

***In Vitro* Rumen Fermentation and Anti Mastitis Bacterial Activity of Diet Containing Betel Leaf Meal (*Piper betle* L.)**

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ABSTRACT

The aims of this experiment was to study the inhibition effect of betel leaf meal (BLM) addition into concentrate diet on mastitis causing bacteria and on rumen fermentation condition. The study consisted of five dietary treatments of BLM level in concentrate feed, i.e., 0%, 2%, 4%, 6%, and 8% and four replicates of each treatment. The treatment diets together with napier grass in ratio of 40 : 60 were fermented using rumen liquor. All treatments were examined their antibacterial activity before and after fermentation. After four hours fermentation, supernatant of each samples were analyzed for VFA, NH_3 , number of bacteria and protozoa. Dry matter (DM) and organic matter (OM) digestibility were analyzed after 48 h fermentation. The results showed that before fermentation, 8% BLM addition caused the biggest ($P<0.05$) inhibition diameter of *Staphylococcus* spp. growth compared to other lower levels. However after fermentation there were no significant differences among the addition levels of BLM. Two per cent of BLM addition produced higher VFA ($P<0.05$) than the other addition levels. Ammonia concentration, dry matter (DM) and organic matter (OM) digestibility were not different among the treatments. Addition of BLM significantly ($P<0.01$) decreased protozoa number, but did not affect bacterial count. It is concluded that the addition of 2% BLM in concentrate feed can be used effectively to inhibit the growth of mastitis causing bacteria (*Staphylococcus* spp.) and does not disturb rumen fermentation condition.

Key words: betel leaf, mastitis, rumen microbe, rumen fermentation, *Staphylococcus* spp.

ABSTRAK

Penelitian ini bertujuan untuk mengkaji pengaruh penambahan tepung daun sirih (*Piper betle* L.) dalam konsentrat terhadap bakteri penyebab mastitis dan kondisi fermentasi rumen. Penelitian ini terdiri atas lima perlakuan pakan (0%, 2%, 4%, 6%, dan 8% level tepung daun sirih dalam konsentrat) dan empat ulangan. Pakan perlakuan beserta rumput gajah dengan perbandingan 40 : 60 kemudian difermentasi menggunakan cairan rumen. Aktivitas antibakteri sebelum dan setelah difermentasi diuji pada setiap perlakuan. Setelah empat jam fermentasi, supernatan setiap sampel diambil untuk pengujian VFA, NH_3 , total bakteri dan protozoa. Selanjutnya, KCBK dan KCBO dianalisis setelah 48 jam. Hasil penelitian ini menunjukkan bahwa sebelum difermentasi semakin tinggi level tepung daun sirih (dari 0% sampai 8%) mengakibatkan diameter hambat yang semakin luas ($P<0,05$) terhadap pertumbuhan *Staphylococcus* spp. Namun setelah difermentasi tidak terdapat perbedaan nyata di antara level daun sirih. Hasil fermentasi *in vitro* menunjukkan bahwa level tepung daun sirih berpengaruh nyata ($P<0,01$) terhadap VFA, tetapi tidak berpengaruh nyata terhadap konsentrasi NH_3 , KCBK, dan KCBO. Level tepung daun sirih nyata menurunkan ($P<0,01$) populasi protozoa, tetapi tidak berpengaruh nyata terhadap total bakteri. Dapat disimpulkan bahwa penambahan 2% tepung daun sirih dalam konsentrat dapat digunakan sebagai penghambat pertumbuhan bakteri penyebab mastitis dan tidak mengganggu kondisi fermentasi rumen.

Kata kunci: daun sirih, mastitis, fermentasi rumen, mikroba rumen, *Staphylococcus* spp.

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INTRODUCTION

In 2004, subclinical mastitis prevalence in Indonesia was recorded for 65%-75% and it continuously increased to 75%-83% in 2006 (Sudarwanto & Sudarnika, 2008) with a decreasing of milk production about 70%. This indicates that eradication and prevention of subclinical mastitis program have not been applied well by the farmers and other stake holders. Mastitis causes a great economic loss such as treatment cost, veterinary time and consultation fees, cost of the drugs, discarded milk, labour cost, fatality, repeated cases of mastitis, decrease in milk yield, milk quality changes, decreased hygienic quality of milk and public health considerations, culling and replacement cost, premium loss and penalties, and post-milking teat disinfection (Seegers *et al.* 2003; Shim *et al.* 2004; Rodrigues *et al.* 2005; Petrovski *et al.* 2006). Antibiotic is usually used for treating and preventing through teat dipping after each milking. However using antibiotic is hazardous for consumer health and it is worldwide refused due to residues appearance in milk and risk factor in creating resistant strain of bacteria. Therefore, natural product which harmless to consumer such as betel leaf is needed to replace role of antibiotic.

