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In vitro assessment of digestibility and rumen fermentation of ammoniated rice straw based beef diet, supplemented with extract of garlic and *Sapindus rarak*

C H Prayitno, Suwarno and A Susanto

Faculty of Animal Science, Jenderal Soedirman University. PO Box 53123 Purwokerto, Indonesia
caribu_prayitno@yahoo.co.id

Abstract

The study was conducted to investigate the effect of garlic extract (GE) and *Sapindus rarak* extract (SRE) on rumen fermentation and rumen microbial structure in vitro experiment. Randomized completely block design was used in this study. There were 6 treatments and 5 blocks included. The materials of this study consisted of rumen fluid of beef cattle. The diet of beef cattle was composed of 40% ammoniated rice straw and 60% concentrates The experimental treatments were, CARS : Concentrate:ammoniated rice straw (60:40), G250 : CARS + 250 ppm GE), G250-SR : CARS+ 250 ppm GE + 0.18 % SRE, G500-SR : CARS + 500 ppm GE + 0.18% SRE, G750-SR : CARS + 750 ppm GE + 0.18% SRE, and G1000-SR : CARS + 1000 ppm GE + 0.18% SRE.

Supplementation of GE and SRE did not affect the pH and DM and OM digestibility and it decreased crude fiber digestibily as much as 13.0% - 16.6%. Supplementation of 250 ppm GE or 250 ppm GE and 0.18% of SRE in beef cattle decreased acetate, protozoa population and increased propionate. Supplementation of 250 ppm GE and 0.18% of SRE in the ammoniated rice straw based beef diet resulted in the increase of rumen fermentation efficiency as indicated by the low production of acetate and methane, and high production of propionate

Key words: *herbal, methane, microbial rumen*

Introduction

Garlic (*Allium sativum*) contains complex mixture of bioactive compounds, including allicin ($C_6H_{10}S_2O$), diallyl sulphide ($C_6H_{10}S$), dialyl disulfide ($C_6H_{10}S_2$) and allyl mercaptan (C_3H_6S). These compounds are able to manipulate rumen fermentation such as decreasing the proportion of acetate and increasing the proportion of propionate and butyrate, inhibiting methanogenesis and decreasing the CH_4:VFA ratio (Busquet et al 2005). Kongmun et al (2010) reported that garlic powder was more effective than coconut oil in reducing protozoa population. Active ingredients in garlic are able to limit the growth of protozoa. Busquet et al (2005) stated that Archaea has unique membrane layer that stabilizes cell membrane function. The synthesis of this membrane is catalyzed by hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase. Garlic is strong inhibitor of HMG-CoA reductase (Gebhardt and Beck 1996), with an effect on isoprenoid synthesis causing Archaea membrane become unstable and the cell die. Kim et al (2012) proved that garlic extract decreased methane production by more than 25%. According to Amagase (2006), bioactive compounds of garlic extract were: alliin, allicin, diallyl disulfide (DADS) and S-Allyl-L-cysteine. Kongmun et al (2010) showed that the molar proportion of

propionate was influenced by garlic supplementation. Supplementation of garlic powder as much as 16 mg/kg DM of feed resulted in the lowest total VFA and highest proportion of propionate and significantly lower CH_4 production.

Guo et al (2008) stated that crude saponins have been shown to inhibit protozoa causing the reduction of methanogens presumably by lowering methanogenic activity of protozoal-associated methanogens. Holtshausen et al (2009) proved that the negative effect of saponin supplementation on IVNDFD increased with the increase of dose rate, which suggests that lower levels of supplementation may attenuate the negative effects on fibre digestion. Cardozo et at (2005) reported that the supplementation of 30 mg/L garlic extract in the diet of beef cattle decreases acetate and increases propionate. Most fibrolitic rumen bacteria, which are generally acetate and butyrate producers, are sensitive to low rumen pH. In contrast, the amylolitic bacteria are acid-tolerant and are responsible for most of the production of propionate in the rumen. Saponins from fruits of *Sapindus rarak* have been reported by Wina et al (2005) and Suharti et al (2010) as defaunating agents. Addition of *S. rarak* saponins through in *vitro* decreased significantly protozoa counts. The symbiotic of protozoa with archea in the rumen is well established (Finlay et al 1994). However, there is only 37% of the archea that live symbiotically with protozoa, the rest live freely in the rumen ecosystem.

