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Isolipidic replacement of krabok oil by whole krabok seed reduces in vitro methanogenesis, but negatively affects fermentation

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Abstract

The background of the current in vitro study involves the issue of methane (CH_4) production inherent to rumen fermentation. One of the dietary strategies to reduce enteric CH_4 production by ruminants involves the supplementation of medium-chain fatty acids in diets. As such, oils containing high amounts of MCFA, such as coconut, palm kernel and krabok oil, are of much interest to formulate energy efficient and environmentally friendly rations for ruminants. Krabok oil (KO) reduces methanogenesis, but the appropriate inclusion level of dietary KO is unclear. We therefore investigated the dose–response relationship between krabok oil and CH_4 production. In practice, the use of whole krabok seed (WKS), instead of KO, is easier, but the efficacy of WKS to inhibit methanogenesis was hitherto unknown. Thus, we also investigated whether WKS provides an alternative tool to inhibit CH_4 production. The experimental substrates contained either KO, WKS, the residue of WKS after fat extraction residue (FER) or FER + KO. Appropriate amounts of WKS or its derivatives were added to a basal substrate so as to attain either a low, medium or high content of KO, that is, 37–46, 90–94 and 146–153 g/kg dry matter respectively. The experimental substrates were formulated to keep the amounts of incubated fat-free OM, crude protein, neutral detergent fibre and acid detergent fibre constant in order to avoid biased results through potential differences in fermentability between WKS and its derivatives, and the basal substrate. The latter resembled the ingredient composition of a total mixed ration commonly used in Thai dairy cows. Fully automated gas production (GP) equipment was used to measure gas- and CH_4 production. Irrespective of the type of substrate ($p \geq .115$), both the absolute (ml/g fat-free OM) and relative (% of total GP) CH_4 production was reduced at the highest inclusion level of WKS or its derivatives ($p \leq .019$). Total GP (ml/g fat-free OM), however, was reduced after incubation of FER, FER + KO, and WKS, but not KO, at the highest inclusion level of the respective substrates ($p = .019$). Volatile fatty acids were likewise affected ($p \leq .001$). Krabok oil can inhibit CH_4 production but only when the dietary KO content is at least 9.4% (DM). Supplementation of KO in the form of WKS, however, is considered not opportune because the fat extracted residue of WKS is poorly degraded during fermentation.

KEYWORDS

in vitro, Krabok oil, krabok seed, medium-chain fatty acids, methane

1 | INTRODUCTION

Ruminants are considered important contributors to the emission of methane (CH_4) into the atmosphere (Moss, Jouany, & Newbold, 2000) and are, therefore, relevant targets to mitigate the emission of greenhouse gasses. Methane also represents a loss of energy otherwise available for intermediary metabolism of the animal (Mitsumori & Sun, 2008). Various strategies have been proposed to reduce enteric CH_4 production by ruminants (Boadi, Benchaar, Chiquette, & Masse, 2004; Morgavi, Forano, Martin, & Newbold, 2010), including the supplementation of medium-chain fatty acids in diets (MCFA,Dohme, Machmüller, Wasserfallen, & Krezer, 2000; Machmüller, 2006). As such, oils containing high amounts of MCFA, such as coconut, palm kernel and krabok oil, are of much interest to formulate energy efficient and environmentally friendly rations for ruminants.

Krabok seeds (*Irvingia malayana* Oliv.ex A. w. Benn) are widely available in South-East Asia and its oil contains approximately 40% lauric and 45% myristic acid (Panyakaew, Goel, Lourengo, Yuangklang, & Fievez, 2013a). Previous research has shown the potential of krabok oil (KO) to inhibit methanogenesis (Panyakaew, Goel, Yuangklang, et al., 2013b). However, the latter results were obtained under in vitro conditions using substrates containing 14.3% (DM basis) supplemental KO. Extrapolation of this in vitro condition (Panyakaew, Goel, Yuangklang, et al., 2013b) to practice may not be warranted because of economical reasons. The potential of KO, however, to reduce CH_4 production at lower levels of supplementation is hitherto unknown, but Panyakaew, Goel, Yuangklang, et al. (2013b) reported a KO induced reduction in *Archaea* numbers when the ration of bulls contained 3.5% supplemental KO. It can be speculated from this observation that CH_4 production also was affected at this low level of KO supplementation.