Betel leaf (*Piper betle* L.) was well known by Indonesian people as medicinal plant and has an immunity stimulant because it contains 4.2% of essential oil and most of them are *chavicol paraallylphenol*, a derivative of *Chavica betel*, *betephenol* as an isomer of *euganol allylpyrocatechine*, *cineol methyl euganol*, *caryophyllen*, *kavikol*, *kavibekol*, *estragol*, and *terpinen* (Sastroamidjojo, 1997). Some of empirical and scientific studies about antibacterial content of betel leaf leaded us to its potential to be used as feed additive for dairy cow and expected to inhibit and kill various bacteria particularly bacteria which cause mastitis such as *Staphylococcus sp.* or *Streptococcus sp.* Previous research of the efficacy of betel leaves extract for teat dipping given after milking showed that *Piper betle* leaves extract have ability to reduce the number of bacteria in milk of mastitis suffered cows (Poeloengan *et al.*, 2005).

Some herbs were used as the sources of antibacterial compounds and effectively decreased microorganism population at *in vitro* fermentation (Pattnaik *et al.*, 1996). Wallace *et al.* (2002) reported that essential oil contained in herbal leaves did not influence volatile fatty acids (VFA) production, NH_3 concentration, protozoa number, and microbial protein flow in rumen. Up to know, betel leaf extract has not ever been used yet as a feed supplement in ruminant ration. This experiment aimed to elaborate the use of betel leaf as anti mastitis feed supplement through *in vitro* study.

MATERIALS AND METHODS

The experiment was divided into 3 stages; 1) Antibacterial activity test of betel leaf meal before and after incubation with rumen fluid, 2) Effect of betel leaf meal on rumen fermentability and digestibility, and 3) Effect of betel leaf meal on viability of rumen microorganism.

Antibacterial Activity Test

Antibacterial activity consisted of 5 treatments of betel leaf level (0%, 2%, 4%, 6%, and 8% in feed concentrate mixed with elephant grass). *Staphylococcus sp.* bacteria was cultured in blood agar media and incubated at 37 °C for 24 h. Colonies formed were removed by sterile ose into a reaction tube which contained 5 mL of phosphate buffer solution (PBS), then incubated at 37 °C for 2 h until turbidity was equal with McFarland 1 standard (3×10^8 cfu/mL). Then, betel leaf meal was mixed with dairy concentrate feed before diluted in 5 mL distilled water and dropped onto a paper disc on agar media. After 4 h *in vitro* fermentation, rumen fluid filtered and dropped onto a paper disc and incubated at 37 °C for 24 h. Parameter observed was diameter of clear zone formed on agar media.

In Vitro Fermentation Test

The effect of BLM was evaluated in an *in vitro* batch fermentation (Tilley & Terry, 1963) trial using a concentrate feed containing betel leaf at levels of 0%, 2%, 4%, 6%, and 8% and napier grass as fiber source in ratio of 40:60. Cattle rumen fluids were collected from a slaughter house using warm (39 °C) thermos which was previously filtered using cheese cloth. Incubations were conducted in 90 mL tube containing 10 mL of rumen fluid, 40 mL of McDougall (pH 8–8.3) and 0.5 g of the experimental diet per tube. Each tube was gassed with CO_2 before sealing with rubber corks with gas release valve. Treatments were tested at an initial pH of 7.0. Incubations were conducted in a water bath at 39 °C for 4 h for antibacterial activity, VFA, NH_3 , number of bacteria and protozoa. To analyze DM and OM digestibility, incubations were conducted for 48 h.

After 4 h incubation, HgCl_2 was added 2-3 drops to kill the rumen microbes and centrifuged at 4000 rpm for 15 min. Liquid samples were withdrawn from each tube to determine pH and analyze ammonia N (Conway 1958) and total VFA concentrations using steam distillation method. Ammonia concentration analysis was initialed with lubricating the Conway using vaseline.