The objective of this study was to evaluate the effects of supplementation of GE and SRE in beef diet on rumen fermentation in order to improve fermentation efficiency.

Materials and methods

This research was conducted experimentally using randomized block design, with 6 treatments and 5 blocks. The treatments were:

- CARS: beef cattle feed (concentrate: ammoniated rice straw, 60:40 with CP: 10.9%, TDN: 63.03%, CF : 23.3%, NDF: 32.04%);
- G250 : CARS + 250 ppm GE;
- G250-SR : CARS + 250 ppm GE + 0.18 % SRE;
- G500-SR : CARS + 500 ppm GE + 0.18% SRE;
- G750-SR : CARS + 750 ppm GE + 0.18% SRE;
- G1000-SR : CARS + 1000 ppm GE + 0.18% SRE.

Location and duration

The experiment was conducted in the laboratory of Feedstuff of Animal Science Faculty, University of Jenderal Soedirman, Purwokerto, Central Java province, Indonesia, from March to July 2013.

Preparation of ammoniated rice straw

Fresh, air-dry rice straw was sampled, weighed and put in an oven (60^{O} C, 24 hours) for dry matter content determination. A certain weight of the air-dry rice straw was chopped 5-7 cm of length, and then it was put into a plastic bag. Urea was weighed (3% of rice straw, dry matter basis), and mixed, diluted thoroughly in water (33% of rice straw, dry matter basis). The urea solution was poured thoroughly on to the chopped rice straw, the bag was sealed and kept in a shaded room for 30 days, until it was ready to use for in-vitro digestibility study.

In vitro fermentation

The rumen fluid for this experiment was collected from a non-fistulated Onggole-Cross cow fed a diet consisted of ammoniated rice straw and concentrate mixture (40:60 %). The rumen fluid

was filtered through double layer cheese cloth for *in vitro* studies. The substrate for *in vitro* rumen fermentation was a mixture of concentrate feed and dried milled ammoniated rice straw. The concentrate mix consisted of rice brand, coconut cake meal, cassava waste, wheat pollard, mineral premix, and NaCl (CP: 12.9%, TDN: 64.03%, CF: 23.3%, NDF: 32.04%).

In vitro fermentation was conducted according to the method of Tilley and Terry (1963). Into each 100 mL fermentation tube, 500 mg substrate, 40 mL McDougall buffer and 10 mL rumen fluid were added. The McDougall buffer contained $NaHCO_3$ (58.8 g), $Na_2HPO_4.7H_2O$ (42 g), KCl (3.42 g), NaCl (2.82 g), $MgSO_4.7H_2O$ (0.72 g), $CaCl_2$ (0.24 g) and H_2O per 6 L. The mixture was stirred and flushed with O_2 –free carbon dioxide and the tubes were then sealed with a rubber cork with the gas release valve. All the fermentation tubes were incubated in a shaker water bath at 39 ºC for 24 h.

Preparation of garlic extract (*GE*) and *Sapindus rarak* extract (SRE) (modified methods of Suharti et al 2010)

Garlics were obtained from a local market in Purwokerto, Central Java, Indonesia. At the first phase, gar

Sapindus rarak fruits were obtained from Surakarta, Central Java province. The first stage of the preparation of SRE, was initiated by separating seeds from the fruit. Then the seed-free fruits were put in an oven at a temperature of 60° C for 4 days, and in warm condition, the *Sapindus rarak* fruits were immediately milled. The SR powder was macerated in methanol (1:4 w / v) overnight. Further the methanol was evaporated in an evaporator-rotary so that the residue was separated from the extractor. The extraction was repeated once more to produce a crude extract. The residue was then dried on a freeze drier and stored at-4ºC. This method would result in SRE yield of 42.5% with 81.9% saponin content.

