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Coconut and to a lesser extent krabok oil, depress rumen protozoa in beef cows

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Abstract

Krabok and coconut oil were assessed for their ability to affect rumen protozoa via a 3×3 Latin square design with three rumen cannulated beef cows. The diets consisted of a TMR supplemented with either 25.5 g/kg of tallow (control) or the same quantity of coconut oil or krabok oil. The animals were fed restricted amounts (DM) of the experimental rations (1.5% of body weight per d) for 28 d per period. The samples of rumen fluid were collected on day 23 and 27 of each period, 0, 3, 6, 9 and 12h after morning feeding. Protozoa numbers decreased by 0.33 log units in the coconut ($P<0.05$) and 0.21 log units in the krabok oil diet ($P<0.05$) compared with the control diet. The ciliate value was not significantly different between treatment but were strongly (R^2 = 0.88) linearly associated with protozoa counts. The concentration of total VFA was not affected (P = 0.804) by the diet. Except propionic acid which showed a trend (P = 0.056), the proportions of the remaining VFA were not significantly ($P>0.10$) different between treatments. The propionate proportion was only reduced by supplementation of coconut oil to the TMR. Neither oils affected amylolytic, cellulolytic or proteolytic bacteria counts. Cluster analysis of the DGGE profile showed two clusters of ciliate communities, one including all the T diet-fed animals. All except one DGGE profile of a cow fed the KO diet group into the second cluster. Coconut oil, and to a lesser extent krabok oil, has a marked effect on the numbers of rumen protozoa.

Keywords: krabok oil, coconut oil, rumen protozoa, ciliate

Introduction

Methane produced during anaerobic fermentation in the rumen represents 2 to 12% gross energy loss to the host animal and contributes 15-20% to the global production of methane (Lila et al., 2003; Asanuma et al., 1999). Methane is produced by Archaea and they exist both as free-living organisms and in a symbiotic relationship with rumen protozoa (Finlay et al., 1994). The latter explains, at least partly, why defaunation of the rumen content is beneficial to mitigate methane emission (Hook et al., 2010). The defaunating effect of lipids depends on its fatty acid composition with medium chain fatty acids being more effective than polyunsaturated fatty acids in controlling the protozoal numbers (Guyader et al., 2014).

Krabok oil is derived from krabok seeds (*Irvingia malayana* Oliv. ex w.Benn) and contains roughly equal amounts of C12:0 and C14:0, i.e. 45% of total fatty acids (Wongsuthavas et al., 2007; Panyakaew et al., 2013a). Previously, Panyakaew et al. (2013a) have shown that, under *in vitro* conditions, krabok oil reduced the production of methane. The mode of action of krabok oil on methanogenesis was, however, not clear (Panyakaew et al., 2013a). In a subsequent *in vivo* study, Panyakaew et al. (2013b) demonstrated that krabok oil *versus* tallow reduced the log copy numbers of protozoa by 32.2%. This result can be interpreted in that the krabok oil reduced methanogenesis attributed, at least partly, to its antiprotozoal effect. Currently, the study of Panyakaew et al. (2013b) is the only study reporting on the defaunating action of krabok oil under *in vivo* conditions and thus

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the aforementioned result needs confirmation. Therefore, in the current study we focus on the potential of krabok oil to reduce the numbers of the ciliate protozoa. Coconut oil, which is particularly rich in lauric (C12:0) and, to a lesser extent, myristic (C14:0) acid, was used as a positive control because it is well known for its methane-suppressing activity (Dohme et al., 1999, Jordan et al., 2006, Machmüller and Kreuzer, 1999, Panyakaew et al., 2013b).

Materials and methods

Animals and experimental design

Three beef cows (Brahman x Thai native crossbreds) average body weight 429 ± 43 kg with permanent indwelling rumen cannulas were used in a 3×3 Latin square design study which was preceded by a 14-d pre-experimental adaptation period. The animals were housed under natural environmental conditions in individual pens (2×4 m^2) with 50% of the floor covered by concrete and the other half by sand. The study was conducted at department of Animal Science, Faculty of Natural Resources, Rajamangala University of technology Isan, Sakon-Nakhon campus. Sakon Nakhon, in the northeast and plateau region of Thailand. Approximately 172 meters above sea level. During this period, the cows were fed a basal ration as a total mixed ration (TMR) consisting of (g/kg TMR): cassava chips, 421.0; rice straw 210.5; dry tomato pomace, 157.9; molasses, 73.7; rice bran, 52.6; soya bean meal, 31.6; urea, 21.0; salt, 10.5; di-calcium phosphate, 7.4; oyster meal, 5.3; mineral premix, 5.3; and sulfur, 3.2. Each experimental period lasted 28 d with cows randomly assigned to each sequence of feeding on the three experimental rations (Table 1). Animals had *ad-libitum* access to water during the pre- and experimental periods and body weights were measured at the beginning of each experiment period.

