nutrients are limited, or other environmental conditions restrict the number of cells that can be supported. Finally, cellular death and a declining population occur when the surroundings cannot maintain the population.

Thus, based on the typical microbial growth pattern in the closed cultures, the microbial growth and biomass decrease, or the microbes reach the death phase when the availability of nutrients is depleted. Thus, feed degradability and gas production in the rumen reflects the intensity of the rumen microbe's activities in digesting feed nutrients and their growth or population. The rate of feed digestion and gas production must be determined by the amount of feed available and its degradability as well as the microbe population, kind, and their activities. The nutrient depletion and then death phase of microbial growth happen earlier when the substrates are easier to be degraded by the microbes. In this experiment, the phenomenon was confirmed by the total gas production and its production rate (Figure 1 and Table 4) as well as DMD and OMD (Table 4). As shown in Table 4, the total gas production and its rate of production, as well as DMD and OMD, were highest in the treatment diets with the highest energy (E_3) and protein (P_3) contents that were in line with the research of Sultan et al. (2010). However, E_3 and P_3 treatments showed the lowest EMPS or microbial biomass. The highest DMD and OMD in the E_3 and P_3 treatments, as shown also by their highest total gas production and its rate of production, resulted in nutrient depletion in E_3 and P_3 treatments that took place earlier than those in the other treatments. Consequently, the microbes reached the death phase earlier and then the EMPS or microbial biomass decreased faster than in the other treatments. Feed degradability and gas production in the rumen reflects the intensity of the rumen microbe's activities in digesting and utilizing feed nutrients for their

growth. In other words, the rate of feed digestion and gas production must be determined by the feed itself (its availability and degradability) and the microbe population, kind, and their activities. In addition, Sauvant and Van Milgen. (1995) reported that microbial protein synthesis would be optimal if the release of N precursors and carbon skeletons in the rumen needed by microbes is aligned or synchronized. EMPS value is influenced by the availability of energy and amino acids used by rumen microbes. Insufficient energy will cause the deamination of amino acids and available carbon chains will ferment into VFAs. Conversely, an excess of amino acids in the rumen will only become NH_3, because some microbes cannot produce amino acids (Bach et al., 2005).

Table 4. The effects of treatment diets on parameters of *in vitro* gas production test

Treatment diets	pH	Temp (ºC)	Total gas prod. (ml/0.5g DM)	Potential of gas prod. (ml/0.5 g DM)	Rate of gas prod. (ml/hour)	DMD (%)	OMD (%)	NH_3 (mM)	EMPS (g N/kg FOM)
Effect of energy content (E)									
E_1	6.89	37.33	104.16^a	109.91^a	0.055	54.67^a	56.22^a	5.44^a	49.50^b
E_2	6.84	37.67	110.62^b	116.77^b	0.057	57.87^a	59.58^b	5.80^{ab}	45.57^{ab}
E_3	6.86	37.78	123.56^c	131.19^c	0.059	61.30^b	63.85^c	6.66^b	39.59^a
SEM	0.03	0.29	9.33	9.74	0.08	4.28	4.50	1.12	5.89
Sign.	n	n	**	**	n	**	**	**	**
Effect of protein content (P)									
P_1	6.89	37.39	108.74^a	116.71^a	0.057^{ab}	54.67^a	56.93^a	4.83^a	48.69^b
P_2	6.84	37.61	112.91^b	119.30^{ab}	0.055^a	57.54^a	59.14^a	6.04^b	46.06^{ab}
P_3	6.86	37.78	116.68^c	121.85^b	0.059^b	61.63^b	63.58^b	7.03^c	39.92^a
SEM	0.03	0.29	9.33	9.74	0.003	4.28	4.50	1.12	5.89
Sign.	n	n	**	**	*	**	**	**	**
Effect of energy and protein content (EP)									
E_1P_1	6.95	37.00	98.93	106.65	0.052	51.25	53.00	4.57	53.77
E_1P_2	6.85	37.50	103.29	108.68	0.055	53.06	54.43	5.45	49.73
E_1P_3	6.87	37.50	110.25	114.39	0.060	59.69	61.23	6.29	45.01
E_2P_1	6.85	37.50	105.95	114.01	0.055	53.97	56.34	4.29	49.98
E_2P_2	6.83	37.50	112.10	118.45	0.056	58.85	59.83	6.06	46.04
E_2P_3	6.85	38.00	113.83	117.85	0.059	60.79	62.57	7.05	40.69
E_3P_1	6.87	37.67	121.34	129.48	0.058	58.79	61.45	5.62	42.31
E_3P_2	6.85	37.83	123.36	130.77	0.060	60.71	63.15	6.62	42.40
E_3P_3	6.85	37.83	125.97	133.32	0.060	64.39	66.96	7.75	34.06
SEM	0.02	0.11	1.91	1.93	0.001	0.99	0.99	0.24	1.35
Sign.	n	n	n	n	n	n	n	n	n

CONCLUSIONS

Based on the results of this research, it can be concluded that the increase of either energy or protein content in the diets increased the rate of feed DM and OM digestibility as well as the rate and total gas production in the rumen *in vitro*, but fastened the decrease of the EMPS or microbial biomass.

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