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Rumen Microbial Population in the *In vitro* Fermentation of Different Ratios of Forage and Concentrate in the Presence of Whole Lerak (*Sapindus rarak*) Fruit Extract

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ABSTRACT : This experiment was designed to investigate the effect of lerak extract on the dynamic of rumen microbes in the *in vitro* fermentation of diet with different ratios of forage and concentrate. *In vitro* fermentation was conducted according to the method of Tilley and Terry (1963). The design of experiment was a factorial block design with 2 factors. The first factor was the ratio of forage and concentrate (90:10, 80:20, and 70:30 w/w) and the second factor was the level of lerak extract (0, 0.6, and 0.8 mg/ml). Total volatile fatty acid (VFA) concentration, proportional VFA and NH_3 concentration were measured at 4 h incubation. Protozoal numbers in the buffered rumen fluid after 4 and 24 h of incubation were counted under a microscope. Bacterial DNAs of buffered rumen fluid were isolated from incubated samples after 24 h of incubation using a QiaAmp kit. Total bacteria, *Fibrobacter succinogenes*, *Ruminococcus albus*, and *Prevotella ruminicola* were quantified using real time polymerase chain reaction (PCR). Lerak extract markedly reduced protozoal numbers in buffered rumen fluid of all diets after 24 h of incubation. Total bacteria did not change with lerak extract addition. While no difference in *F. succinogenes* was found, there was a slight increase in *R. albus* number and a significant enhancement in *P. ruminicola* number by increasing the level of lerak extract in all diets. Propionate concentration significantly increased in the presence of lerak extract at level 0.8 mg/ml. It was concluded that the addition of lerak extract could modify rumen fermentation and had positive effects on rumen microbes. (**Key Words :** *Sapindus rarak*, Rumen Microbial, Fermentation, Forage to Concentrate Ratio)

INTRODUCTION

Low beef cattle production in developing countries may be caused by inadequate nutrient supply in the high-forage based ration. The smallholder farmers have limited feed resources available for feeding their livestock. To overcome these problems, a strategy to maximize the utilization of available feed resources in the rumen is required; i.e by providing an optimum condition for bacterial growth using a feed supplement as a defaunating agent.

It is known that the activity of rumen bacteria in digesting feed fiber is usually affected by protozoa. This is due to the predation of some bacteria by protozoa (Gutierrez, 2007). *In vitro* studies suggest that predation and digestion of bacteria by protozoa is by far the most important cause of microbial protein turnover in the rumen, with autolysis, other lytic factors, and endogenous proteolysis being of minor importance (Wallace and McPherson, 1987). Therefore, suppression of the protozoal population has been suggested as an alternative way to optimize bacterial growth in the rumen.

Saponins or saponin-like substances have been reported to have a potency to suppress growth of the protozoa and change fermentation patterns in the rumen system (Makkar and Becker; 1997; Wang et al., 1998; Benchaar et al., 2008). Lerak (*Sapindus rarak*) pericarp extract contains high level of saponin and has been reported to have a defaunating effect in the sheep rumen (Wina et al., 2006). However, the effectiveness of saponin to suppress protozoa may depend on the diet composition. Thus, the interaction between the diet composition and the microbes initially present in the rumen may play a key role in the effectiveness of defaunating agents.

Previous research showed that total bacterial number increased when protozoa was eliminated or reduced from

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the rumen ecosystem (Hsu et al., 1991; Thalib et al., 1995; Ivan et al., 2000). Hu et al. (2005) reported that the addition of tea saponins can affect the rumen fermentation *in vitro*, inhibited the rumen protozoal growth and increased microbial mass yield. However, few reports about effect of saponin on the population and numbers of specific rumen bacteria are available. In this experiment, different ratios of forage and concentrate in the diets were used as substrates and were incubated in the presence of different levels of whole lerak fruit extract. The number of different bacterial species was detected and quantified by using a real-time PCR.

