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ADDITION OF ESSENTIAL OIL SOURCE, *Amomum compactum* Soland ex Maton, AND ITS EFFECT ON RUMINAL FEED FERMENTATION IN-VITRO**

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ABSTRACT

Essential Oil (EO), as feed additive, is known to increase the feed efficiency and reduce the methane production among ruminants. This research was done to study the effect of Java cardamom (*Amomum compactum* Soland ex Maton) essential oil as feed additive on ruminal feed fermentation. The in vitro gas production technique was used in this research to determine the effect of cardamom on nutrient digestibility or fermentation in the rumen. Cardamom meal was added into the feed sample to get end concentration of EO in the fermentation medium as much as 0, 25, 50, 75 and 100 mg/L. The substrate consisted of *Pennisetum purpureum*, rice bran and wheat pollard. The addition of cardamom did not significantly affect the digestibility of dry matter except at 100 mg/L in which it decreased. Protein digestibility decreased when the diet was added with cardamom, whereas organic matter and crude fiber digestibility increased up to 13.5% and 24% level of EO100 mg/L, respectively. The production of volatile fatty acid (acetate, propionate, butyrate), pH and microbial protein synthesis except the ammonia concentration, were not affected by cardamom addition. Similarly, the methane production and protozoa population did not significantly change. The utilization of Java cardamom as feed additive positively affected the ruminal feed fermentation by increasing the organic matter and crude fiber digestibility and reducing the protein digestibility.

Keywords: *Amomum compactum* Soland ex Maton, essential oil, methane, ruminal fermentation

INTRODUCTION

The digestive system of ruminants is distinctly different from those of monogastric animals. As a part of the ruminansia system, the rumen is a unique organ which functions as a large natural fermenter. Bacteria, fungi, and protozoa habitated the rumen (Nagaraja 2016) and play a main role in the ruminal feed fermentation of particularly plant materials including fibrous plants cell wall (Wang & McAllister 2002). During the fermentation process, the energy supplied for the host animal is the volatile fatty acid (Choudhury *et al.* 2015), and from those processes other useful by-products are produced including the high quality protein from non-protein nitrogen in the form of bacterial cells, and vitamins, particularly vitamins B, besides several wastes of gaseous CO_2 and CH_4. Methane emission from enteric rumen fermentation implies an ineffective feed energy utilization. About 6 to 10% (Eckard *et al.* 2010) and 2-15% (Kumar *et al.* 2009) of the total gross energy of consumed feed is released and lost through the breath as methane. Methane is a potent greenhouse gas with a global warming potency 25 times that of CO_2 (Eckard *et al.* 2010). Researches have been done to increase the efficiency of rumen fermentation and reduce the methane production through rumen microbes and rumen fermentation manipulation, like those on antibiotic utilization, ionophore, for the modification of rumen fermentation (Russell & Strobel 1989).

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Monensin, one of ionophore antibiotic has increased the efficiency of feed utilization by decreasing 30% of ruminal methanogenesis, thereby reducing the ammonia concentration in the rumen by interfering with proteolytic bacteria, mostly deamination bacterial activities. The reduction of ammonia in the rumen resulted in a protein loss in urine. Among the growing cattle, monensin has increased the rumen VFA concentration, digestibility and protein retention, thus improving food use and weight gain (Salles *et al.* 2008). Ionophore in the feedlot of cattle has increased the daily gain (1.6%) and feed efficiency (7.5%) (Jouany & Morgavi 2007). However, the use of antibiotic was recently banned in several countries due to the presence of antibiotic residues in animal products and the emergence of resistant bacteria. Hence, a safe and organically produced feed additive as an antibiotic alternative is needed. Essential oils have antimicrobial activities that are currently generally considered safe for human and animal consumption (US Food & Drug Administration 2017).