In Vitro Digestibility

After 48 h incubation DM and OM digestibility were determined (Tilley & Terry, 1963). The fermentor's tube rubber corks were unsealed. Saturated mercury chloride (HgCl_2) was added 2-3 drops to kill microbe and centrifuged 4000 rpm for 10 min to separate sediment and supernatant. Supernatants were discarded and centrifuged 4000 rpm for 15 min. 50 mL of HCl-pepsin solution 0.2% added and incubated again for 48 h without rubber corks. Residues were filtered with filter paper (Whatman No. 41) and enamel cup which was already known weight using vacuum pump. Filtrates were placed in the porcelain dish and dried to analyze dry matter in the oven at 105 °C for 24 h for determining DM. Filtrates were ashed at 450-600 °C for 8 h in electrical furnish for determining OM digestibility.

Fermentation residues were used without samples as a blanco. Dry and organic matter digestibility were then calculated.

Viability Test of Bacteria and Protozoa

Bacterial population was determined by counting the viable bacterial colonies (Ogimoto & Imai, 1981). Brain Heart Infusion (BHI) media was used to count total bacteria. Brain Heart Infusion was made by mixing such as 3.7 g of BHI powder, 0.05 g of glucose, 0.05 g of sellulobiose, 0.05 g of starch, 0.05 g of cystein, 100 µL of hemin, 0.005 ml of resazurin and 1.5 g of agar and then stored in sterile tube. The mixture was heated until the color changed from yellowish brown to reddish brown and changed back to yellowish brown. Then it was chilled and flowed by CO₂. Anaerobe BHI media was stored in hungate tube which previously was filled with 0.150 g of bacto agar until the volume reached 4.9 mL of each tube. Rumen fluid which had been treated and incubated for four hours was pipetted for 0.05 mL and mixed with 4.95 mL dilution media. This was repeated to form serial dilutions (10⁻², 10⁻⁴, 10⁻⁶, 10⁻⁸). Each dilution tube was then taken 0.1 mL and mixed in agar media and rolled while it was given water in the roller until became solid form. Then bacteria were incubated for 24 h and each colony was counted at the tube using marker.

For protozoa counting (Ogimoto & Imai, 1981), 1 mL of rumen fluid samples were taken immediately after 4 h incubation and stored in sample bottles which had been filled 1 mL of trypan blue formaline Saline (TBFS). Rumen fluid was taken using syringe and injected between counting chamber and cover glass. Direct counts of total bacteria were made using a cell chamber (0.0625 mm², 0.02 mm² deep; Brand, Germany) under light microscope (Olympus Optical Co., Japan).

Statistical Analysis

Data obtained were analyzed using analysis of variance (ANOVA). Antibacterial activity data were analyzed according to completely randomized design, and VFA, NH₃, DM, and DO digestibility, number of bacteria and protozoa were analyzed according to block randomized designs. Differences between means were tested using the Duncan multiple range test and declared at P<0.05 (Gasperz, 1994).

RESULTS AND DISCUSSION

Antibacterial Activity of Betel Leaf Meal on *Staphylococcus sp.*

Antibacterial activity test of BLM with level 0%, 2%, 4%, 6%, and 8% of concentrate diet on *Staphylococcus sp.* resulted in inhibition diameter zone of 0.0, 3.9, 15.9, 18.1, and 22.0 mm, respectively. The high inhibition diameter associated with the high levels of BLM in concentrate diet. Inhibition diameter occurred due to bioactive compounds contained in BLM which had an-

tibacterial activity. BLM treatments significantly (P<0.05) inhibited *Staphylococcus sp.* before *in vitro* fermentation (Table 1). This in line with the report of Poeloengan *et al.* (2005) that *Piper betle* leaves in concentration of 50%; 25%; 12.5%; and 6.25% have an antibacterial effect on *Streptococcus agalactiae*, *Staphylococcus aureus* dan *Staphylococcus epidermidis*. Benchaar *et al.* (2008) found that carvacrol contained within essential oil could kill *Staphylococcus aureus* and *Pseudomonas aeruginosa*. However, after *in vitro* fermentation, all addition levels of BLM showed higher (P<0.05) inhibition compared to control (0%) (Table 1), but did not different among addition levels (2%–8%). This similar response might occurred due to the adaptation of rumen bacteria to the high level of BLM. Adaptable bacteria can reduce the activity of essential oil after rumen fermentation because the bacteria degraded the high level of BLM. However, when the BLM was given in low and medium levels, it could stimulate rumen bacteria and increased the antibacterial activity of BLM after fermentation (Acamovic & Brooker, 2005; Patra & Yu, 2012). McIntosh *et al.* (2003) reported the list resistantcy and sensitivity of bacteria to essential oil-containing media, *Streptococcus bovis* was the most resistant bacteria while *Prevotella ruminicola*, *Clostridium sticklandii*, and *Peptostreptococcus anaerobius* were the most sensitive and *Prevotella bryantii* adapted to grow in media containing high concentration of essential oil. High level addition of BLM was related to the presence of the adaptable bacteria such as *Prevotella bryantii*.