The use of whole krabok seed (WKS) instead of KO as a source of MCFA is preferred in the practice of ration formulation and preparation because it is more readily available, easier to use and economically more attractive. However, to the authors' knowledge, the potential of WKS instead of KO to reduce methanogenesis is currently unknown. The latter appears to be relevant because Martin, Rouel, Jouany, Doreau, and Chilliard (2008) showed that the inhibitory effect of linseed oil on absolute CH_4 production was 2.5 times greater compared to whole linseed. The main objectives of the current study were, therefore, to evaluate the effect of KO on CH_4 production at three different levels and to investigate the potential of WKS, instead of KO, to inhibit CH_4 production.

2 | MATERIALS AND METHODS

2.1 | Preparation of experimental substrates

Whole krabok seed was purchased from a local market (PuPan mountain) and ground at 0°C to pass a 1-mm screen, using a Willey mill (Peppink 100 AN). Thereafter, the ground WKS was thoroughly mixed and divided into two portions with one portion subjected to fat extraction to yield both krabok oil (KO) and fat extracted residue (FER). Fat was extracted from WKS by means of the Sohxlet method using hexane as a solvent (AOAC, 1990). Then, the basal substrate, WKS and FER were chemically analysed (Table 1), and appropriate amounts of KO, WKS and FER were added to a basal substrate to attain experimental substrates containing either a low, medium or high content of KO (Table 2), respectively, ranging from 37–46, 90–94 and 146–153 g/kg dry matter (DM).

The experimental substrates were formulated to keep the amounts of incubated fat-free organic matter (OM), crude protein (CP), neutral detergent fibre and acid detergent fibre constant within the three inclusion levels of FER, FER + KO and WKS and the basal substrate versus the three inclusion levels of KO (Table 2). The underlying rationale of the substrate formulations concern the fact that KO as such cannot be fermented and thus not yield any gas during fermentation. Moreover, addition of WKS increases the amount of, at least potentially, fermentable OM and thus causes, if any, greater GP and CH_4 production, thereby, hindering correct interpretation of the results. The latter is avoided when WKS, FER and FER + KO are compared. The ingredient composition of the basal substrate (Panyakaew, Goel, Lourengo, et al., 2013a) resembled that of a total mixed ration commonly used in Thai dairy cows and consisted of (g/kg fresh product): cassava chips, 421; rice straw, 211; dry tomato pomace, 158; molasses, 73.7; rice bran, 52.6; soybean meal, 31.6; urea, 21; salt, 10.5; di-calcium phosphate, 7.4; oysters meal, 5.3; mineral premix, 5.3; sulphur, 3.2.

2.2 | In vitro gas and CH_4 production

Gas production profiles of the experimental substrates were determined using fully automated GP equipment (Cone, Gelder, Visscher, & Oudshoorn, 1996) with GP being measured over 48 hr. Samples of each substrate were ground through a 1-mm sieve using a Wiley mill (Peppink 100AN, Olst, The Netherlands). Precise amounts (0.5 g) of each experimental substrate (Table 2) were weighed into 250 ml fermentation bottles (Schott). Each substrate was weighed in triplicate bottles. Bottles of blanks (rumen fluid without sample) were run in duplicate. Rumen fluid was obtained from two rumen cannulated Holstein-Friesian cows 2 hr after the morning feeding at 8:00 hr. Approximately 250 ml rumen fluid was collected from the front ventral, middle ventral and cranial dorsal sac from each individual cow. Then, the rumen fluid was pooled and filtered through cheesecloth and subsequently mixed (1:2 v/v) with an anaerobic buffer/mineral solution (Cone et al., 1996) under continuous flushing with CO_2. Prior to inoculation, the fermentation bottles were placed in a shaking water bath kept at 39°C and pre-flushed with CO_2. The bottles were then inoculated with 60 ml of buffered rumen fluid and connected to fully automated GP equipment. Ten µL of the headspace gas was collected from the bottles at distinct incubation times (0, 2, 4, 8, 12, 24, 30, 36 and 48 hr) and directly injected into a gas chromatograph to determine the CH_4 concentration (Pellikaan, Hendriks, Bongers, Becker, & Cone, 2011).

