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REGULAR ARTICLES

Supplementation of *Flemingia macrophylla* and cassava foliage as a rumen enhancer on fermentation efficiency and estimated methane production in dairy steers

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Abstract Four rumen-fistulated dairy steers, 3 years old with 180 ± 15 kg body weight (BW), were randomly assigned according to a 4 × 4 Latin square design to investigate on the effect of *Flemingia macrophylla* hay meal (FMH) and cassava hay meal (CH) supplementation on rumen fermentation efficiency and estimated methane production. The treatments were as follows: T1 = non-supplement, T2 = CH supplementation at 150 g/head/day, T3 = FMH supplementation at 150 g/head/day, and T4 = CH + FMH supplementation at 75 and 75 g/head/day. All steers were fed rice straw ad libitum and concentrate was offered at 0.5 % of BW. Results revealed that supplementation of CH and/or FMH did not affect on feed intake ($P > 0.05$) while digestibility of crude protein and neutral detergent fiber were increased especially in steers receiving FMH and CH+FMH ($P < 0.05$). Ruminal pH, temperature, and blood urea nitrogen were similar among treatments while ammonia nitrogen was increased in all supplemented groups ($P < 0.05$). Furthermore, propionic acid (C3) was increased while acetic acid (C2), C2:C3 ratio, and estimated methane production were decreased by dietary treatments. Protozoa and fungi population were not affected by dietary supplement while viable bacteria count increased in steers receiving FMH. Supplementation of FMH and/or FMH+CH increased microbial crude protein and efficiency of microbial nitrogen supply. This study concluded FMH (150 g/head/day) and/or CH+FMH (75 and 75 g/head/day) supplementation could be used as a rumen enhancer for increasing nutrient digestibility, rumen fermentation efficiency, and microbial protein synthesis while decreasing estimated methane production without adverse effect on voluntary feed intake of dairy steers fed rice straw.

Keywords Cassava hay · Dairy steers · *Flemingia macrophylla* · Methane · Rice straw · Fermentation efficiency

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Introduction

Manipulation of rumen microbial ecosystem for enhancing fibrous feed digestibility, reducing methane (CH_4) emission, and nitrogen excretion by ruminants to improve their performance are some of the most important goals for animal nutritionists. Legumes provide high quality forages for animals with a positive effect on the environment due to reduction in the use of inorganic N fertilizer thanks to their N2 fixation ability (Lüscher et al. 2014). The presence of condensed tannins (CT) in legume, tree, and shrub foliage has been found to increase rumen fermentation efficiency and decrease CH_4 (Williamsa et al. 2011). Calabrò et al. (2011) and Guglielmelli et al. (2011) reported that if CT in the feed exceeded 6 % of dry matter (DM), feed intake and digestibility would be dramatically reduced. If CT level was between 2 and 4 % DM, it would help to protect protein from rumen digestion, thereby increasing bypass protein or rumen undegradable protein (RUP).

Cassava hay (CH) can be processed from whole crop of about 4 months, containing high level of CP (25 %) and CT (3–4 %). Ampapon et al. (2016) reported that supplementation of CH for buffaloes fed rice straw improved rumen ecology and increased fermentation end products and microbial protein synthesis while reducing protozoal populations and

methane production. On the other hand, *Flemingia* (*Flemingia macrophylla*) has a high protein at 25.8 % and CT at 5.8 % (Kang et al. 2016) with fresh edible biomass of 45 to 64 tons/ha/year (Binh et al. 1998) and is therefore of interest as a supplementary feed. Kang et al. (2016) reproted that supplementation of *Flemingia* hay meal (FMH) could enhance in vitro fermentation and reduce CH_4 production. However, the leaves are reported to contain a high proportion of tannins and are not preferred by livestock during the wet season although they are acceptable during the dry season, when alternative feeds are less available. Therefore, this study was to determine effects of CH and FMH supplementation on feed intake, nutrient digestibility, and rumen fermentation in dairy steers fed rice straw based diet.