Essential oil, a plant secondary metabolite, is a natural product which inhibits the activity of a wide range of microorganisms, including bacteria, protozoa and fungi (Chao *et al.* 2012; Cosentino *et al.* 1999; Deans & Ritchie 1987; Sivropoulou *et al.* 1996). Naturally, the essential oil is part of a plant defense mechanism against predator; as antibacterial, antifungals, antivirals and as insecticides (Bakkali *et al.* 2008). Thus, the essential oil has been used as an alternative antibiotic to modify rumen microbes and ruminal fermentation.

Several researches on essential oil utilization as feed additive have reported positive results particularly in increasing the productivity and decreasing methane production by in vitro and or in vivo studies (Bodas *et al.* 2012; Geraci *et al.* 2012). Mixed oil of cinnamaldehyde, eugenol, and capsicum in cattle feedlot produced similar effects as monensin (Geraci *et al.* 2012). It has increased the growth and health performance, optimized the feed fermentation in the rumen and increased the immune system (Compiani *et al.* 2013). Hence, the prospect of using essential oil as alternative antibiotic for feed additives is very promising (Khorrami *et al.* 2015).

Essential oils from rosemary, oregano, ceylon cinnamon, dill seeds, cinnamon leaves, cinnamon bark, and eucalyptus, at level 1,125 ml/L of fermentation media, had reduced methane production and ammonia concentration in rumen medium with no detrimental effect on neutral detergent fibre (NDF) degradability, except the eucalyptus. Individually and in combination of several essential oils, oregano had reduced the abundance of archaea (Cobellis *et al.* 2016a).

Essential oil of clove, eucalyptus, garlic, oregano and peppermint at concentration of 0.25, 0.5 and 1 g/L of medium had reduced methane production with the increasing essential oil level, reduced population of protozoa and archaea. However, NDF digestibility also decreased except in garlic oil due to the diminishing cellulolytic bacteria. The combination of those essential oils did not affect the volatile fatty acid production except when clove and oregano oil were added (Benchaar & Greathead 2011; Calsamiglia *et al.* 2007).

Biological activities of essential oils in rumen fermentation vary as the effects of essential oils also depend on their chemical compositions. Same essential oil obtained from different plants in the same genus may have opposite effect, stimulatory or inhibitory (Ferme *et al.* 2004; Patra 2011). Its purity and dose also influenced the activity of essential oil (Macheboeuf *et al.* 2008).

Amomum compactum Soland ex Maton (Java cardamom) of the Zingiberaceae family is commonly called Java cardamom, or false cardamom. In Indonesia, Java cardamom is a commonly used spice in several dishes and is also part of a traditional medicine called jamu. The active components of Java cardamom essential oil comprise the following: 98% of the total oil consist of 1,8-cineole (38.7%), β-pinene (13.6%), α-terpineol (12.6%), spathulenol (8.3%), 4-terpineol (4.5%), germacrene D (3.0%), α-pinene (2.8%) and β- selinene (2.7%) (Chempakam & Sindhu 2008). In another study, the major active components of cardamom essential oil are 1,8-cineole (30.2%) and a-terpinyl acetate (46.6%) (Sardar *et al.* 2013). This study determined the in vitro effect of Java cardamom mixed diet on the nutrient digestibility, methane production and other parameter of ruminal fermentation.

MATERIALS AND METHODS

Feed, Treatments and In Vitro Fermentation

This study determines the effect of Java cardamom on nutrients digestibility, ruminal fermentation and methane production using the batch culture of in vitro gas production technique. Feed sample for in vitro fermentation consisted of *Pennisetum purpureum* which was cut before flowering stage, rice bran and wheat pollard obtained from feed shop, at a ratio of 60:20:29 based on dry matter. Java cardamom meal was prepared by initially drying its seed in dryer incubator at 55°C and by grinding to pass through a 1 mm pore-size sieves. The addition of Java cardamom was based on the final concentration of essential oil in the fermentation media, i.e. 0, 25, 50, 75 and 100 mg/L.