TABLE 1 Analysed composition of the feedstuffs used to formulate the experimental substrates[a]

	Basal substrate[b]	Whole krabok seed	Extracted residue[c]	Krabok oil
Dry matter (DM), g/kg	952	974	937	1,000
	g/kg DM			
Crude ash	107	21	38	–
Crude protein	183	133	246	–
Ether extract	54	564	190	1,000
Neutral detergent fibre	358	270	464	–
Acid detergent fibre	275	225	416	–
Acid detergent lignin	68	81	155	–
Selected fatty acids	mg/100 mg			
C10:0	0.02	2.68	2.47	2.61
C12:0	0.16	45.28	43.19	45.17
C14:0	0.46	42.67	41.85	43.24
C16:0	19.11	4.18	4.63	4.17
C18:0	5.47	0.37	0.43	0.37
C16:1 *(cis)*	0.31	0.75	1.19	0.71
C18:1 *(cis)*	25.31	2.64	4.20	2.32
C18:2, *n*−6 *(cis, cis)*	39.89	0.42	0.62	0.37
C18:3, *n*−3 *(cis, cis)*	1.67	0.05	0.07	0.11
Detected, not specified	3.74	0.60	0.94	0.53
Undetected	3.84	0.36	0.41	0.41

[a]Krabok oil was assumed to be 100% ether extract/dry matter.

[b]Basal substrate consisted of (g/kg fresh product): rice straw, 211; cassava, 421; tomato pomace, 158; molasses, 73.1; rice bran, 52.6; soybean meal, 31.6; urea, 21.0; salt, 10.5; Ca-phosphate, 7.4; oyster shells, 5.3; premix, 5.3; sulphur, 3.2.

[c]Residue after fat extraction (hexane) from whole krabok seed.

2.3 | Gas and CH_4 curve fitting

Cumulative gas and CH_4 production data were fitted using the model described by Cone et al. (1996) and Groot, Cone, Williams, Debersaque, and Lantinga (1996). The non-linear least squares regression procedure was used (SAS Institute Inc., 2010), and the data were fitted according to the following equation:

$$Y = \sum_{i=1}^{n} \frac{Ai}{1 + (Bi/t)^{Ci}},$$

where Y = cumulative gas or CH_4 production (mL/g fat free-OM incubated), n = total number of phases, i = number of phases, Ai = estimated asymptotic GP in phase i (mL/g of incubated fat-free OM), Bi = incubation time (h) where half of phase i gas or CH_4 production has been reached, Ci = sharpness of the switching characteristic for phase i, and t = time of incubation (h). Gas production was fitted using a tri-phasic model following the procedure as described by Cone, Gelder, and Driehuis (1997), where phases 1 and 2 are assumed to be related with the fermentation of the soluble and non-soluble fraction, respectively, while phase 3 is assumed to be related with microbial turnover (Cone et al., 1997). The time points related to the asymptotes of GP in phases 1, 2 and 3 (A1, A2 and A3 respectively) were set at 3 hr for A1, 17 hr later for A2 and 28 hr later (relative to A2) for A3 to enable the estimation of the parameters Bi and Ci, more easily, as described by Van Gelder et al. (2005).

Data on CH_4 production were fitted according to the model already described with n = 1. The cumulative amount of CH_4 produced was obtained as described in detail by Pellikaan et al. (2011). Briefly, CH_4 concentrations in the headspace in individual bottles were determined at specific time points. The cumulative CH_4 production was calculated as the sum of the headspace CH_4 concentration and the amount of CH_4 vented from the bottle between two successive CH_4 measurements.

The maximum rate of gas or CH_4 production (R_{max}, mL/g fat-free OM/h) and the time at which this maximum rate of gas or CH_4 production is reached (TR_{max}, h) were calculated using the following equations (Yang, Tamminga, Williams, Dijkstra, & Boer, 2005):

$$R_{max} = \frac{Ai \times Bi^{Ci} \times Ci \times (TR_{max})^{(-Ci-1)}}{\left[1 + Bi^{Ci} \times (TR_{max})^{-Ci}\right]^2},$$

$$TR_{max} = Bi \times \left[\frac{Ci-1}{Ci+1}\right]^{1/Ci},$$

where i, Ai, Bi and Ci are defined as previously described. If $Bi \leq 1$ then R_{max} occurs at t = 0 hr.