MATERIALS AND METHODS

Preparation of whole fruits lerak extract

The lerak fruits were collected from Central Java, Indonesia and dried in an oven at 60°C until they consisted of 90% dry matter. After drying, the whole fruits (including seed) were ground immediately. Whole fruits lerak meal was soaked overnight in methanol (1:4, w/v). After the particles settled, the extract was separated and the extraction was repeated with an equal volume of fresh methanol. The methanol fractions were then pooled and evaporated in a rotary evaporator. The residual fraction was freeze dried and kept in airtight bags at freezer (-4°C) until further use (Wina et al., 2006). The freeze-dried extract was dissolved in distillate water just before used as extract of whole fruit lerak.

In vitro fermentation

The rumen fluid for this experiment was collected just before morning feeding from a rumen fistulated Ongole crossbred beef animal. This animal was fed a diet consisted of native grass and commercial concentrate with 50%:50% ratio. The rumen fluid was filtered through a double layer of cheesecloth for *in vitro* studies. The substrate for *in vitro* rumen fermentation was a mixture of concentrate feed and dried milled native grasses at different ratios. The concentrate mix consisted of soybean meal, coconut cake meal, cassava waste, wheat pollard, molasses, dicalcium phosphate (DCP), NaCl and $CaCO_3$ (CP 19%, TDN 75%). Native grasses were harvested from the surrounding area of Bogor Agricultural University Farm (Indonesia), dried in the oven 65°C over night and then milled.

In vitro fermentation was conducted according to the method of Tilley and Terry (1963). The experiment was conducted in a factorial block design with 2 factors (3×3) and 3 replications. The first factor was the ratio of forage and concentrate (90:10, 80:20, and 70:30 w/w) and the second factor was the level of lerak extract (0, 0.6, and 0.8 mg/ml). The experimental diets were mixed with respective doses of lerak extract and used as a substrate in *in vitro* studies. Into each 100 ml fermentation tube, 500 mg substrate, 40 ml McDougall buffer and 10 ml rumen fluid were added. The McDougall buffer, per 6 liters contained $NaHCO_3$ (58.8 g), $Na_2HPO_4 \cdot 7H_2O$ (42 g), KCl (3.42 g), NaCl (2.82 g), $MgSO_4 \cdot 7H_2O$ (0.72 g), $CaCl_2$ (0.24 g) and H_2O. The mixture was stirred and flushed with O_2-free carbon dioxide and the tubes were then sealed with a rubber cork fitted with the gas release valve. All fermentation tubes were incubated in a shaker waterbath at 39°C for 24 h.

Sampling and measurement

The number of protozoa in the buffered rumen fluid after 4 and 24 h of incubation were counted under a microscope. The contents of the fermentation tubes were mixed and a 0.5 ml aliquot was mixed with 0.5 ml methyl green formaldehyde saline solution containing 35% formaldehyde, distilled water, methylgreen and NaCl. The stained sample was kept at room temperature and the population of protozoa was counted directly by using a counting chamber (0.1 mm) under a microscope (40×).

Total VFA concentrations and molar proportions of VFA at 4 h fermentation were analyzed by using gas chromatography (Chrompack CP9002, Netherlands, flame ionized detector, Capillary column type WCOT Fused Silica 25 m×0.32 mm, oven temperature: conditioning at 60°C and running at 115°C and nitrogen as gas carrier). Before analysis, the pH of the rumen fluid aliquots from *in vitro* incubation was adjusted to 3-4 with H_2SO_4. Thus, 1.5 ml of the adjusted rumen fluid aliquot was mixed with 30 mg sulfosalicylic acid ($C_7H_60_60_6S.2H_20$) and centrifuged at 12,000 rpm×10 min (7°C) then 0.5 μl of the mixed solution was injected to the GC. Ammonia ($N\text{-}NH_3$) concentrations at 4 h fermentation were determined by using the micro diffusion method (Conway, 1962).