Protozoa and bacteria counts

After 24 h incubation, 1 mL of aliquot of each treatment was taken for protozoa and bacteria counts. One mL of aliquot was mixed with 1 mL of methyl green formal dehyde (35% of formaldehyde, distilled water, methylgreen and NaCl) (Ogimoto and Imai 1981). The stained sample with methyl green formaldehyde was kept in cool temperature (17 ºC) and the protozoa population was directly counted in a counting chamber (0.1 mm) with a microscope (40 x magnitudes) and then was exposed to a 30x20 cm screen. Protozoa population, Colony Forming Unit (CFU), was calculated using a formula:

$$P = \frac{1 \times 1000 \times C \times DF}{0.1 \times 0.065 \times 16 \times 5}$$

P: Protozoa population
C: protozoa number, colony.
DF: dilution factor (=2)

To count the number of bacteria population, the dilution method was used at 24-hour incubation. As much as 0.05 mL of aliquot was added to the 4.95 ml diluted medium. A serial dilution 10^{-6}, 10^{-7} and 10^{-8} was made using of Brain Heart Infusion (BHI) medium (Gerard-Champod et al 2009). The count unit of bacteria in term of colony forming unit, was calculated using a formula:

$$B = \frac{\text{colony number}}{0.05 \times 10^{x} \times 0.1}$$

B: Bacteria population

X: dilution factor.

Measurement of total gas was based on the method of Menke and Steingass (1988) and the phases of measurements were as follows: (1) Piston was smeared with vaseline before inserting it into the tube, (2) A total of 230 mg of feed treatment was put into Menke tube basin, (3) Preparation of medium that was made of 400 mL of distilled water; 0.1 mL micromineral solution; 200 mL of buffer solution; 200 mL macromineral solution; rezazurin 0.1 mL and 40 mL of reducing solution, (4) one section of rumen fluid was mixed with 2 parts of medium, stirred at 39^{o}C with magnetic stirrer, (5) A total of 30 mL solution hose was inserted into Menke tube and locked with a plastic clip, (6) Incubated for 24 h and the scale was read every 6 h.

Methane (CH_4) production

The CH_4 production was calculated from stoichiometry of the main VFA formed during fermentation, i.e: acetate (C_2), propionate (C_3), and butyrate (C_4) as follows:
$CH_4 = 0.45\ C_2 - 0.275C_3 + 0.40\ C_4$ (Moss et al 2000).
C_2 = concentration of acetate, C_3= concentration of propionate, C_4= concentration of butyrate.

Statistical analysis

The data was analyzed by one-way ANOVA (Steel and Torrie 1995).

Results and discussion

The results (Table 1) showed that GE and SRE supplementation on feed resulted in similar digestibility of dry matter and organic matter as those of control feed. The means ranges of dry and organic matter digestibility were 60.4%-67.5% and 61.6%-67.5%, respectively. This result showed that the increasing garlic extract supplementation did not result in the decrease of microbe's activitiy of digesting feed. The results of this study was similar to the findings of Manasri et al (2012), who found that there were no significant differences in IVDMI, IVDMD, IVOMD, IVCPD, and pH of beef cattle feed supplemented with garlic pellet. Anassori et al (2012) report, their in-vitro study using sheep rumen fluid found out that dietary garlic oil decreased OMD. Kongmun et al (2010) found an increase of IV (True) D when 16 mg of garlic powder was included in an in-vitro study using rumen fluid of swamp buffaloes.

Table 1. Effect of supplementation of GE and SRE on digestibility, VFA, total gas and rumen microbes

Items	Treatments							Prob
	CARS	G250	G250-SR	G500-SR	G750-SR	G1000-SR	SEM	
pH	6.80 ^{a}	6.70 ^{b}	6.50 ^{b}	6.60 ^{b}	6.50 ^{b}	6.60 ^{b}	.10	.000
IVDMD (%)	65.6^{a}	64.6 ^{a}	64.9 ^{a}	62.9 ^{b}	61.40 ^{b}	60.42 ^{c}	2.29	.000
IVOMD (%)	65.8 ^{a}	63.4 ^{a}	64.4 ^{a}	63.5 ^{a}	61.8 ^{b}	61.6 ^{b}	2.14	.015
IVCFD (%)	77.8 ^{a}	74.9 ^{b}	70.08 ^{c}	71.8 ^{c}	70.8 ^{c}	72.8 ^{bc}	2.30	.000
TVFA (%)	160 ^{a}	150 ^{c}	146 ^{c}	153.2 ^{b}	140^{d}	160 ^{a}	5.02	.000
Gas production (ml)	10.02 ^{a}	6.82 ^{c}	2.13 ^{d}	11.09 ^{a}	7.11 ^{c}	9.10 ^{b}	0.55	.000
Protozoa (10^6 cell/ml)	31.5 ^{a}	15.00 ^{c}	10.42 ^{d}	11.4 ^{d}	16.00 ^{bc}	19.00 ^{b}	0.64	.000
Bacteria (log 10 cell/ml)	9.46 ^{a}	9.69 ^{b}	9.57 ^{b}	9.75 ^{c}	9.94 ^{c}	9.38 ^{a}	0.17	.000