TABLE 2 Ingredient and analysed composition of the basal substrate supplemented with either whole krabok seed (WKS), krabok oil (KO), fat extracted residue of WKS (FER) or FER + KO

Inclusion level	Low				Medium				High			
Supplemental substrate	**FER**	**KO**	**FER + KO**	**WKS**	**FER**	**KO**	**FER + KO**	**WKS**	**RS**	**KO**	**FER + KO**	**WKS**
Incubated ingredients, mg dry matter (DM)[a]												
Basal	501.2	501.9	501.1	501.3	500.2	500.6	501.5	500.6	499.7	501.4	499.9	500.4
KO	–	24.2	16.2	–	–	51.7	46.7	–	–	89.1	86.0	–
FER	18.0	–	18.6	–	52.2	–	52.2	–	95.7	–	93.7	–
WKS	–	–	–	36.1	–	–	–	95.3	–	–	–	174.4
KO content, % of DM	0.7	4.6	3.7	3.8	1.8	9.4	9.4	9.0	3.1	15.1	15.3	14.6
Incubated macronutrients, mg												
Dry matter	519.2	526.1	535.9	537.4	552.4	552.3	600.4	595.9	595.4	590.5	679.6	674.8
Organic matter	465.2	472.6	481.8	483.3	497.1	499.0	545.0	540.6	538.6	537.1	622.8	617.9
Fat-free organic matter	434.9	421.6	435.2	436.0	460.4	420.4	461.5	460.0	493.6	421.2	492.2	492.6
Chemical composition												
EE, mg/g DM	58.4	97.1	86.8	87.8	66.4	142.3	139.1	135.3	75.6	196.4	192.2	185.5
C12:0, mg/g DM	2.8	19.8	15.7	16.4	7.4	40.3	40.2	38.9	12.6	64.8	65.1	62.8
C14:0, mg/g DM	2.8	19.1	15.2	15.6	7.3	38.7	38.7	36.8	12.3	62.2	62.6	59.3
CP, mg/g fat-free OM	221.4	218.2	221.5	221.8	227.0	218.4	227.1	227.0	233.4	218.2	233.0	233.3
NDF, mg/g fat-free OM	432.1	426.5	432.2	434.2	441.8	426.5	441.6	445.7	452.6	426.4	452.1	459.4
ADF, mg/g fat-free OM	333.6	326.9	333.9	334.4	345.4	327.1	345.4	345.4	358.6	326.9	358.0	358.7

Abbreviations: ADF, acid detergent fibre; CP, crude protein; DM, dry matter; EE, ether extract; NDF, neutral detergent fibre; OM, organic matter

[a]Basal substrate consisted of (g/kg fresh product): rice straw, 211; cassava, 421; tomato pomace, 158; molasses, 73.1; rice bran, 52.6; soybean meal, 31.6; urea, 21.0; salt, 10.5; Ca-phosphate, 7.4; oyster shells, 5.3; premix, 5.3; sulphur, 3.2.

2.4 | Chemical analysis

The DM content was determined after drying at 103°C overnight (ISO 6,496; ISO, 1999) and ash content after incineration for 3 hr at 550°C (ISO 5,984; ISO 2002). Nitrogen (N) was measured by the Kjeldahl method (AOAC, 1990) and a factor of 6.25 was used to convert N into CP. Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were analysed as per the standard methods, Van Soest, Robertson, and Lewis (1991) and Van Soest, (1973) respectively. Neutral detergent fibre was analysed after a pre-treatment with a heat-stable amylase. Acid detergent fibre (ADF) was analysed according to the method described by Van Soest et al. (1973). Both NDF and ADF are expressed exclusive of residual ash. Ether extract and fatty acids were analysed as described by Panyakaew, Goel, Lourengo, et al. (2013a). Volatile fatty acids (VFA) were analysed using gas chromatography (Thermo) equipped with a flame ionization detector with an HP-FFAP column (30 m × 0.53 mm i.d., 1.00 µm film thickness) (Agilent) using hydrogen as carrier gas (Dieho, Dijkstra, Schonewille, & Bannink, 2016).

2.5 | Calculations and statistical analysis

Branched-chain volatile fatty acids (BCVFA) in fermentation fluid were calculated as the sum of isobutyric acid and isovaleric acid. The non-glucogenic to glucogenic ratio (NGR) was also calculated (Ørskov, 1977):

$$NGR = (HAc + 2HB + HV) / (HP + HV),$$

where HAc is acetic acid, HB is butyric acid, HP is propionic acid and HV is valeric acid.