Populations of bacteria were quantified with a real time PCR machine (Rotor Gene Q 1.7.94, QIAGEN, USA). Species targeted primers were used to amplify 16S DNA from DNA extracted from the original rumen liquid. The primers for this work were designed according to Denman and McSweeney (2006) for total bacteria, Tajima et al. (2001) for *P. ruminicola* and *F. succinonegens*, and Koike and Kobayasi (2001) for *R. albus*. The primers of specific target were synthesized by Hokkaido System Science Co. Ltd. (http://www.hssn.co.jp), Japan. The nucleotide sequences of the primers used in this study are shown in Table 1. PCR amplification for standard preparation was performed by using Mastercycler personal (Eppendorf 5332) with the following condition: 5 min at 94°C, 30 s at 94°C, 45 s at 55°C, 1 min at 72°C for 35 cycles and a final extension of 5 min at 72°C. Then, the PCR product of each specific target run under electrophoresis to check band

Table 1. The nucleotide sequences of the primers used in this study

Spesies	Forward primer	Reverse primer
Total bacteria[a]	5' CAA CGA GCG CAA CCC3'	5'CCA TTG TAG CAC GTG TGT AGC C3'
F. succinogenes[b]	5'CGT ATG GGA TGA GCT TGC3'	5'GCC TGC CCC TGA ACT ATC3'
R. albus[c]	5'CCC TAA AAG CAG TCT TAG TTC G3'	5'CCT CCT TGC GGT TAG AAC A3'
P. ruminicola[b]	5'GGT TAT CTT GAG TGA GTT3'	5'CTG ATG GCA ACT AAA GAA3'

[a] Denman and McSweeney (2006). [b] Tajima et al. (2001). [c] Koike and Kobayashi (2001).

number and size. The PCR product was purified using a QIAquick PCR purification kit (QIAGEN, USA) and quantified using spectrophotometry. For each sample derived standard, the copy number concentration was calculated based on the size of the PCR product and the mass concentration (Ozutsumi et al., 2006). Five-fold serial dilutions of linearized plasmid DNA was used to generate a standard curve for quantitative assessment.

For quantification, bacterial DNAs from buffered rumen fluid were isolated from incubated samples after 24 h of incubation. The sample (1.5 ml) from each tube of the *in vitro* fermentation was centrifuged for 5 min at 10,000 g. The microbial pellet was used for DNA extraction (Qiamp stool kit, Qiagen). The different microbial groups, such as total bacteria, *Fibrobacter succinogenes*, *Ruminococcus albus* and *P. ruminicola* were determined in the samples using quantitative PCR assay. The qPCR conditions for bacteria specific targets used were based on qPCR machine (Rotor gene, Qiagen) protocol with the following conditions: initial denaturation at 95°C for 5 minutes, amplification at 95°C for 10 seconds and combined annealing and extension at 60°C for 30 s. There were 40 cycles of amplification. The population of different microbial groups was determined as relative to the total bacterial populations. Relative population sizes of *R. albus*, *F. succinogenes*, and *P. ruminicola* were expressed as a proportion of total rumen bacterial 16S rDNA.

Statistical analysis of the data was carried out by ANOVA using General Linear Procedure. Computation was performed using SPSS 13.0 for windows evaluation version.

RESULTS

Protozoal numbers

The numbers of protozoa were markedly reduced when lerak extract at levels of 0.6 and 0.8 mg/ml was added to the buffered rumen fluid in all diets fermentation at 24 h of incubation. The protozoal numbers increased with the increasing ratio of concentrate to forage in the ration. Protozoal numbers were reduced after 24 h of incubation at higher concentrate ratio in the diet (Table 2). There was no interaction between ratio of forage to concentrate and the level of lerak extract in the all parameters measured.

