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Methane Production and Methanogen Population in Rumen Liquor of Swamp Buffalo as Influenced by Coconut Oil and Mangosteen Peel Powder Supplementation

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Abstract: An *in vitro* study was conducted to evaluate effect of coconut oil and Mangosteen Peel Powder (MPP) supplementation on methane production and methanogen population in rumen liquor of swamp buffalo. Completely randomized design was used with nine treatments including control and supplementation with coconut oil (3, 6% DM) and/or MPP (2, 4% DM). Supplementation of coconut oil and MPP significantly reduced methane production when compared with control group ($p<0.05$) however, increasing coconut oil and MPP levels decreased proportion of methane reduction. Population of methanogen was unchanged by supplementations ($p>0.05$) in contrast, the methanogen diversity slightly differed among treatments, especially when combinations with coconut oil and MPP used. The UPGMA dendrogram showed supplementation of coconut oil and MPP combination had pronounced effect on gel profile than that of single supplementation, especially combination of 6% of coconut oil with 2% of MPP which resulted in lowest band numbers. In addition, supplementation by high level of coconut oil or MPP did not show positive effects. Therefore, suitable level should not exceed than 6% for coconut oil and 4% DM for MPP supplementations, respectively.

Key words: Coconut oil, mangosteen peel powder, methane, methanogen, swamp buffalo, rumen liquor

INTRODUCTION

Ruminant animals are one of the largest sources of methane emission with 81-92 million tons produced per year globally which is equivalent to 23-27% of total anthropogenic methane (IPCC, 2007). Methane produced during ruminal fermentation represents a loss of 2-15% of gross energy intake and thus decreases the potential conversion of digesta to metabolisable energy (Giger-Reverdin and Sauvant, 2000).

Fat inclusion in the diet causes a marked decrease in methane production by rumen fluid with the effect being at least partly governed by the fat source used (Soliva *et al*., 2003; Machmuller, 2006). Most recently, Kongmun *et al*. (2010) reported that supplementation of coconut with garlic powder could improve *in vitro* ruminal fluid fermentation in terms of volatile fatty acid profile, reduced methane losses and reduced protozoal population. Moreover, there have been reports of decreased methane emission by ruminants consuming plant secondary compounds (Carulla *et al*., 2005; Puchala *et al*., 2005). Supplementation of pellets containing condensed tannins and saponins (MP and soapberry fruit) influenced rumen ecology by significantly lowering methane concentration in rumen atmosphere and reduced methanogen population (Poungchompu *et al*., 2009). Thus, the objective of the present study was to investigate effect of coconut oil and MPP supplementation on methane production and methanogens population in *in vitro* by used rumen fluid of swamp buffalo.

MATERIALS AND METHODS

Experimental design and gas production technique: An *in vitro* study was conducted to evaluate effect of coconut oil and MPP supplementation on methane production and methanogens population. Completely randomized design was used with nine treatments including control and supplementation with coconut oil (3, 6% DM) and/or MPP (2, 4% DM). The method used for *in vitro* fermentation based on the technique described by Menke *et al*. (1979).

Sample collection and analysis: The gas samples were collected at 6, 12, 24, 48 and 72 h of incubation and stored for methane concentration analyzed by using Gas Chromatography with a Flame Ionization Detector

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(GC/FID). The fermented content were sampled at 12 h of fermenting and was fixed in 1% formalin for microbial population analyzed using molecular techniques.

Community Deoxyribonucleic Acids (DNA) extraction: Community DNA was extracted from 2 mL of rumen fluid by the RBB+C method described by Yu and Morrison (2004). In brief, the cell lysis is achieved by bead-beating in the presence of 4% (w/v) Sodium Dodecylsulfate (SDS), 500 mM NaCl and 50 mM EDTA.

After bead-beating, most of the impurities and the SDS are removed by precipitation with ammonium acetate and then the nucleic acids are removed by precipitation with isopropanol. Genomic DNA can then purified via sequential digestion with RNase A and proteinase K and the DNA are purified using columns from QIAgen DNA Mini Stool Kit (QIAGEN, Valencia, CA).