The effects of the various substrates on GP, kinetics and fermentation parameters were evaluated by subjecting the data to ANOVA (SAS Institute Inc., 2010) using the model:

$$Yij = \mu + S_i + IL_j + (S \times IL)_{ij} + e_{ij},$$

where Y_{ij} = response variable (e.g., CH_4, GP, kinetics and fermentation parameters), µ = overall mean, S_i = incubated type of supplemental substrate (i = FER, KO, FER + KO or WKS), IL_j = inclusion level of KS or KS derivatives (j = low, medium or high), $(S \times IL)_{ij}$ = interaction term between type of supplemental substrate and inclusion level of KS or KS derivatives and e_{ij} = residual error. Tukey's t test was used to identify treatments with different effects on the variable involved. Throughout, the level of statistical significance was declared at $p < .05$.

3 | RESULTS

The results of in vitro gas and CH_4 production and associated kinetic parameters are provided in Tables 3 and 4. Although being less relevant due to their impact on the gas and CH_4 production curves, the

TABLE 3 Main effects on in vitro gas production and associated kinetics parameters of a basal substrate supplemented with either whole krabok seed (WKS), krabok oil (KO), fat extracted residue of WKS (FER) or FER + KO at three inclusion levels

	Type of supplemental substrate (*S*)				Inclusion level (IL)				P-values		
Parameter	FER	KO	FER + KO	WKS	Low	Medium	High	*SEM*	*S*	IL	*S* × IL
GP48, ml/g fat-free OM	279.9^{ab}	293.8^{a}	255.3^{c}	267.6^{bc}	287.9^{d}	272.4^{de}	257.3^{e}	5.16	<0.001	0.004	0.019
A1, ml/g fat-free OM	53.5^{b}	65.5^{a}	53.8^{b}	62.1^{ab}	60.4	58.2	57.1	2.77	0.010	0.716	0.734
A2, ml/g fat-free OM	187.0^{b}	208.2^{a}	188.0^{b}	190.2^{b}	196.2	195.6	186.5	2.90	<0.001	0.117	0.005
B1, h	3.1^{a}	2.4^{ab}	2.7^{ab}	2.1^{b}	2.9	2.4	2.5	0.21	0.027	0.128	0.551
B2, h	6.8	7.5	7.2	7.1	7.2	6.9	7.5	0.38	0.655	0.342	0.545
C1	2.2	2.6	2.6	2.2	2.5	2.1	2.6	0.45	0.844	0.451	0.604
C2	2.3^{c}	2.4^{bc}	2.6^{a}	2.5^{ab}	2.3^{e}	2.5^{de}	2.6^{d}	0.06	0.001	0.003	0.061
R_{max1}, ml/g fat-free OM/h	12.2^{b}	21.5^{a}	15.7^{ab}	21.0^{a}	16.7	17.4	19.1	1.60	0.002	0.484	0.946
R_{max2}, ml/g fat-free OM/h	19.4	20.1	20.1	19.8	19.6	20.6	19.1	0.88	0.778	0.263	0.259
TR_{max1}, h	1.7	1.6	1.8	1.2	1.7	1.3	1.7	0.34	0.588	0.504	0.529
TR_{max2}, h	4.5	5.2	5.3	5.0	4.8	4.9	5.4	0.27	0.255	0.122	0.773

Note: ^{a,b,c,d} and ^{e} means within a row with different superscripts differ significantly within *S* and IL respectively. B1 and B2, incubation time at which half of maximum gas production has been formed in phase 1 and phase 2, respectively; C1 and C2, the sharpness of the switching characteristic for the profile in phase 1 and phase 2 respectively.

GP48, Cumulative gas production after 48 hr of incubation; A1 and A2, asymptote of gas production in phase 1 and phase 2 respectively; OM, organic matter; $R_{max}1$ and $R_{max}2$, maximum gas production rate in phase1 and phase2 respectively; $TR_{max}1$ and $TR_{max}2$, time occurrence of R_{max} in phase 1 and phase 2 respectively.