The inoculum for the in vitro gas production was obtained from two ruminally canulated Ongole grade cattle whose fed diet consisted of *Pennisetum purpureum* and beef cattle concentrate at 60:40 DM bases. Rumen fluid was collected before the morning feeding, squeezed through a polyester cloth into a vacuum flask thermos, and immediately sent to the laboratory.

Serum bottles of 125 mL volume were used for the in vitro incubations. Three sets of bottles were prepared. One set is for determining dry matter digestibility (DMD) and organic matter digestibility (OMD), gas and methane production, one set for crude protein digestibility (CPD), and the third set for the rumen fermentation parameters. The day before the incubation, sufficient anaerobic media were prepared based on Theodorou *et al.* (1994). Sixty-three milliliters of media were added into the serum bottles which were previously filled with 700 mg of substrate and Java cardamom powder according to the treatments and were continuously flushed by oxygen free carbon dioxide. The bottles were sealed immediately with butyl rubber stopper and aluminum crimp cap and pre-warmed overnight at 39°C. In the morning, the rumen fluid was collected, and 7 mL were added into each bottle using 10 mL plastic syringe. The bottles were then incubated for 24 h at 39°C. The bottle head gas pressure space was zeroed/released before incubation by inserting a 0.6 mm needle attached to a pressure transducer.

At the end of the incubation period, the gas was collected using a calibrated syringe and 5 mL of gas were transferred into 5 mL plain vacuum tube (Becton Dickinson Vacutainer System) for methane analysis. DMD, OMD and CPD were determined by filtering the bottle content, and the residual feed were collected for nutrients analysis, including DM, OM and CP, according to AOAC (2005). Samples for protozoa calculation were prepared by pipetting 1 mL of bottle content and by adding 0.8 mL of formaldehyde saline (1 mL of 37% formaldehyde + 9 mL 0.9% NaCl). One microliter sample was then transferred to haemocytometer for direct calculation under a microscope based on Abreu *et al.* (2004).

For ammonia measurement, 1 mL of bottle content was preserved with 1 mL NaCl 20% and frozen until a later analysis of ammonia based on phenol hypochlorite reaction as explained by Chaney and Marbach (1962). Media, of as much as 1 mL for volatile fatty acid (VFA) analysis were added into tube containing 1 mL of 20% metha-phosphoric acid and stored in freezer for further analysis using the gas chromatography. Prior to ammonia sampling, the VFA, microbial protein and protozoa, and pH media were measured. The Rumen microbial protein was determined by the Lowry method (Alexander & Griffiths 1993). Microbial cells were separated from residual feed by centrifugation using 1.5 mL of bottle content at 500 g. The cells were precipitated from supernatant by spinning down at 15,000 g while the pellets were re-suspended in physiology solution and recentrifuged. Re-suspension was repeated twice. The last suspension was subjected for protein determination.

Calculation and Statistical Analysis

The parameters observed and computed were the nutrients digestibility including dry matter digestibility (DMD), organic matter digestibility (OMD), crude protein digestibility (CPD in %), total VFA, acetate, propionate and butyrate concentration (in mmol/100 mL),

rumen microbial protein, ammonia concentration (in mg/100 L), methane production as mL/g DM digested, and protozoa number. Data were subjected to a one-way analysis of variance with the different level of Java cardamom as the treatment and the means were compared using Duncan Multiple Range test.

RESULTS AND DISCUSSION

Nutrient Digestibility

The role of rumen microbes in nutrient digestion is very critical for ruminant production. Rumen microbes help the ruminant, the host animal, to extract energy and serve protein by digesting and fermenting the feeds. Feeds for ruminant are commonly fibrous material that cannot be used by monogastric animal. Rumen fermentation was modified to achieve a higher nutrient utilization by: improving fiber digestion, reducing feed protein degradation to increase the availability of amino acid absorbed in small intestine, reducing the degradation rate of readily fermentable carbohydrate, and by shifting methane production to propionate (Jouany & Morgavi 2007). Hence, the essential oil compounds are added particularly to modify the feed fermentation in rumen because of their effects on the growth of bacteria, fungi, and protozoa.