IVDMD= In vitro dry mater digestibility, IVOMD = In vitro organic mater digestibility, IVCFD= In vitro crude fiber digestibility, TVFA= total volatile fatty acids.
^{abc} *means in the same row without common letter are different at P<0.05*

In general, the results of this study showed that the supplementation of GE, either alone, or in

combination with SRE, decreased crude fibre digestibility as much as 7.4 % (Table 1). The decrease in crude fibre digestibility was supposedly associated with the reduced fibre-degrading bacteria population; this is shown with the reduction of acetate concentration up to the supplementation level of 750 ppm. The reduction of acetate will limit the synthesis of methane due to the limited hydrogen ion. Anassori et al (2012), reported that the addition of garlic essential oil in feed immersed in sheep rumen fluid, and Manasri et al (2012) stated that inclusion of garlic pellet in feed incubated in beef cattle rumen fluid decreased IVNDFD and IVADFD, which was in line with the results of this study.

An in-vitro study by Kongmun et al (2010) reports that inclusion of garlic powder in feed immersed in the rumen fluid of swamp buffaloes decreased total volatyle fatty acids (TVFA), relative to the control. However, Kim et al (2012) describes that TVFA production and rumen pH were not affected by garlic extract (GE) inclusion in feed that was incubated in Holstein cow rumen fluid. The results of this study showed that the increase of GE concentration up to 750 ppm, decreased the VFA concentration.

The result showed that the increase of GE supplementation was followed by the decrease of acetate concentration, except at 1000 ppm GE; it increased the concentration of VFA. These results showed that GE supplementation was able to change rumen fermentation from acetogenic to glucogenic, as it was showed by the increase in propionate concentration as much as 9.23%.

Generally, the supplementations decreased acetate:propionate ratio (Table 2). The decrease in acetate:propionate ratio was easily understandable (made sense) since the acetate concentrations decreased, in contrast, propionate concentration increased by the supplementation treatment. The decrease in acetate:propionate ratio by the supplementation was expected in this study, because this in-vitro study was targeted to obtain a proper, appropriate feed for beef cattle, and propionate is a simple precursor for the bio-chemical formation of meat-amino acids and protein. This result was similar with the finding of Kongmun et al (2010) that the inclusion of garlic powder in feed incubated in swamp buffalo rumen fluid, decreased acetate:propionate ratio, and that of Kim et al (2012) that the inclusion of GE (20mg/ml) decreased acetate:propionate ratio from 1.79 (control feed) to 1.32 (control feed + GE).

The results showed that the treatment significantly lowered total gas respectively for G250, G250-SR, G500-SR, G750-SR, G1000-SR, amounted to 29.03%, 38.1%, 80.76%, 0.60%, 35.5%, 17.5% (Table 1). These results indicated that the increased concentrations of the GE were not in line with the decrease in total gas. The highest decrease in the concentration of total gas due to the supplementation of 250 ppm GE + 0.18% SRE in feed was 80.76%. Increased concentrations of extract of GE above 250 ppm only lost a total gas of 28.5%. The decrease in total gas in this study illustrated that the tested treatment was able to improve rumen fermentation, because 30% of the total gas as a result of rumen fermentation, is methane. Anassori et al (2012) found a similar results; the addition of garlic oil in sheep feed, decreased rumen gas production. On the contrary, Kim et al (2012) found out that the inclusion of garlic whole plant methanol extract in feed immersed in rumen fluid of fistulated Holstein cow, increased gas production. This decrease in gas production due to the increasing levels of garlic was also found by Kongmun et al (2010).