TABLE 4 Main effects on methane (CH_4) production and associated kinetics parameters of a basal substrate supplemented with either whole krabok seed (WKS), krabok oil (KO), fat extracted residue of WKS (FER) or FER + KO at three inclusion levels

	Type of supplemental substrate (S)				Inclusion level (IL)				P-value		
Parameter	FER	KO	FER + KO	WKS	Low	Medium	High	*SEM*	*S*	IL	*S* × IL
CH_4, ml/g fat-free OM	55.9	55.9	52.9	47.6	57.2^{d}	56.5^{d}	43.3^{e}	2.05	0.115	0.001	0.231
CH_4, % of total gas	20.1	19.2	20.6	17.8	20.0^{de}	20.8^{d}	17.1^{e}	0.80	0.133	0.019	0.146
A, ml/g fat-free OM	61.7^{a}	61.2^{ab}	57.1^{ab}	51.6^{b}	62.6^{d}	61.4^{d}	47.4^{e}	2.09	0.050	<0.001	0.208
B, h	8.6^{a}	7.4^{ab}	7.4^{ab}	6.3^{b}	7.9^{d}	7.9^{d}	6.1^{e}	0.33	0.004	0.006	0.149
C	1.4^{b}	1.6^{ab}	1.6^{ab}	1.8^{a}	1.5^{e}	1.5^{e}	1.8^{d}	0.05	0.013	0.002	0.110
R_{max}, ml/g fat-free OM/h	4.5^{b}	5.2^{a}	4.8^{ab}	5.1^{a}	5.0	4.8	5.0	0.12	0.001	0.186	0.079
TR_{max},h	2.4^{b}	2.7^{a}	2.8^{a}	2.8^{a}	2.5^{e}	2.7^{d}	2.8^{d}	0.05	<0.001	0.004	0.460

Note: ^{a,b,c} and ^{d} means within a row with different superscripts differ significantly within *S* and IL respectively.

A, asymptote of CH_4 production; B, incubation time at which half of maximum CH_4 production has been formed; C, the sharpness of the switching characteristic for the profile of CH_4 production; OM, organic matter; R_{max}, maximum rate of CH_4 production; TR_{max}, time occurrence of R_{max}.

values on the switching characteristics of the GP curves related to the soluble fraction (C1) and the non-soluble fraction (C2) and the C value related to the CH_4 curve are presented in the tables. The various C values are neither specifically addressed nor further discussed.

3.1 | Gas production

Except for the cumulative GP measured after 48 hr and its associated asymptote GP of the non-soluble fraction (A2), all other relevant kinetic parameters were not affected by *S* × IL ($p \geq .259$). Therefore, only the main effects of *S* and IL were presented in Table 3. The inclusion level of the various substrates influenced only GP 48-hr, but all other relevant kinetic parameters were found to be not significantly affected ($p \geq .122$).

GP 48-hr, expressed as ml gas/g fat-free OM, was affected by both type ($p < .001$) and inclusion level ($p = .004$) of supplemental FER, FER + KO or WKS. The lowest values were observed after incubation of the basal substrate containing either FER, FER + KO or WKS at the highest inclusion level with the respective substrates ($p = .019$) with the average GP 48-hr of 244 ml/g fat-free OM for the three substrates (i.e., FER, FER + KO, WKS; $p = .150$). This value was found to be 22.4% lower ($p \leq .037$) than the value found when only KO was added at the highest inclusion level to the basal substrate, that is, 315 ml/g fat-free OM. The latter value was not different ($p = .329$) to the GP 48-hr values measured at the lowest inclusion level of the experimental substrates, irrespective ($p = .149$) whether FER, KO, FER + KO or WKS was incubated. The latter results already implicate that the supplementation of only KO to the basal substrate did not affect GP 48-hr, that is, the dose of supplemental KO explained only ~1% of the variation in GP-48h ($p = .315$).

The asymptote of GP of the soluble fraction (A1) was different between the experimental substrates ($p = .010$), and the lowest values were observed after the incubation with supplemental FER and FER + KO. The associated half-time of A1 was likewise affected ($p = .027$) and was prolonged when basal substrate containing FER and FER + KO were incubated. These observations are in line with the values on the rate of maximum GP of the soluble fraction (Rmax1), but its associated time occurrence was found to be not different ($p = .529$) between treatments.

The asymptote GP of the non-soluble fraction (A2) differed significantly by the type of supplemental substrate ($p < .001$) and A2 was lowest when FER, FER + KO and WKS were incubated. For the latter three treatments combined, A2 was found to be 9.5% lower compared to KO. However, the depressant effect of either FER, FER + KO or WKS versus KO was 2.2 times greater ($p = .005$) at the highest inclusion level. Neither the half-time of the asymptote GP of the non-soluble fraction (B2) nor the associated values on maximum rate of GP (*R*max2) and its time occurrence (TRmax2) were influenced by any treatment ($p \geq .255$).