VFA Production

Betel leaf meal addition resulted (P<0.01) in the variation of total VFA among treatments. Total VFA production were higher (P<0.05) for the diet containing 2% of BLM than those of diet with higher level of BLM addition (4%, 6%, and 8%) and control diet (Figure 1). Control diet and BLM addition of 2%, 4%, 6%, and 8% had similar total VFA production.

Decreasing VFA production at 4%, 6%, and 8% dietary BLM treatments were due to the increase in bioactive compounds in the diets, one of which was eugenol which had antibacterial activity (Benchaar *et al.*, 2007). Benchaar *et al.* (2008) reported that high doses of antibac-

Table 1. Inhibition zone diameter of betel leaf meal (BLM) on *Staphylococcus sp.*

Before ruminal fermentation		After ruminal fermentation	
BLM level (%)	Inhibition diameter (mm)	BLM level (%)	Inhibition diameter (mm)
0	0.00±0.00 ^a	0	0.00±0.00 ^a
2	3.90±3.62 ^a	2	16.10±1.92 ^b
4	15.90±3.64 ^b	4	15.30±1.64 ^b
6	18.10±1.40 ^{bc}	6	15.80±1.14 ^b
8	22.00±2.88 ^c	8	14.60±0.87 ^b

Note: Means in the same column with different superscripts differ significantly (P<0.05).

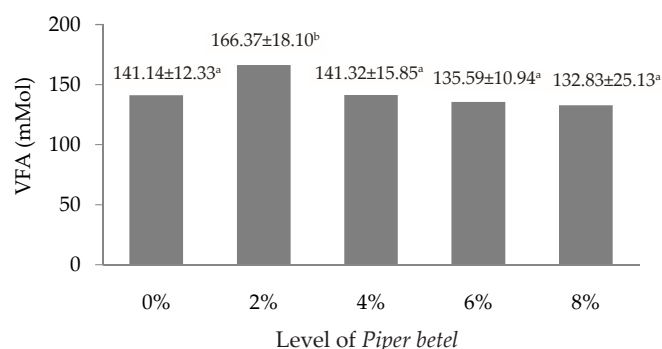


Figure 1. Total VFA concentration resulted in *in vitro* fermentation from elephant grass and feed concentrate added with different level of betel leaf meal

terial compounds of BLM did not affect total VFA. On the other hand, Castillejos *et al.* (2006) reported that eugenol decreased concentration of total VFA and change VFA profile but increased propionate concentration.

High doses of bioactive compounds contained in BLM changed rumen ecology by inhibiting fibrolytic bacteria in particular *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* resulting in low fermentation product (Lin *et al.*, 2012). Total VFA production as response to the addition of BLM varied according to the addition level of BLM. High level of BLM addition decreased rumen population of bacteria. On the other hand low level of BLM addition modulated the bacteria growth led to high VFA production. The result suggested that low level of BLM in the diet may improve ruminal fermentation characteristics.

NH₃ Concentration

NH₃ production *in vitro* as the response to dietary addition of BLM were shown in Figure 2. Dietary addition of BLM did not significantly affect NH₃. Different BLM addition levels produced statistically similar ($P>0.05$) NH₃ concentration. However, there was a tendency that 2% of BLM addition had higher NH₃ concentration compared to those of control and other levels of BLM addition. Eventhough Benchaar *et al.* (2008)

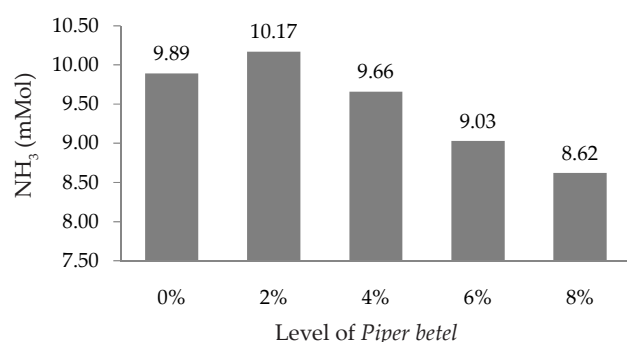


Figure 2. NH₃ concentration resulted in *in vitro* fermentation from elephant grass and feed concentrate added with different level of betel leaf meal