Quantitative analysis of methanogen populations: Real-time PCR amplification and detection was performed using a Choromo4 detection system (Bio-Rad, Hercules, CA). The reaction was conducted in a final volume of 10 μL containing the following: 5.1 μL Quatimix EASY SYG Kit (BIOTOOLS B and M Labs, S.A.), 0.408 μL as a forward primer, 0.408 μL as a reverse primer, 2.244 μL distilled water and 2.0 μL of DNA solution of unknown concentration. PCR primers used for methanogen are F 5'-TTCGGTGGATCDCARAGRGC-3' and R 5'-GBARG TCGWAWCCGTAGAATC C-3' (Denman *et al*., 2007). Regular PCR conditions for were as follows: 30 sec at 94°C for denaturing, 30 sec at 60°C for annealing and 30 sec at 72°C for extension (48 cycles), except for 9 min denaturation in the first cycle and 10 min extension in the last cycle.

Denaturing Gradient Gel Electrophoresis (DGGE)-analyses: The PCR amplification for methanogen was conducted in a total volume of 50 μL containing 0.5 μM of primers (F 5'-GC-clamp-ACGGGGYGCA GCAGGCGCGA-3' and R 5'-GWATTAC CGCGGCKGCTG-3'; Bano *et al*., 2004), 80 μM of dNTP mixed, 1.75 mM of $MgCl_2$, 1×PCR buffer 1×GC buffer and 1.25 U of platinum taq DNA polymerase.

The DNA templates were first subjected to an initial denaturation at 95°C for 5 min, 30 sec at 95°C for denaturing, 30 sec at 61°C and decrease 0.5°C $cycle^{-1}$, 1 min at 72°C (10 cycles), 30 sec at 95°C for denaturing, 30 sec at 56°C for annealing, 1 min at 72°C for extension (25 cycles) and 72°C for 30 min. The products were resolved on 80 g L^{-1} polyacrylanide gel (37.5:1) with a 300-600 g L^{-1} denaturing gradient for 5 min at 200 voltage and then for 16 h at 85 voltage. After the run, gels were stained for 30 min in 0.5×Tris-borate-EDTA buffer containing SYBR Gold nucleic acid stain (Molecular Probes, Eugene, OR) and gel images were captured using Photo documentation (Vilber Lourmat, France). The gel images were analyses by using the Phoretix 1D advanced analysis package.

Profiles were compared by hierarchical clustering to join similar patterns into groups (Fromin *et al*., 2002). To this end all the images of DGGE gels were matched using the internal control sample and the bands were quantified after local background subtraction. The similarity among profiles was calculated with the Pearson product-moment correlation coefficient and the clustering was done with the Unweighted Pair-Group method using Arithmetic averages (UPGMA; Mehmood *et al*., 2009). A significance test based on pairwise similarity measures was used to compare the community profiles of different groups of samples (Kropf *et al*., 2004).

Statistical analysis: Methane production and methanogen numbers were analyzed by using the General Linear Models (GLM) procedure (SAS Institute, 1996). Data were analyzed using the model:

$$Y_{ij} = \mu + T_i + \varepsilon_{ij}$$

Where:

Y_{ij} = Observation from treatment i, j, the replication
μ = Overall mean
T_i = Mean of treatment
ε_{ij} = Residual effect

Mean separations with a significant F ($p<0.05$) for treatment will be statistically compared using the orthogonal contrast.

RESULTS AND DISCUSSION

Methane production: Methane production has been shown to be affected by feed supplementation ($p<0.05$; Table 1). Supplementation of coconut oil and MPP reduced methane production in various time, especially at 48th h after fermentation which was significantly different when compared with control group ($p<0.01$). Combination of coconut oil with MPP decreased methane production in all sampling times ($p<0.05$) however, increasing both coconut oil and MPP levels decreased proportion of methane reduction ($p<0.05$). These results agreed with previous study in *in vitro* (Kongmun *et al*., 2010) and *in vivo* (Machmuller and Kreuzer, 1999). Moreover, other studies have shown that dietary supplementation with medium chain fatty acid, rich in coconut oil, depressed methane production (Yabuuchi *et al*., 2006; Odongo *et al*.,

Table 1: Effect of feed supplementation on methane production and Log quantity of methanogen population determined by real-time PCR