Table 1. Sequence of experimental treatments in a 3×3 Latin square design experiment with 3 treatments (supplementation (25 g/kg DM) with either beef tallow, coconut oil or krabok oil) and 3 fistulated beef cows (B) in each of the three periods, the amount of oil/fat added to each treatment and the supply of total medium chain fatty acids (C10:0+C12:0+C14:0).

Parameter	Tallow	Coconut oil	Krabok oil
Period			
1	B1	B2	B3
2	B2	B3	B1
3	B3	B1	B2
Fat/oil source (g/kg DM)			
Tallow	25	0	6.3
Coconut oil	0	25	0
Krabok oil	0	0	19.2
Fatty acids (g/kg DM)*			
C10:0	0.02 ± 0.00	1.10 ± 0.58	0.44 ± 0.13
C12:0	0.03 ± 0.01	11.0 ± 0.38	8.85 ± 0.70
C14:0	0.89 ± 0.08	4.61 ± 0.45	8.53 ± 0.50
Total C10:0+C12:0+C14:0	0.94 ± 0.07	16.7 ± 0.55	17.8 ± 0.36
C12:0 to C14:0 ratio	0.04 ± 0.01	2.40 ± 0.23	1.04 ± 0.14

*average ± standard deviation of four batches.

Experimental rations

The three isolipidic experimental rations were a TMR with either: (1) 25.5 g/kg DM tallow (T), (2) 25.5 g/kg DM coconut oil (CO), or (3) 19.2 g/kg DM krabok oil +6.3 g/kg DM tallow (KO). Treatment 3 was designed to provide similar amounts of MCFA compared to treatment CO (Table 1). Besides C12:0 and C14:0, C10:0 was also balanced between the experimental ration because the latter has been shown to effectively reduce methane production and/or the number of protozoa and methanogens (Dohme et al., 2001, Goel et al., 2009). Dietary inclusion of oil supplements in the KO treatment was based on fatty acid compositions of the oils used in the previous experiments of Panyakaew et al. (2013a,b). All the oils were from the same batch as the latter study but were stored in airtight containers for 3 months longer. The fatty acid composition of the oil/fat is provided in Table 2. The animals were fed restricted amounts (DM) of the experimental rations (1.5% of body weight) to ensure a constant intake of non-variable nutrients. The rations were offered daily in two equal portions at 07:30 and 16:00h, and feed refusals, if any, were recorded.

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Table 2. Fatty acid composition (g/100 g fatty acids) of the coconut oil, krabok oil, tallow and total mixed ration (TMR).

Fatty acid	Tallow	Coconut oil	Krabok oil	TMR
C8:0	0.01	0.21	0.00	0.00
C10:0	0.05	2.10	1.58	0.28
C12:0	0.12	45.8	42.0	0.12
C14:0	3.97	20.5	46.4	0.38
C16:0	26.8	11.1	4.49	15.7
C18:0	25.7	3.22	0.41	6.42
C18:1 c9	23.5	13.1	2.57	25.9
C18:1 c11	1.16	0.42	0.43	0.96
C18:2 n-6	0.53	2.63	0.32	42.2
C18:3 n-3	0.39	0.02	0.04	0.00
C18:2 c9t11	0.37	-	-	0.23

Rumen sample collection

Rumen contents (~200 ml) were collected on day 23 and 27 of each period at 0, 3, 6, 9 and 12h after the morning feeding by sampling in different directions of the rumen. After thorough mixing, the samples were divided into three portions: the first aliquot of ~10 ml was stored at -80 °C until DNA extraction; the second aliquot of ~10 ml was acidified with 0.2 ml phosphoric:formic (10:1) and stored at -20 °C until analysis of volatile fatty acids (VFA). The third aliquot of ~1 ml was fixed with 10% formalin saline solution (37% to 40% formalin in 0.9% (w/v) normal saline solution, 1:9) and stored at 4 °C for microscopical protozoa counting. For the VFA analysis, acidified rumen fluid samples were mixed per day (0, 3, 6, 9 and 12h) per cow. Rumen fluid samples from day 27 were pooled per cow before DNA extraction. Individual rumen fluid samples were used for protozoa and bacteria counts.