Material and methods

Animal, diets, and experimental design

Four rumen-fistulated dairy steers (75 % Holstein Friesian × 25 % Thai native breed), 3 years old with 180 ± 15.0 kg body weight (BW) were randomly assigned according to a 4 × 4 Latin square design to receive four dietary treatments and were as follows: T1 = non-supplement, T2 = CH supplement at 150, T3 = FMH supplement at 150, and T4 = CH + FMH supplement at 75 and 75 g/head/day, respectively. All steers were fed rice straw ad libitum with concentrate mixture at 0.5 % BW twice per day at morning (07:00) and afternoon (16:00) feeding time. Feed ingredients and chemical compositions are illustrated in Table 1. The experiment was conducted for four periods and each period lasted for 21 days (14 days for feed adaptation and 7 days for sample collection).

Data collection, sampling procedures, and chemical analysis

Feeds offered and refusals were recorded daily the experimental period for dry matter (DM) intake measurement. Samples of feed and feces were collected daily during the last 7 days of each period using total collection method. All samples were divided into two parts; the first part was for analysis of DM (AOAC 2012) daily during the collection days and the second was kept in refrigerator and pooled by steer (feces) and by period (feed) at the end of each period. Samples were dried at 60 °C for 48 h and ground (1 mm screen using a Cyclotech Mill, Tecator, Sweden) for analysis of DM, crude protein (CP), ash, acid detergent fiber (ADF) (AOAC 2012), and neutral detergent fiber (NDF) (Van Soest et al. 1991). The sample of FMH and CH were analyzed for condensed tannins (CT) using the Vanillin-HCL method (Burns 1971; modified by Wanapat and Poungchompu 2001).

Ruminal pH and temperature were measured by using a portable pH temperature meter (HANNA Instruments HI 8424 microcomputer, Singapore) at 0, 2, 4, and 6 h post morning feeding through rumen fistula on the last day of each period. Approximate 200 ml of rumen fluid was taken from the middle part of the rumen by using a 60-ml hand syringe at each sampling time. Fluid samples were subsequently strained through four layers of cheesecloth and divided into three parts. The first 45 ml of rumen fluid sample was collected and kept in a plastic bottle to which 5 ml of 1 M H_2SO_4 were added to stop the fermentation process of microbe activity and then centrifuged at 3000×*g* for 10 min. About 20–30 ml of supernatant were collected and analyzed for volatile fatty acids (VFA) by high performance liquid chromatography (HPLC; Samuel et al. 1997) and ammonia nitrogen (NH_3-N; AOAC 2012). The second portion of 1 ml rumen fluid was collected and kept in a plastic bottle to which 9 ml of 10 % formalin solution (1:9 *v/v*,

Table 1 Feed ingredients and chemical compositions of dietary feed

Items	Concentrate	Cassava hay meal	*Flemingia* hay meal	Rice straw
Ingredients, % dry matter				
Cassava chip	69.0			
Rice bran	9.0			
Palm kernel meal	8.0			
Coconut meal	9.0			
Minerals	1.0			
Salt	1.0			
Molasses	3.0			
Sulfur	1.0			
Chemical composition, %				
Dry matter	88.1	89.9	94.2	91.5
% dry matter				
Organic matter	92.7	87.4	94.7	89.6
Crude protein	14.5	22.3	25.8	2.7
Neutral detergent fiber	30.2	45.9	53.1	85.1
Acid detergent fiber	15.1	29.2	31.1	45.8
Condensed tannin	–	4.6	5.8	–

rumen fluid/10 % formalin) was added and stored at 4 °C for measuring of microbial population (bacteria, fungal zoospores, and protozoa) using total direct count method according to Galyean (1989) by hemacytometer (Boeco, Singapore). The third portion (10 ml of fluid) was kept to study of viable bacteria counts (cellulolytic, proteolytic, amylolytic, and total viable bacteria) using roll-tube technique of Hungate (1969).

Blood samples (about 10 ml) were drawn from the jugular vein and kept into the tubes to which EDTA was added. Samples were centrifuged at 3500×*g* for 20 min and plasma was collected and stored at −20 °C for analysis of blood urea nitrogen (BUN) according to Crocker (1967).