Population of total and some bacteria

Total bacteria and 3 different types of bacteria were quantified by using real time PCR. Table 2 shows that total bacteria did not increase with different types of diet or level of lerak extract. Both *F. succinogenes* and *R. albus* represented the main fibrolytic bacteria in the rumen. Higher inclusión of concentrate in the diet mixture reduced the population of *F. succinogenes* but did not change the population of *R. albus*. Addition of lerak extract at different concentrations did not affect the population of *F. succinogenes*. However, the population of *R. albus* seemed to increase in the presence of lerak extract. *P. ruminicola* population did not show any significant increase with different types of diets but significantly increased in a dose dependent manner with the addition of lerak extract.

Volatile fatty acids and ammonia concentrations

Total VFA production but not molar proportion of

Table 2. Population of protozoa and some specific bacteria in buffered rumen of *in vitro* fermentation using different ratios of forage and concentrate in the presence of different levels of lerak extract

Parameters	F:C ratio			Lerak extract (mg/ml)			SEM
	90:10	80:20	70:30	0	0.6	0.8	
Protozoal numbers, log 10							
4 h	3.62	3.95	4.18	4.32	3.83	3.60	0.17
24 h	3.56^{ab}	3.29^{b}	4.18^{a}	4.39^{x}	3.42^{y}	3.22^{y}	0.20
Total bacteria, log10/ng DNA	8.74	8.74	8.63	8.66	8.77	8.67	0.03
F. succinogenes (% TB), $\times 10^{-2}$	11.24^{a}	8.07^{ab}	3.46^{b}	7.5	8.25	6.51	1.53
R. albus (% TB), $\times 10^{-2}$	4.63	4.47	4.43	1.99	6.05	5.58	0.79
P. ruminicola (% TB), $\times 10^{-2}$	3.4	5.3	3.8	2.32^{y}	3.25^{xy}	7.25^{x}	0.93

Different superscripts on the same row represents a significant difference ($p<0.05$). F = Forage. C = Concentrate feed. TB = Total bacteria.

Table 3. Effect of lerak extract on volatile fatty acids and ammonia productions in the different ratios of forage and concentrate diet

Parameters	F:C ratio			Lerak extract (mg/ml)			SEM
	90:10	80:20	70:30	0	0.6	0.8	
Total VFA (mM)	34.9[b]	55.4[a]	54.8[a]	43.9	46.6	52.1	3.15
Proportion of VFA (mol/100 mol)							
Acetate	68.5	86.8	68.2	68.2	69.5	67.5	0.62
Propionate	17.7	17.1	18.1	16.4[y]	17.6[y]	18.9[x]	0.33
Isobutyrate	2.2	3.0	2.7	3.0	2.4	2.6	0.22
Butyrate	10.3	8.9	9.0	9.5	9.2	9.5	0.37
Isovalerate	2.2	2.0	1.6	2.1	1.7	2.0	0.12
Acetate:Propionate (A:P)	3.9	4.1	3.8	4.2[x]	4.0[xy]	3.6[y]	0.098
N-NH_3 (mM)	12.3	12.3	11.3	11.3	11.9	12.8	0.55

Different superscripts on the same row represents a significant difference ($p<0.05$). F = Forage, C = Concentrate feed.

individual volatile fatty acid or A/P ratio was significantly affected by forage: concentrate ratio in the diet. Production of total VFA increased when the level of concentrate in the ration increased. Addition of lerak extract did not affect the total VFA but at the level 0.8 mg/ml, it significantly increased the molar proportion of propionic acid ($p<0.05$) and reduced the ratio of acetate to propionate (Table 3). Although it was not significantly different, the molar proportions of isobutyrate and isovalerate reduced when lerak extract was added into the *in vitro* fermentation. The concentration of N-NH_3 slightly increased with the increasing level of lerak extract although the increase was not statistically significant.