Volatile fatty acids

Samples for the analysis of VFA were pooled within a day per cow, and then centrifuged at 15,000×*g* (4 °C) for 10 min. Thereafter, the supernatant was filtered through a nylon membrane (0.2 µm) and transferred into vials. The VFA was analyzed by gas chromatography (Schimadzu GC-14A, Belgium) as described by Van Ranst et al. (2010).

Microbiota counts

Total protozoa were counted using a haemacytometer (Boeco, hamburg, Germany) of 0.1 mm depth and a microscope (Model Olympus BX50). Twenty aliquots per sample were counted (Galyean, 1989). The media of Hobson (1969) were used to determine amylolytic, cellulolytic and proteolytic bacteria groups using the roll tube method (Hungate, 1969)

Quantitative polymerase chain reaction (qPCR)

Ciliate protozoa and total bacterial rRNA gene copies present in DNA extract of each ruminal digesta sample were quantified as described by Boeckaert et al. (2008) and Boon et al. (2003). The QIAamp Stool Kit was used to extract total DNA from a 0.4 g rumen sample following the protocol for 'Isolation of DNA from the stool for Pathogen Detection' in the handbook supplied by Qiagen Ltd. (Crawley, UK), according to the manufacturer's recommendation. *Butyrivibrio* rRNA gene copies present in DNA extract of each sample were quantified using an ABI Prism SDS 7000 instrument (Applied Biosystems, Lennik, Belgium) following the principle of Boeckaert et al. (2007). Dilutions (1:20) of DNA from all samples were added to amplification reactions (25 µl) containing 12.5 µl SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK), 6 µl RNA free water, 0.75 µl B395f primer (10 µM stock), 0.75 µl B812r primer (10 µM stock) and 5 µl DNA. Cycling conditions were 1 cycle of 50 °C for 2 min and 95 °C for 10 min and 40 cycles of 95 °C for 1 min; 54 °C for 30 s and 60 °C for 1 min. Measurements were done in triplicate for each run. A standard curve for qPCR was constructed using six different DNA concentrations (n=3), ranging from 2.67 copies to 2.67 × 108 copies of DNA per µl. A *Butyrivibrio* 417 bp PCR fragment inserted in a TOPO vector was used as a template for the standard curve. The slope of the standard curve was -3.42 (R^2=0.99).

Diversity of ciliate protozoa

Total DNA was extracted from 0.5 g of rumen sample following the method of Boeckaert et al. (2007). A nested PCR approach was used to amplify a fragment of the 18S rRNA gene of ciliates for DGGE (Boeckaert et al., 2007). In the first PCR, the general eukaryotic primer Euk1A and the ciliate specific primer 539r were combined. In the second PCR run, the ciliate specific primer 316f and the general eukaryotic primer Euk516r-GC were used (applied from Yu and Morrison, 2004). By using this specific combination of primers, most of the degeneracies present in the ciliate specific primers

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were avoided for DGGE analysis. The final concentrations of the different components in the Master Mix were according to the manufacturer's instructions (Promega, Madison, USA) and contained 1 µl DNA extract (first run) or 1 µl of PCR product (second run), 1 µl of each primer (10 µM stock), 1 µl dNTP mix (0.2 mM), 10 µl GoTaq® Reaction buffer with 1.5 mM MgCl2 (1 x), 0.25 µl GoTaq® DNA polymerase (1.25 u) and DNase-, RNase-free filter-sterilized water (Sigma, Bornem, Belgium) to a final volume of 50 µl. Amplification conditions used were initial denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min, extension at 72 °C for 2 min; final extension at 72 °C for 12 min.

Denaturing gradient gel electrophoresis (DGGE) analysis

Ciliate DGGE analysis was performed using the Bio-Rad D gene system (Bio-Rad,hercules, CA). PCR fragments were loaded onto a 7% (w/v) polyacrylamide gel (77.8% acrylamide 40%, 22.2% bis-acrylamide 2%) in 1×TAE buffer (40 mM Tris, 20 mM acetate, 2 mM EDTA, pH 8.5) with denaturing gradient ranging from 40% to 60%. The electrophoresis was run for 16h at 60 °C and 45 V. DGGE patterns were visualized by staining with SYBR Green I nucleic acid gel stain (Molecular Probes, Eugene OR, USA). The obtained DGGE patterns were analysed with the BioNumerics software version 3.5 (Applied Maths, Kortrijk, Belgium). Similarities were calculated by the Pearson correlation, which takes into account band intensity and band position. The clustering algorithm of Ward was used to calculate dendrograms (Boeckaert et al., 2007).