Urine samples were collected using total collection method during the last 7 days by plastic container fixed with sulfuric acid (10 %) to protect nitrogen (N) loss. The urinary samples were analyzed for total N according to AOAC (2012) for determining N utilization. The allantoin in the urine was determined by HPLC as described by Chen et al. (1993). The amount of purines derivative (PD) absorption (X mmol/day) corresponding to the PD excretion (Y mmol/day) was calculated basing on the relationship derived by Chen and Gomes (1995): $Y = 0.85X + (0.385\ W^{0.75})$. The supply of microbial N (MN) was estimated by urinary excretion of PD according to Chen and Gomes (1995): MN (g/day) = 70X / (0.116 × 0.83 × 1000) = 0.727X; where X is PD absorption in mmol/day, digestibility of microbial purine is 0.83, the N content of purines is 70 mg N/mmol, and the ratio of purine-N:total N in mixed rumen microbes is 11.6:100. The efficiency of microbial N synthesis (EMNS) to denote the microbial N supplied to the animal per unit of digestible organic matter apparently fermented in the rumen (DOMR) was calculated using the following formula: EMNS=MN (g/day) / DOMR (assuming that rumen digestion was 650 g/kg OM of digestion in total tract, DOMR = DOMI × 0.65; DOMI = digestible organic matter intake).

Calculation of ruminal CH_4 production were estimated using VFA proportions according to Moss et al. (2000) as follows: CH_4 production = 0.45(acetate, C_2) − 0.275 (propionate, C_3) + 0.4 (butyrate, C_4).

Statistical analyses

All data were subjected to ANOVA according to a 4 × 4 Latin square design using the general linear models (GLM) procedures (SAS 2013). The results were presented as mean values with the standard error of the means. Differences between treatment means were determined by Duncan's new multiple range test (Steel and Torrie. 1980). Differences among the means with $P < 0.05$ were accepted as representing statistically significant differences.

Results and discussion

Feed intake and nutrient digestibility

In Table 2, feed intake and digestibility of DM, OM, and ADF were not affected by dietary supplements ($P > 0.05$) while digestibility of CP and NDF were increased ($P < 0.05$) in steers received FMH and/or CH+FMH. On the other hand, Fagundes et al. (2014) reported that feeding *Flemingia* foliage resulted in lower digestibility of goat. This could be explained by the high level of CT (10.5 %) in *Flemingia* foliage in the study of Fagundes et al. (2014) which was harvested at dry season compared to CT of FMH (5.8 %) in the present study. Calabrò et al. (2011) and Guglielmelli et al. (2011) reported that if CT in the feed exceeded 6 % of DM, feed intake and digestibility would be dramatically reduced while CT level between 2 and 4 % DM, it would help to protect protein from rumen digestion, thereby increasing bypass protein or rumen undegradable protein.

Table 2 Effect of legume foliage supplementation on feed intake and nutrient digestibility in dairy steers

Items	Control	CH	FMH	CH+FMH	SEM	*P* value
Total intake						
kg/day	2.4	2.5	2.5	2.7	0.06	0.08
% BW	1.5	1.6	1.7	1.6	0.06	0.14
g/kg $BW^{0.75}$	54.2	58.1	60.6	58.0	0.17	1.72
Rice straw intake						
kg/day	1.6	1.7	1.8	1.8	0.35	0.67
% BW	1.0	1.2	1.3	1.3	0.05	0.13
g/kg $BW^{0.75}$	36.4	40.5	43.3	40.1	1.81	0.14
Apparent digestibility, %						
Dry matter	60.8	60.0	62.2	63.5	3.28	0.08
Organic matter	63.2	67.2	66.5	67.0	3.95	0.54
Crude protein	61.1[a]	62.8[ab]	67.1[b]	65.2[c]	1.73	0.02
Neutral detergent fiber	46.0[a]	55.1[b]	57.7[b]	63.2[c]	2.62	0.05
Acid detergent fiber	42.2	44.3	50.7	53.4	4.24	0.11

[a, b, c] Means in the same row with different superscripts differed ($P < 0.05$)

CH cassava hay meal, *FMH Flemingia* hay meal, *SEM* standard error of the means

Table 3 Effect of legume foliage supplementation on fermentation characteristics, blood urea nitrogen, and methane production in dairy steers