The addition of Java cardamom significantly reduced the dry matter digestibility ($P<0.05$) at levels 100 mg/L by as much as 12.29% compared to control (Table 1). A slight increase of dry matter digestibility (10.42%) occurred at an addition level of 25%, while addition of 50 and 75 mg/L did not significantly change the dry matter digestibility.

The organic matter digestibility was also significantly affected by the different addition levels of Java cardamom, except at 25 mg/L (Table 1). Addition at level 50 mg/L and up had increased organic matter digestibility by 7.46, 7.27 and 13.05% for Java cardamom addition at levels 50, 75 and 100 mg/L, respectively. Increased dry matter and organic matter digestibility using Chinese herbal addition was also reported by Wang and Wang (2016). However, Cobellis *et al.* (2016a) reported that the addition of essential oils in *in vitro* ruminal fermentation i.e. essential oils from dill seed, cinnamon leaves, cinnamon bark, ceylon cinnamon bark, eucalyptus leaves, oregano leaves and rosemary leaves at level 1,125 mg/L or their combination in lower concentration (800 mg/L) had reduced the dry matter digestibility, but had no effect on neutral detergent fiber digestibility. The major component of those essential oils was carvone in dill seed oil; trans-cinnamaldehyde in cinnamon leaves, cinnamon bark, and Ceylon cinnamon bark; 1.8-cineole in eucalyptus leaves and rosemary leaves; and carvacrol, in oregano leaves. Even though the main component of Java cardamom is 1.8 cineole, the same main component of essential oil from eucalyptus and rosemary leaves, but their effects on nutrient digestibility were different. This may be due to the addition of different essential oils source. The addition of 1,8-cineole in *in vitro* rumen fermentation at level 50 mg/75 mL medium corresponding to 666 mg/L had no effect on organic matter digestibility (Araujo *et al.* 2011). Mixture of thymol, limonene and guaiacol at lower level, 1.5 mg/L, had no effect on dry matter, organic matter neutral detergent fiber, acid detergent fiber and crude protein digestion (Castillejos *et al.* 2005). Moreover, the addition of thymol at level 5 and 50 mg/L have no effect on dry matter, organic matter, neutral detergent fiber and acid detergent fiber digestibility, but at high level, 500 mg/L, the nutrient degradability was reduced (Castillejos *et al.* 2006). The addition of eugenol up to 500 mg/L did not affect the digestion of dry matter, organic matter, neutral detergent fiber and acid detergent fiber. The effect of essential oils in rumen fermentation was determined by their chemical composition (Ferme *et al.* 2004; Patra & Saxena 2009). Purity and doses also influenced the efficacy of essential oil (Macheboeuf *et. al* 2008).

Table 1 Effect of Java cardamom essential oil on ruminal *in vitro* nutrient digestibility

Parameters	Level of essential oil added (mg/L)				
	0%	25%	50%	75%	100%
True Nutrient Digestibility (%)					
Dry matter*	49.31^{ab}	54.45^{b}	47.24^{ab}	47.03^{ab}	43.25^{a}
Organic matter*	47.45^{b}	45.59^{b}	50.99^{ab}	50.90^{ab}	53.64^{a}
Crude protein**	56.02^{c}	44.21^{ab}	32.80^{a}	48.17^{bc}	36.42^{ab}
Crude fiber**	31.30^{a}	39.69^{b}	43.88^{c}	46.82^{c}	51.79^{d}

Notes: ^{a,b,c,d}Different superscript in the same row differ significantly
*($P<0.05$)
**($P<0.01$)