reported that chemical composition and dose of essential oil affect N metabolism in rumen, but in the present result the difference was not significant. Ammonia is the main nitrogen sources of microbes to synthesize amino acid (Sitoresmi *et al.*, 2009) for their growth. Factor affecting the reduction of NH₃ was low degradation of feed protein in rumen. The decrease of NH₃ concentration and deaminase activity was due to the decrease of bacteria growth, in particular hyper-ammonia production (HAP) bacteria (Russell & Houlihan, 2003; Patra & Yu, 2012). Wallace (2002), reported that *Prevotella spp.* and *Ruminobacter amylophilus* were HAP bacteria and most sensitive to essential oil whereas they have ability to produce high ammonia from amino acid sources. On the other hand, a decrease in feed protein degradation by essential oil would increase the by-pass of protein and thus increase the total protein supply to ruminant (Lin *et al.*, 2012).

Dry and Organic Matter Digestibility

Dry and organic matter digestibility were not significantly different among the treatments (Table 2). Dry and organic matter digestibility results had similar pattern with that of NH₃ concentration data, i.e., there was a tendency that 2% of BLM addition had higher dry and organic matter digestibility concentration compared to those of control and other levels of BLM addition.

The same results were reported by Castillejos *et al.* (2006) that eugenol had no effect on DM, OM, NDF, and ADF digestion. Patra & Yu (2012) also reported that dry matter and NDF degradability were not affected by essential oil, but it would decrease DM digestibility in high doses. It appears that addition of BLM had not negative effect on rumen degradability especially in low doses.

Rumen Microorganism and Rumen pH

Total viable bacteria were not affected by BLM addition (Table 3). The present results were lower than the results of Koike & Kobayashi (2009) who reported that the total bacteria were 10¹⁰–10¹¹ per mL of rumen content. The contrary results were reported by Patra & Yu (2012) that essential oil use increased bacteria at low and medium doses, but it tended to decrease bacteria at high doses. This was further explained by Acamovic & Brooker (2005) who reported that secondary metabolite such as essential oil could interact to various cell components which modulate the response of their targets. Therefore, BLM addition in low level could stimulate the

Table 2. *In vitro* digestibility with different level of betel leaf meal (BLM) at *in vitro* fermentation

<i>In vitro</i> digestibility	Level of BLM (%)				
	0	2	4	6	8
DM, %	48.65	53.21	51.54	52.67	50.78
OM, %	46.79	51.79	50.38	51.10	49.79

Table 3. pH, number of bacteria, and protozoa of different level of betel leaf meal (BLM) at *in vitro* fermentation

Parameter	Level of BLM (%)				
	0	2	4	6	8
pH	6.7	6.7	6.7	6.7	6.7
Bacteria (cfu/mL)	7.5x10 ⁶	1.2x10 ⁷	6.4x10 ⁶	2.8x10 ⁶	3.8x10 ⁶
Protozoa (cfu/mL)	2.3x10 ^{5a}	7.9x10 ^{4b}	8.3x10 ^{4b}	5.5x10 ^{4b}	4x10 ^{4b}

Note: Means in the same column with different superscripts differ significantly ($P < 0.05$).

number of rumen bacteria. The present result showed that level of 2% of BLM addition had slightly higher rumen bacteria than control and other addition levels, but the difference was not strong enough.

The effect of BLM addition on number of protozoa is shown in Table 3. Addition of BLM significantly ($P < 0.01$) decreased number of protozoa. The increase BLM level decreased linearly protozoa. The decrease of protozoa approved that BLM contained the chemical compound which was toxic to the protozoa. Defaunation already became object of research of microbiologist several years ago (Wallace *et al.* 2002). Lin *et al.* (2012), reported that essential oil combination would decrease 50%-90% of protozoa and fungi. It showed that BLM can be utilized as a defaunating agent. Furthermore, it is expected that methane production could be reduced with the addition BLM in concentrate diet, since methanogenic bacteria were associated with protozoa.

Rumen pH of each treatment was similar, i.e., 6.7 (Table 3) indicating that rumen pH was unaffected by BLM addition. This pH value supported a conducive environment for growth of rumen microbes. The same results were reported by Lin *et al.* (2012) that essential oil type and dose did not affect rumen pH.

CONCLUSION

Betel leaf meal addition in concentrate diet has antibacterial activity on mastitis causing bacteria (*Staphylococcus* spp.) either before or after *in vitro* fermentation. Two per cent addition of BLM in concentrate diet has the optimum effect on VFA production, NH₃ concentration, dry matter and organic matter digestibility, and number of bacteria and protozoa.

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