Items	Control	CH	FMH	CH+FMH	SEM	*P* value
Ruminal pH	6.8	6.8	6.5	6.6	0.16	0.10
Temperature	38.8	38.9	38.6	38.9	0.05	0.26
Ammonia nitrogen, mg/dl	8.51[a]	9.06[b]	9.28[b]	9.12[b]	0.20	0.04
Blood urea nitrogent, mg/dl	10.2	9.5	10.8	10.4	0.42	0.86
Total volatile fatter acid, mmol/l	121.4	124.3	120.5	126.7	2.35	0.49
mol/100 mol						
Acetic acid (C_2)	68.7[a]	65.1[b]	64.8[ab]	62.2[b]	1.35	0.04
Propionic acid (C_3)	20.1[a]	25.4[b]	24.3[b]	27.6[b]	1.03	0.03
Butyric acid (C_4)	8.7	8.1	8.9	8.4	0.79	0.87
C_2:C_3	3.4[a]	2.6[b]	2.7[b]	2.3[b]	0.12	0.05
Estimated CH_4 production[d], mmol/l	22.0[a]	19.1[b]	18.8[b]	17.0[b]	0.29	0.02

[a, b, c] Means in the same row with different superscripts differed ($P < 0.05$)

[d] Calculated according to Moss et al. (2000): Methane = 0.45 (C_2) − 0.275 (C_3) + 0.4 (C_4)

CH cassava hay meal, *FMH Flemingia* hay meal, *SEM* standard error of the means

Rumen characteristic and blood metabolites

Table 3 shows that dietary treatment supplement did not change ruminal pH, temperature, and BUN ($P > 0.05$) while ruminal NH_3-N was increased ($P < 0.05$) in steers consumed FLM and/or CH. It was reported that CT in the diet was beneficial by providing protein-tannin complexation, decreasing of feed protein for ruminal degradation, and ammonia nitrogen release. In the present study, total VFA and butyric acid were similar among groups while C_2 was reduced and C_3 was increased in steers receiving CH and/or FLM supplement. Makkar et al. (1995) found that supplementation of CT improved C_3 and decreased C_2 concentration. Beauchemin et al. (2007) reported that supplementation of quebracho tannin extract did not change proportions of C_3, but decreased C_2 in cattle. In addition, Naumann et al. (2013) reported that effect of CT on C_2 inhibition could occur by decreasing the availability of nutrients to microorganisms in the rumen which resulted in decreasing of C_2/C_3 ratio resulting from an increased transfer of hydrogen to C_3 (Dschaak et al. 2011). Another possibility is that CT is hydrogen acceptors and reduces the amount of hydrogen available in the rumen.

Furthermore, estimated ruminal CH_4 production by equation of Moss et al. (2000) was decreased ($P < 0.05$) as a result of dietary supplementation. Moss et al. (2000) reported that prediction of CH_4 production by using a proportion of VFA could change in rumen ecology. In the rumen, during the process of fermentation, enteric CH_4 is produced from the disposal of metabolic hydrogen. Reducing equivalents that are not consumed during the formation of useful products such as VFA could be transformed into CH_4 representing a loss of as much as 15 % gross energy to the animal. Hess et al. (2003) suggested that the decline of CH_4 emission by diets including the *Calliandra* and *Flemingia* foliage in in vitro study was obtained. It has been suggested that many factors can affect such as type and dose of CT (Naumann et al. 2013), while CT may directly inhibit the growth of methanogen in the rumen. Inhibition of methanogenesis by CT may also result in decreasing the acetate to propionate ratio, resulting from an increased transfer of hydrogen to propionate (Dschaak et al. 2011). Another possibility of CH_4 production could be due to the decreasing protozoal population by CT supplement in the diet.