Statistical analysis

Parameters were statistically analysed using a mixed model, with period and fat source (tallow, coconut, krabok oil) and cow as a fixed and random factor, respectively. Rumen fatty acid and qPCR data were analysed using the MIXED procedure using IBM SPSS Statistics 23 package. The model for the rumen fatty acid data included the fixed effect of day, time of sampling and their interaction and the random effect of cow assuming an autoregressive order one covariance structure fitted based on Akaike information and Schwarz Bayesian model fit criteria. The statistical model for qPCR data included the fixed effect of day and the random effect of cow assuming the covariance structure as described before. Least squares means are reported and significance was declared at $P<0.05$, and a trend at $0.05 \geq P<0.10$.

Results

Animals and feed intake

All the animals remained healthy throughout the experiment and consumed all their daily feed allocation (no feed refusals were collected). The average bodyweight of the animals for experimental period 1, 2 and 3 were 429, 415 and 422 kg, respectively.

Selected indices of rumen fermentation and protozoa counts

The concentration of total VFA (Table 3) was not affected ($P = 0.804$) by the diet. Except for propionic acid which showed a trend ($P = 0.056$), the proportions of the remaining VFA (Table 3) were not significantly ($P>0.05$) different between treatments. Also a trend ($P = 0.070$) was observed for the acetate to propionate ration to be lower after feeding of the TMR containing coconut oil compared to the other two diet. The KO diet had an acetate to propionate ration comparable to the T diet.

Total protozoa counts per unit rumen fluid were significantly ($P = 0.01$) affected by the dietary treatment. Compared to the T diet, the CO and KO diets had significantly reduced protozoa counts; 65% and 40%, respectively. Statistically significant differences, however, between the experimental rations could not be detected ($P = 0.448$) for the ciliates. There was however, a strong positive ($R^2 = 0.88$) linear ($y = 1.869x - 3.038$) association between the total protozoa counts (y) and ciliate numbers (x). There were no significant differences in the counts of amylolytic ($P = 0.472$), cellulolytic, ($P = 0.152$) and proteolytic ($P = 0.872$) bacteria (Table 3).

DGGE analysis

The DGGE profile of one sample of a cow fed the KO diet showed no ciliate communities to be present. The remaining eight DGGE profiles indicated two clusters of ciliate communities to be present (Figure 1). One cluster included all the T diet-fed animals while the other cluster contained all except one cow fed the KO diet.

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Table 3. Selected indices of rumen fermentation and protozoa counts after the feeding of the experimental rations.

Parameter	Experimental ration			SEM	P-value
	Tallow	Coconut oil	Krabok oil		
Total VFA (mmol/l)	70.4	71.9	73.3	3.1	0.804
Individual VFA (mol/100 mol)					
Acetic acid	66.7	62.8	64.9	1.2	0.131
Propionic acid	18.3$^{(b)}$	21.7$^{(a)}$	18.6$^{(b)}$	1.0	0.056
Butyric acid	11.9	12.6	13.8	0.9	0.343
Iso-valeric acid	0.20	0.17	0.15	0.30	0.563
Valeric acid	0.12	0.13	0.12	0.10	0.840
Acetate/propionate	3.70$^{(a)}$	3.00$^{(b)}$	3.54$^{(ab)}$	0.20	0.070
Microbiota					
Total protozoa (× 10^5 cell /ml)	5.09^{a}	1.80^{b}	3.04^{b}	0.38	0.010
Ciliates (log copy/g rumen fluid)	4.11	2.48	3.60	1.78	0.448
Amylolytic bacteria (× 10^7 cell/ml)	5.74	6.37	6.49	0.42	0.472
Cellulolytic bacteria (× 10^9 cell/ml)	6.73	7.28	7.69	0.27	0.152
Proteolytic bacteria (× 10^7 cell/ml)	4.87	5.54	5.74	0.36	0.872

[a,b]Treatment means within the same row with different superscript are significantly different or show a trend (superscript between brackets) to differ (P<0.1).
VFA=volatile fatty acids.