The crude protein digestibility had significantly decreased when Java cardamom oil was added ($P<0.01$) (Table 1). Crude protein digested by rumen microbes were lower in all levels of Java cardamom addition. Contrary to the crude protein digestibility, the crude fiber digestibility increased with increasing Java cardamom level ($P<0.01$). Compared to control, the increases were 26.81%, 40.19%, 49.58% and 65.46% at addition levels of 25, 50, 75 and 100 mg/L, respectively. Essential oil may inhibit the colonization of proteases bacteria in rumen as indicated by the lower activity of protease (Wallace *et al.* 2002). Lowering the crude protein digestibility in the rumen is an advantage since the escaped feed protein from rumen microbial degradation will be flushed into abomasum and small intestine for further digestion by indigenous animal proteases and absorbed for animal metabolism. Inside the rumen, the feed protein is digested and broken down into small peptides, further into amino acid then ammonia. The ammonia which do not incorporate in the microbial protein but is absorbed across the rumen wall and pass in the bloodstream, is then converted to urea in the liver and excreted through the urine (Moran 2005).

Previous researches show that essential oil did not affect fiber digestion in rumen (Cobellis *et al.* 2016a; Wallace *et al.* 2002). However, in this research, the addition of Java cardamom positively affected the fiber digestion (Table 1) which is one main goal of rumen modification (Jouany & Morgavi 2007).

Rumen Fermentation Processes and Composition

Volatile fatty acid (VFA) production and composition as well as the acetate: propionate ratio did not change with the treatment even though the crude fiber digestibility had increased (Table 2). The VFA in the rumen resulted from digestion and fermentation of carbohydrate by the rumen microbes (Moran 2005). The effects of single and mixed source essential oils on the VFA production in the rumen are similar. The use of blended essential oils of oregano, cinnamon, thyme, orange peel resulted in decreasing VFA concentrations (Spanghero *et al.* 2008). The VFA production also decreased with the use of single essential oils from dill seeds, cinnamon leaves, cinnamon bark, Ceylon cinnamon bark, eucalyptus leaves, oregano leaves and rosemary leaves (except eucalyptus leaves) (Cobellis *et al.* 2016a). This study results also showed changes in VFA components and acetate to propionate ratio. The reduction in VFA was also reported by Castillejos *et al.* (2006), when thymol and eugenol were added at high level 500 mg/L. In contrast, thymol and eugenol at lower levels of 5 and 50 mg/L neither changed the VFA concentration nor its composition.

Several individual essential oils added in several doses (Busquet *et al.* 2006) showed that addition at 0 to 30 mg/L did not change the VFA production, while at level 300 mg/L some essential oils had reduced or slightly increased VFA production. And at addition level of 3,000 mg/L, almost all essential oil changed the VFA production and composition depending on the essential oil source. Addition of spices as source of essential oil at low level showed that cinnamon did not affect VFA, while clove and coriander lowered VFA, and cumin and turmeric increased the VFA and all treatments except cumin and turmeric reduced the acetate-propionate ratio (Chaudhry *et al.* 2012).

Moreover, eucalyptus and rosemary essential oil with majority compound 1,8-cineole, showed

Table 2 Effects of Java cardamom essential oil on some parameters of ruminal in vitro fermentation

Parameters	Level of essential oil (mg/L)				
	0	25	50	75	100
Total VFA (mmol/100 mL)	18.28	17.19	18.80	18.41	19.77
Asetat	13.77	12.79	13.69	13.78	13.97
Propional	2.64	2.84	3.22	2.83	3.80
Butirat	1.87	1.55	1.89	1.80	2.00
Acetate:Propionate	5.22	4.57	4.52	5.00	3.97
Protozoa (x 104)	9.42	12.15	10.67	9.61	10.38
Microbial protein (mg/100 mL)	241.73	247.84	278.95	299.16	255.29
NH_3 concentration (mg/100 mL)**	25.79[a]	26.60[a]	25.77[a]	30.11[b]	26.13[a]
Methane/DM Digested (mL/g)	42.15	50.06	44.52	43.89	42.74
pH	6.78	6.77	6.78	6.79	6.78

Notes: [a,b]Different superscript in the same row differ significantly
*($P<0.01$)

different effect from Java cardamom on VFA production. At the same level, eucalyptus oil did not affect VFA, while rosemary had reduced VFA (Cobellis *et al.* 2016a). Essential oils may also alter the VFA profile, even when essential oils are added at doses below their capacity to depress VFA production (Spanghero *et al.* 2008). Other observed effects of essential oils on VFA compositions were reduced acetate and increased butyrate proportions (Castillejos *et al.* 2006), increased acetate proportion (Castillejos *et al.* 2005, 2006; Spanghero *et al.* 2008) and increased propionate proportion (Busquet *et al.* 2006; Cardozo *et al.* 2006).