Rumen microorganisms population

Table 4 shows that bacterial direct counts was the highest in steers received CH+FMH ($P < 0.05$). On the other hand, protozoal populations was reduced by dietary treatments while fungal zoospores was similar among groups ($P > 0.05$). This might be due to CT in CH or FLM affected on cell membrane of protozoa probably because of the presence of cholesterol in eukaryotic cells membranes, but not in prokaryotic bacterial cells, as CT exhibit an affinity toward cholesterol (Klita et al. 1996). Poungchompu et al. (2009) found that dairy heifers fed with tannins or saponins had lower populations of protozoa and fungi

Table 4 Effect of legume foliage supplementation on rumen microbial population in dairy steers

Items	Control	CH	FMH	CH+FMH	SEM	*P* value
Ruminal microbes, cell/ml						
Bacteria, $\times 10^9$	2.7[a]	3.3[b]	3.7[b]	4.2[c]	0.64	0.02
Protozoa, $\times 10^5$	8.4[a]	5.1[b]	5.7[b]	5.3[b]	0.24	0.02
Fungi, $\times 10^5$	2.3	2.5	2.5	2.6	0.12	0.83
Viable bacteria, CFU/ml						
Amylolytic, $\times 10^8$	4.3	5.5	6.1	4.6	0.25	0.88
Proteolytic, $\times 10^8$	2.0	2.4	2.1	2.3	0.56	0.35
Cellulolytic, $\times 10^8$	7.2[a]	8.5[b]	9.8[c]	8.7[b]	1.45	0.02
Total, $\times 10^9$	5.0[a]	5.6[b]	6.1[b]	6.5[b]	1.34	0.04

[a, b, c] Means in the same row with different superscripts differed ($P < 0.05$)

CH cassava hay meal, *FMH Flemingia* hay meal, *SEM* standard error of the means

Table 5 Effect of legume foliage supplementation on urinary purine derivatives and microbial nitrogen supply in dairy steers

Items	Control	CH	FMH	CH+FMH	SEM	*P* value
Purine derivatives, mmol/day						
Allantoin excretion	24.5	27.4	26.5	31.3	0.63	0.24
Allantoin absorption	75.6	88.1	87.4	98.7	0.16	0.91
MCP, g/day	325[a]	387[a]	452[b]	482[b]	1.14	0.02
EMNS, g/kg OMDR	23.1[a]	25.4[a]	28.7[b]	30.2[b]	2.56	0.04

[a, b] Means in the same row with different superscripts differ ($P < 0.05$)

CH cassava hay meal, *FMH Flemingia* hay meal, *SEM* standard error of the means, *MCP* microbial crude protein, *EMNS* efficiency of microbial N supply, *OMRD* organic matter digested in the rumen

compared to non-supplemented. Moreover, supplementation with FMH resulted in the highest population of cellulolytic bacteria ($P < 0.05$) while amylolytic and proteolytic bacteria were unchanged. In contrast, Hess et al. (2003) found that supplementation of saponin-rich fruits did not change total bacterial counts. Inhibitory activity of tannins against bacteria has been implicated owing to the ability of tannins to form complexes with the cell wall and membrane of bacteria causing morphological changes of the cell wall and the extracellular enzymes secreted.

Purine derivatives and efficiency of microbial protein synthesis

The excretions and absorption of allantoin in urine were not affected by dietary supplement ($P > 0.05$) while microbial crude protein (MCP) and EMNS were increased by FLM and FLM+CH supplement ($P < 0.05$; Table 5) which was also confirmed by Cutrignelli et al. (2007). However, McSweeney et al. (2001) reported that MPS did not change by supplementation of tropical forage *Calliandra*-containing CT in sheep. McNeill et al. (1998) also found that microbial flow from the rumen was not inhibited by CT presented in *Leucaena leucocephala* (73 g/kg CT). In addition, Newbold et al. (1997) reported that suppression or elimination of protozoa may enhance the flow of microbial protein from the rumen, increase the efficiency of feed utilization, and improve the nutrition of the animals which could confirm to the present result that protozoa population in supplemented steer were lower than non-supplemented.

Conclusions and recommendations

This study concluded that supplementation with FMH and CH could increase nutrient digestibility, rumen fermentation efficiency, and microbial protein synthesis, while decreased estimated methane production. It is suggested that supplementation of FMH (150 g/head/day) and/or CH+FMH (75 and 75 g/head/day) could be used as a rumen enhancer without adverse effect on voluntary feed intake of dairy steers fed rice straw.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Informed consent Informed consent was obtained from all individual participants included in the study.

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