The results showed that the supplementation treatment effectively decreased protozoa population (Table 1). The decrease of protozoa population relative to the control were 52.38%, 66.92%, 63.81%, 49.21%, and 39.68% relatively for G250, G250-SR, G500-SR, G750-SR, and G1000-SR. The decrease in protozoa population in this study indicated that the supplementation treatments were able to represent defaunating agents. This shows that GE and SRE contain compound that have the ability to reduce protozoa population. Some of the natural compounds that have characteristics of defaunating agents are saponins and tannins. Cheeke (1999) describes the mechanism of saponin in inhibiting the growth of protozoa and methanogens.

Newbold et al (1995) explain that between 15-37% of methanogens lives symbiotically with protozoa, therefore, the decrease in protozoa population will be followed by the decrease in methanogens population. Hart et al (2006) explains that allicin of garlic has the characteristic as an anti-methanogenic, which means that there is about 63% of the methanogens that is targeted by the allicin. Supplementation of 250 ppm GE and 0.18% SRE was the most effective in decreasing protozoa population relative to other treatments, which meant that the combination of 250 ppm of GE (allisin source) and 0.18% SRE (saponin source) was the most effective for the inhibition of protozoa growth. Kongmun et al (2010) also found similar results that protozoa counts decreased from 14.3 ($x10^5$) CFU in control feed to 6.4 ($x10^5$) CFU in control feed supplemented with 16 mg garlic powder.

The results showed that the treatment affect the concentrations of the tested rumen bacteria (Table 1). The limitations of this study was because the study did not observe the species of the bacteria, it only observed the total strains of bacteria. In previous study in dairy cows, the results showed that the combination of garlic extract and *Sapindus rarak* affected bacteria populations (Prayitno et al 2013). The dominant bacteria in the rice straw-based feed were fibre-degrading bacteria. It is reported by Kongmun et al (2010), inclusion of a mixture of garlic powder 4 mg, and coconut oil at 8 mg, increased *Ruminococcus albus*, a cellulolytic rumen bacteria, from 18.3 ($x10^5$) CFU for control feed, to 33.8 ($x10^5$) CFU for control feed plus the mixture of GP and CO. Kim et al (2012) also found similar results when GE (20 mg/ml) was included in feed; the treatment decreased methanogenic bacteria and increased fibrolytic bacteria. Manasri et al (2012) found rather different results than those of Kim et al (2012), when garlic pellet in feed, immersed in beef cattle rumen fluid, decreased methanogenic and total bacteria counts.

Table 2. Effect of supplementation of GE and SRE on volatile fatty acids

Items	Treatments							Prob.
	CARS	G250	G250-SR	G500-SR	G750-SR	G1000-SR	SEM	
Proportion of VFA (mM)								
Acetate	48.9 ^{b}	39.1 ^{d}	40.3 ^{d}	44.4 ^{c}	42.07 ^{dc}	47.6 ^{b}	3.21	.000
Propionate	27.4 ^{c}	29.6 ^{b}	29.9 ^{b}	29.2 ^{b}	29.7 ^{c}	31.09 ^{a}	1.69	.000
Butyrate	9.89 ^{b}	9.89 ^{b}	9.96 ^{b}	10.7 ^{b}	9.83 ^{b}	12.3 ^{a}	2.98	.000
Acetate:propionate (A:P)	1.79 ^{a}	1.32 ^{d}	1.35 ^{d}	1.52 ^{c}	1.52 ^{c}	1.53 ^{c}	0.16	.000
Methane (mM)	18.4 ^{a}	13.4 ^{c}	13.9 ^{c}	16.2 ^{b}	15.2 ^{b}	17.8 ^{a}	3.02	.000

^{abcd} *Means in the same row without common letter are different at P<0.05*

The results of this study showed that GE supplementation as well as GE + SRE supplementation decreas

Conclusions

- Based on this experiment, it could be conclude that supplementation of 250 ppm garlic extract, 0.18% SRE in the ammoniated rice straw based beef diet resulted in the increase of rumen fermentation efficiency as indicated by the low production of acetate and methane, and high production of propionate.

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