3.2 | Methane production

Methane production and associated kinetic parameters (Table 4) were not affected by *S* × IL ($p \geq .079$). Irrespective of the type of supplemental substrate ($p \geq .115$), both absolute (ml/g fat-free OM) and relative (% of total GP) CH_4 production were found to be reduced at the highest inclusion level of the supplemented substrates ($p \leq .019$). The asymptotic CH_4 production (A) was found to be lowest on the highest inclusion level of any experimental substrate ($p < .001$). The incubation of supplemental WKS versus FER resulted in a 16.4% lower A value ($p = .050$). Values for the half-time of the asymptotic CH_4 production (B) were affected in a similar manner as the asymptotic CH_4 production ($p \leq .006$). The maximum rate of CH_4 production (R_{max}) was only affected by the type of substrate, and the

lowest value was found when the experimental substrate contained FER (p = .001). The time occurrence of the maximum rate of CH_4 production was significantly affected by the type of supplemental substrate and inclusion level, but the differences were small and the relevancy of such differences can be disputed.

3.3 | Volatile fatty acids

The concentration of total VFA, as well as total VFA expressed as μmol/mg fat-free OM, was influenced (p < .001) by inclusion level x type of substrate (Table 5). At the lowest inclusion level, VFA concentrations expressed as μmol/mg fat-free OM were similar between the experimental substrates but VFA values decreased with increasing inclusion levels of FER, FER + KO and WKS (p ≤ .001). The proportion of HAc was neither affected by inclusion level x type of substrate (p = .383) nor by type of supplemental substrate (p = .158). In contrast, the lowest proportions of HAc were found at the highest inclusion level of the experimental substrates (p < .001), while group mean values of the HP proportions were found to be reciprocal of HAc (R^2 = 0.937, p < .001). Consequently, HAc/HP ratio was significantly affected by the experimental treatments and the lowest values were found when FER + KO or WKS were incubated at the highest inclusion level (p = .017). The proportions of BCFA and NGR were likewise affected (p ≤ .001). Upon ANOVA, both *S* and IL significantly affected the proportion of butyrate, but the differences were found to be quite small and can be considered negligible.

4 | DISCUSSION

4.1 | Dose of krabok oil and CH_4 production

Methane production reduced with increasing levels of WKS and its associated derivatives and thus with increasing levels of KO (Table 2). Regressing CH_4 production against the content of dietary KO (Figure 1) indicated a quadratic dose–response relationship (p ≤ .008). The response of KO on CH_4 production is in line with Panyakaew, Goel, Lourengo, et al. (2013a) who also demonstrated a KO-induced CH_4 mitigating effect in case the incubated substrate contained 14% (DM) supplemental KO. On the other hand, the current observation is not in line with the idea that 4.6% (DM) supplemental KO inhibits CH_4 production and this appears to be in contrast with the observation of Panyakaew, Goel, Yuangklang, et al. (2013b) who found a KO-induced reduction in *Archaea* numbers when the ration of bulls contained 3.5% supplemental KO. The results of Panyakaew, Goel, Yuangklang, et al. (2013b) can be interpreted in that a dose of 3.5% supplemental KO would effectively reduce CH_4 production. The apparent discrepancy in results

TABLE 5 Selected indices of in vitro fermentation of a basal substrate supplemented with either whole krabok seed (WKS), krabok oil (KO), fat extracted residue of WKS (FER) or FER + KO at three inclusion levels