	Methane production (mL)					Methanogen no.
Treatments	6 h	12 h	24 h	48 h	72 h	12 h
Control	8.60	16.30	21.1	22.1	23.3	4.94
CO (3%)	7.10	15.4	20.4	15.8	22.6	5.04
CO (6%)	7.50	13.3	18.2	12.1	15.0	5.00
CT (2%)	6.50	9.70	17.0	22.6	22.4	5.16
CT (4%)	7.50	7.70	20.4	21.8	21.6	4.94
CO (3%) MP (2%)	8.00	15.5	18.5	21.3	22.4	5.19
CO (3%) MP (4%)	7.30	8.80	16.1	20.4	20.4	5.03
CO (6%) MP (2%)	7.50	13.6	18.1	20.6	21.6	4.96
CO (6%) MP (4%)	7.80	13.9	17.6	14.9	20.6	4.73
SEM	0.24	0.64	1.18	1.29	1.39	0.11
Contrast						
Control vs. supp	**	**	**	**	**	NS
Control vs. CO	*	**	**	**	NS	NS
Control vs. MP	NS	NS	**	**	**	NS
Control vs. COMP	**	**	**	**	*	NS
CO vs. MP	NS	**	NS	**	**	NS
CO (3%) vs. CO (6%)	NS	**	NS	**	**	NS
MP (2%) vs. MP4 (%)	NS	**	NS	**	NS	*

CO (3%) = Supplemented with 3% DM of coconut oil, CO (6%) = Supplemented with 6% DM of coconut oil, MP (2%) = Supplemented with 2% DM of mangosteen peel powder, MP (4%) = Supplemented with 4% DM of mangosteen peel powder; *p<0.05, **p<0.01, NS = Non Significant, SEM = Standard Error of the Mean

2007). Machmuller (2006) stated that the methane suppressing effect of coconut oil is directly inhibit rumen methanogenic archaea and may change their metabolic activity. Methane production was also decreased with MPP supplementation and agreed with Poungchompu *et al.* (2009) who found that methane emission was depressed by inclusion of MPP and soapberry fruit pellet in dairy heifer diet. In this study, there was no MPP effect on dry matter or organic matter disappearance thus, the methane suppressing-effects of saponins were presumably a direct action against rumen microbes involved in methane formation including methanogens and protozoa (Sliwinski *et al.*, 2002). Tavendale *et al.* (2005) proposed two mechanisms whereby condensed tannins reduced methane emissions from ruminants: indirectly through a reduction in fiber digestion which decreases H_2 production and directly through an inhibition of the growth of methanogens. Moreover, Guo *et al.* (2008) concluded saponin appeared to reduce methane production by inhibiting protozoa and presumably lowering methanogenic activity of protozoal associated methanogens. In contrast, Beauchemin *et al.* (2007) failed to reduce enteric methane emissions from growing cattle by feeding the dietary DM as quebracho tannin extract. Reduction of methane production by tannin or saponins containing plant could be due to both kind of plant and proportion that animal received.

Methanogen population: Methanogen population was not impacted by feed supplementation (p>0.05; Table 1) while methane production was decreased (p<0.05). However,

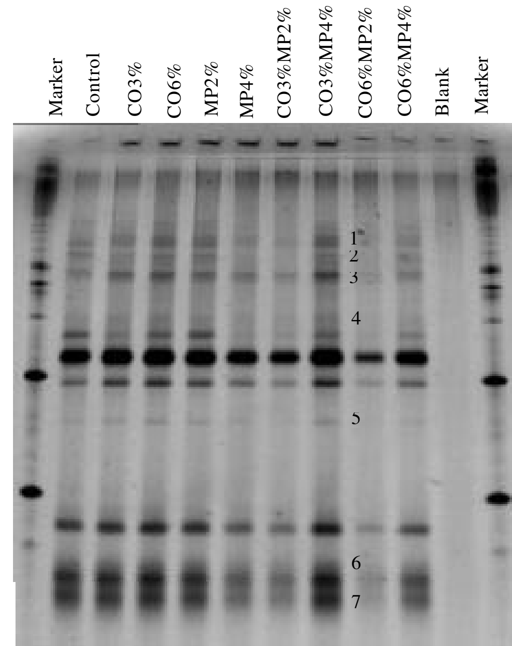

Fig. 1: Photographed gel after DGGE electrophoresis of methanogen 16s rDNA fragments from 9 treatments of rumen fluid. CO3% = supplementation of coconut oil at 3%, CO6% = supplementation of coconut oil at 6%, MP2% = supplementation of mangosteen peel powder at 2%, MP4% = supplementation of mangosteen peel powder at 2%