The effects of essential oils on the population of rumen microbe protozoa and bacteria did vary. The addition of Java cardamom did not affect the protozoa number and microbial protein synthesis. The effects of herb extract and essential oil on protozoal numbers also differed. Anise extract reduced the protozoa number while capsicum and blend of cinnamon and eugenol did not (Cardozo *et al.* 2006). The effect of essential oil on protozoa number also depended on the source and doses (Patra & Yu 2012). Eucalyptus and clove oils had stimulatory effect at level 250 mg/L, and at 500 and 1,000 mg/L these inhibited the protozoa, while garlic, oregano and peppermint inhibited the protozoa at level 250 up to 1,000 mg/L. Individual essential oil had varied effects while their combination had reduced the protozoa number (Cobellis *et al.* 2016a). Similar tendency was observed in the effect of essential oil on microbial synthesis. Other researches showed stimulation (Fraser *et al.* 2007), no effect (Castillejos *et al.* 2005; Cobellis *et al.* 2016b) and decreased protozoa number (Wanapat *et al.* 2012). Even though essential oils have the ability to inhibit rumen microbes (Chao *et al.* 2012; Cosentino *et al.* 1999; Deans & Ritchie 1987; Sivropoulou *et al.* 1996) their activities still depend on doses and functional group of compounds. The antimicrobial activity of functional groups from the strongest to the weakest are phenol followed by cinnamaldehyde, alcohol, aldehyde, ketone, ether and hydrocarbon, respectively (Kalemba & Kunicka 2003). Phenol is a major component of essential oils with the broadest spectrum activity (Kalemba *et al.* 2012). Generally, its main activity is the disturbance of the cytoplasmic membrane, disruption of the proton motive force, electron flow, active transport, and the coagulation of cell contents (Kotzekidou *et al.* 2008).

The ammonia at treatment level 75 mg/L had different effects ($P<0.01$) from other treatments (Table 2). The higher ammonia might be due to the higher protein digestibility. In several studies, the addition of herb and essential oil had reduced the ammonia concentration (Chaudhry *et al.* 2012; Cobellis *et al.* 2016a; Fraser *et al.* 2007; Wanapat *et al.* 2013) as a result of a reduced peptidolytic activity of ruminal bacteria (Busquet *et al.* 2006). These compounds also inhibited the growth of hyper ammonia producing bacteria, a ruminal bacteria involved in ammonia production (McIntosh *et al.* 2003; Newbold *et al.* 2004). Addition of Java cardamom in this research reduced digestibility of protein but was not accompanied by reduction of ammonia concentration. It seems that Java cardamom does not have effect on ammonia producing bacteria, but only on peptidolitic.

Methane production was not affected by the treatments (Table 2). The existence of protozoa which was not influenced by the treatment might be one of the reasons. Most methanogens archaea in the rumens are associated with protozoa by endosymbiosis (Belanche *et al.* 2014). Hence, defaunation (elimination of protozoa number) decreased the methane production (Morgavi *et al.* 2010).

The pH of the *in vitro* medium with the added Java cardamom ranged from 6.77 to 6.79 (Table 2), which were the optimum range for microbial activities that support rumen feed metabolism. The physiological pH range was between 5.5 and 6.9, and it is one of the most variable factors in the rumen environment (Choudhury *et al.* 2015).

CONCLUSION

Java cardamom is a potential rumen modifier when used as feed additive. Its utilization in *in-vitro* rumen fermentation increased the feed efficiency by increasing the organic matter and crude fiber digestibility and by reducing the ruminal feed protein digestion.

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