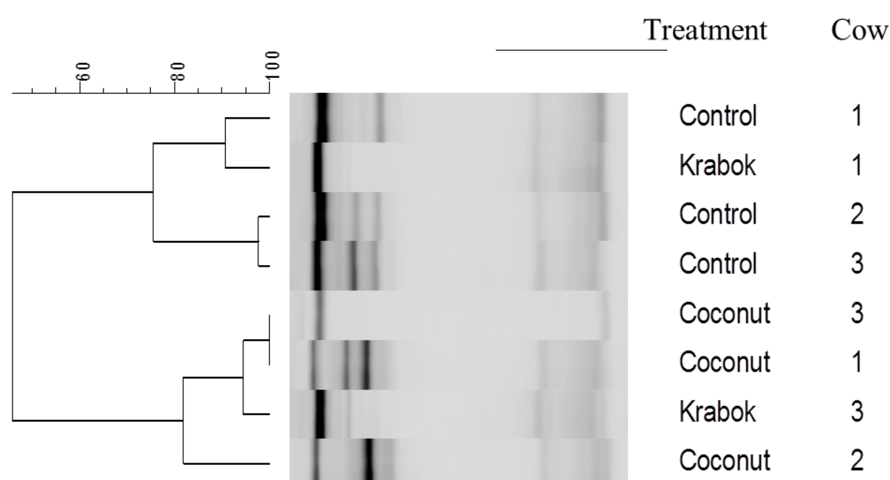

Figure 1. Cluster analysis of the of the denaturing gel electrophoresis profile of ciliate protozoa present in the rumen of beef cows fed a total mixed ration supplemented with either tallow (control), coconut oil or krabok oil+tallow.

Discussion

The present study indicates that coconut oil has a marked effect on numbers and the community of protozoa in the rumen. The number of protozoa decreased (P=0.010) by 65% and 40% in the CO and KO diet, respectively, when compared to the control. Similar effects of coconut oil and krabok oil have been reported by Panyakaew et al. (2013a), when higher amounts of C12:0+C14:0 (35 g/kg of supplemented fat) were fed to bulls. The latter authors found that the number of protozoa was decreased by 31 and 51%, respectively. Moreover, similar defaunating effects of coconut oil have been reported by Machmüller et al. (2003) and Machmüller (2006). A reduction in ciliate protozoa counts of 88 and 97% was reported by the latter author when 3.5 and 7.0% of coconut oil were added to a basal diet. An earlier study from the same group (Machmüller et al., 2003) reported a 65% decrease in ciliate protozoa numbers when sheep were fed with coconut oil instead of protected fat at 50 g/kg DM, double the amount used in the present study. Although protozoa numbers were decreased in the present study, ciliates were not affected (Table 3) due to high variability. However, there was a strong (R^2=0.88) positive linear association between total protozoa counts and the ciliate values.

The effect of coconut oil and krabok oil on the number of protozoa has been reported to be negatively correlated with propionate with krabok oil

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having more potential to reduce ruminal methanogenesis (Panyakaew et al., 2013b). In the present study, however, krabok versus coconut oil had a lesser potential to reduce the protozoa numbers, which agrees with the propionate levels being not affected by krabok oil. The difference in results between the two studies may be due to the difference in the dietary fat content which was ~ 30% lower the current study. The T diet in both studies yielded almost identical value for acetate and propionate concentration indicating that the differences were caused by coconut oil and krabok oil supplementation. The amounts of C12:0 and C14:0 supplemented in the present study were lower in both the C and KO diets indicating that reduction in the protozoa number is more sensitive in C12:0 than C14:0. This reasoning is in line with the *in vitro* results reported by Soliva et al. (1998). Moreover, the proportion of C12:0/C14:0 with twice of C (1:2.23) higher than KO (1:0.9) (calculated from table 2) diets in this studies, may cause of less effect of KO on the ciliate number and DGGE profile (Soliva et al., 2003; Dohme et al., 2001). From this result, it can be inferred that in practice a dose of, at least, 35 g of krabok oil per kg of diet should be used to reduced methane emission by beef cattle.

Unfortunately, one of the DGGE gels of a cow fed the KO diet did not yield valid results. The cluster analysis on the DGGE profiles of rumen ciliates showed two clusters of ciliate communities. One included all the T diet-fed animals and the other all the CO and KO diet-fed animals except one. The DGGE profiles indicate, therefore, that the supplementations of C12:0 + C14:0 affected ciliate communities. This is in line with the protozoa counts and the strong positive linear association between total protozoa counts and ciliate numbers. The DGGE profiles also corroborate the stronger effect of coconut oil compare to krabok oil on ciliate communities.

Conclusions

Krabok oil like coconut oil reduced the rumen protozoa population but both oils did not decrease ruminal ciliates numbers as measured by qPCR. The propionate proportion was only reduced by supplementation of coconut oil to the total mixed ration. Neither oils affected amylolytic, cellulolytic or proteolytic bacteria counts in the rumen fluid when supplementing the total mixed ration with 17 g/kg DM of total C10:0 + C12:0 + C14:0. Cluster analysis of the denaturing gel electrophoresis profile of ciliate communities showed a clustering of the coconut oil containing diet and the tallow containing diet. Coconut oil, and to a lesser extent krabok oil, affected the numbers of rumen protozoa.

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Animal Welfare Statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The authors confirm that they have followed EU standards for the protection of animals used for scientific purposes.

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