Soliva *et al.* (2003) reported that methane production and declined with an increasing proportion of luaric acid and confirmed the presumptions Dohme *et al.* (1999) who revealed that the methane suppressing effect of coconut oil was the results of a direct inhibition of ruminal methanogens. It indicated that feed supplementation likely effect on CO_2 or H_2, the precursor of methane synthesis or methanogenesis process greater than direct effect on methanogen population. The result analyzed by PCR-DGGE technique showed that the methanogen diversity appeared slightly differed among treatments especially when combinations of coconut oil and MPP were used (Fig. 1). There were seven difference bands separate from DGGE image was number of band was higher in supplementation of coconut oil than those in MPP groups. However, the UPGMA dendrogram show supplementation of coconut oil and MPP combination had more effect on gel profile than that single supplementation, especially combination of 6% of coconut oil with 2% of MPP which appeared lowest bane numbers (Fig. 2). However, coconut oil did not affect on DGGE band patterns information by Kongmun *et al.* (2011) and Hristov *et al.* (2009). This research found fifteen bands from DGGE gel image which were quite

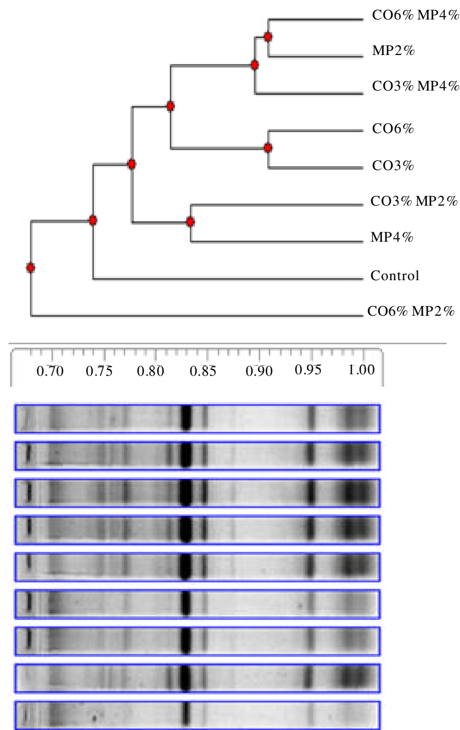

Fig. 2: The UPGMA dendrogram of methanogen 16sDNA fragments from 9 treatments of rumen fluid. CO3% = supplementation of coconut oil at 3%, CO6% = supplementation of coconut oil at 6%, MP2% = supplementation of mangosteen peel powder at 2%, MP4% = supplementation of mangosteen peel powder at 2%

similar with the research of Kongmun *et al*. (2011) who found almost thirteen separated bands from DGGE gel of rumen fluid from swamp buffaloes. However, earlier research of the team (Wanapat *et al*., 2011) found only seven separated bands in the rumen of the same animal species (*Bubalus bubalis*). On the other hand, it was found 9-10 bands in lactating cow (Hristov *et al*., 2009), 14 bands (4-9 bands per animal) in sheep (Wright *et al*., 2007) and 12-23 bands in feedlot cattle (Wright *et al*., 2008). However, increasing of MPP level (4% DM) resulted in inverse effect which presented more ban in the DGGE gels. This may cause by other composition in the MPP such as crude protein and fiber, nitrogen and carbon sources for microbe (Hungate, 1966). In addition, identification of methanogen species by gene sequencing technique could provide more useful information concerning the bacterial diversity in the rumen.

CONCLUSION

Coconut oil and mangosteen peel powder supplementation decreased methane production by changing methanogen population. Although, methanogen population was not changed, methanogen diversity was relatively changed by dietary supplementation. Combination of coconut oil and mangosteen peel powder had pronounced effect than those of single supplementation. Supplementation by high level of coconut oil or mangosteen peel powder did not show positive effects. Thus, appropriate level should not exceed 6% DM for coconut oil and 4% DM for mangosteen peel powder supplementations.

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