DISCUSSION

The main component in the extract of lerak fruit pericarp was identified previously and consisted of several compounds of saponin (Hamburger et al., 1992; Wina et al., 2005a). It was assumed that the same compounds were also present in the whole lerak fruit extract. The protozoal numbers reduced when the extract of lerak fruit pericarp or lerak whole fruit was added regardless of different ratios of forage: concentrate in the diet. From our previous work (under process of publication), the addition of 1.0 mg/ml, but not at the level 0.1 mg/ml, of lerak extract depressed protozoal numbers in the *in vitro* system. The saponin content in the whole lerak fruit extract may be lower than in the fruit pericarp, therefore its ability to reduce protozoal numbers was lower at the first hours of fermentation as compared to that reported by Wina et al. (2005a) using the methanol extract of fruit pericarp.

The activity of saponin in lerak extract to defaunate rumen protozoa is not affected by the diet composition. Under different diets, decreased protozoal counts occur with either supplementation of saponin rich extracts (Kamra et al., 2000; Goel et al., 2008; Guo et al., 2008) or saponin rich forages (Hess et al., 2003). Saponins reduce the viability of protozoa by its binding capacity to sterol in the protozoal membrane or by its ability to alter the cell-membrane permeability of protozoa (Francis et al., 2002), hence, disintegrating the protozoal cell membranes. Teferedegne et al. (2000) reported that saponins tended to have a more dramatic effect on the activity of rumen protozoa than its degraded saponin product, sapogenin, confirming the importance of the glycoside in the activity of saponins in the rumen.

Since saponin reduces protozoal numbers, the microbial composition of the rumen would be changed. The result of denaturing gradient gel electrophoresis (DGGE) in our previous study of the effects of saponin (under process of publication) showed a new band and two other bands increased their intensity indicating the increase in several specific bacterial populations. Some of those bacteria, stimulated by lerak extract had high similarity to *Prevotellas* sp. In the present experiment, the quantification of bacterial populations with real time PCR showed a significant increase in *P. ruminicola* numbers. Wallace et al. (1994) reported that the growth of *P. ruminicola* was enhanced when 1% of saponin from *Yucca shidigera* was added to the pure cultures. Karnati et al. (2009) reported that defaunating the rumen by using unsaturated fat increased *Prevotella*, *Eubacterium*, and some *Butyrivibrio*.

Fibrolytic microbes respond differently to different saponins. In the present experiment, the relative abundance of *R. albus* slightly increased in response to lerak saponin ($p>0.05$), but its growth was reported to be negatively affected by Yucca saponin (Wang et al., 2000). *Ruminococcus flavefaciens* was slightly affected by tea saponin (Mao et al., 2010) while *Fibrobacter succinogenes* was relatively stable in the present experiment and in the work reported by Wina et al. (2005). *Fibrobacter succinogenes* is a gram negative bacteria possessing two membranes in its cell wall. These membranes may protect the bacteria from saponins. On the other hand, gram positive bacteria are very sensitive to saponins (Wallace et

al., 1994).

When lerak saponin depressed the growth of protozoa, it was likely that some methanogens associated with protozoa were also reduced. Under these conditions the use of H_2 in the rumen would be shifted from producing methane to propionate as the increased numbers of *P. ruminicola* would utilize the greater level of H_2 to form higher concentrations of propionate. Goel et al. (2008) also reported that saponin and saponin-like substances increased propionate concentration and its relative ratio in total VFA in the rumen, especially when high concentration of saponin was supplied. The saponin extracted from *S. rarak* pericarp was also found to accelerate propionate production without decreasing total VFA production (Wina et al, 2005). As propionate is used for generating energy via gluconeogenesis in host animals (Yost et al., 1977; Murray et al., 2006), the increase in propionate production should improve feed efficiency. Ammonia production in the rumen was not affected by level of lerak extract or diet composition. Addition of *S. rarak* saponin do not affect the protein degradation in *in vitro* (Muetzel et al., 2005).

IMPLICATIONS

The addition of lerak extract 0.8 mg/ml could modify rumen fermentation by reducing protozoa and increasing *Prevotella* sp. Saponin of lerak extract has the potential to be used as a natural propionate enhancer.

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