		Total VFA		Individual VFA (mol/100 mol)					
Inclusion level	Substrate	mM	μmol/mg fat-free OM	Acetic acid	Propionic acid	Butyric acid	BCVFA	AP	NGR
Low	FER	94.9^{ab}	13.1^{a}	64.5^{ab}	18.7^{cd}	12.3^{b}	2.9^{a}	3.4^{ab}	4.5^{ab}
	KO	92.4^{bcde}	13.1^{a}	64.2^{ab}	18.5^{cd}	12.9^{ab}	2.8^{abc}	3.5^{ab}	4.6^{ab}
	FER + KO	94.9^{abc}	13.1^{a}	64.3^{ab}	18.5^{cd}	12.8^{ab}	2.8^{ab}	3.5^{ab}	4.6^{ab}
	WKS	93.2^{bcd}	12.8^{ab}	64.7^{a}	18.3^{d}	12.6^{ab}	2.8^{abc}	3.5^{a}	4.6^{a}
Medium	FER	96.8^{a}	12.6^{b}	63.8^{abc}	19.0b^{cd}	12.7^{ab}	2.9^{a}	3.4^{abc}	4.4^{abc}
	KO	90.8^{de}	13.0^{ab}	63.8^{abc}	18.8^{cd}	13.0^{a}	2.8^{abcd}	3.4^{abc}	4.5^{ab}
	FER + KO	91.9^{cde}	11.9^{c}	63.6^{abc}	19.1^{bcd}	13.0^{ab}	2.7^{bcd}	3.3^{abc}	4.4^{abc}
	WKS	91.7^{de}	12.0^{c}	63.8^{abc}	19.0^{bcd}	12.9^{ab}	2.6^{cd}	3.4^{abc}	4.4^{abc}
High	FER	96.6^{a}	11.7^{c}	64.0^{abc}	19.0^{bcd}	12.6^{ab}	2.9^{ab}	3.4^{abc}	4.4^{abc}
	KO	91.3^{de}	13.1^{a}	63.2^{abc}	19.5^{bc}	13.0^{a}	2.6^{de}	3.2^{bcd}	4.3^{bc}
	FER + KO	90.7^{de}	11.1^{d}	62.5^{c}	20.8^{a}	12.6^{ab}	2.4^{e}	3.0^{d}	4.0^{d}
	WKS	89.8^{e}	10.9^{d}	63.1^{bc}	20.0^{ab}	12.8^{ab}	2.4^{e}	3.2^{cd}	4.2^{cd}
SEM		0.60	0.07	0.32	0.21	0.14	0.04	0.05	0.05
P-value	*S*	<0.001	<0.001	0.158	0.014	0.005	<0.001	0.051	0.024
	IL	0.001	<0.001	<0.001	<0.001	0.057	<0.001	<0.001	<0.001
	IL × *S*	0.001	<0.001	0.383	0.002	0.641	0.004	0.017	0.002

Note: ^{a,b,c} and ^{d} means within a column with different superscripts differ significantly.

Abbreviations: AP, acetate-to-propionate ratio; BCVFA, branched-chain VFA; NGR, non-glucogenic to glucogenic ratio; OM, organic matter; VFA, volatile fatty acids.

cannot be easily explained by the type of basal substrate because the ingredient composition of the ration provided to the bulls (Panyakaew, Goel, Yuangklang, et al., 2013b) was identical to that of the basal substrate used in the current study. Unfortunately, actual CH_4 production was not measured in the study of Panyakaew, Goel, Yuangklang, et al. (2013b) and this lack of information hinders further interpretation. Nevertheless, it appears that KO might reduce CH_4 production only when the substrate contains > 9.4% KO, at least under in vitro conditions. Needless to say that further research is warranted to substantiate our observations on the relationship between supplemental KO and CH_4 production.

The VFA pattern shifted from HAc to HP with increasing IL of WKS and its associated derivatives. This observation is in line with results from our previous study, showing that the anti-methanogenic effect of MCFA was accompanied by a dose-dependant shift in VFA from HAc to HP (Panyakaew, Goel, Yuangklang, et al., 2013b). Such a shift in VFA pattern is in line with the synthesis of CH_4 being prohibited because the synthesis of HP provides an alternative sink for hydrogen (McAllister & Newbold, 2008).

4.2 | Physical form of krabok oil

The results indicate that the physical form of KO does not affect CH_4 production. In other words, free KO and WKS were potentially equally effective to reduce CH_4 production. This result appears to be in contrast with that of Martin et al. (2008) who demonstrated that linseed oil was superior over crude linseed in inhibiting CH_4 production. An unambiquous explanation for the discrepancy in results is difficult to provide, but it may be related to the physical form of the oilseeds itself. Martin et al. (2008) used crude linseed in their in vivo experiment with dairy cows, while in the current in vitro study grinding of krabok seed was deemed necessary because of the experimental setup. It might, therefore, be speculated that the extent by which the oil was released from linseed (Martin et al., 2008) differed from that of the krabok seed used in the current study. In view of the current results on CH_4 production, it can be speculated that krabok oil was fully released from the ground krabok seed during the process of fermentation. Thus, at first sight, ground WKS appears to be an attractive tool to mitigate CH_4 production. However, FER, FER + KO and WKS, but not KO, caused a severe reduction in both GP 48-hr (Table 3) and total VFA (Table 5) at the highest inclusion level of the respective substrates. These observations can be interpreted in that the digestibility of the fat-free residue of krabok seed is relatively low. This is in line with the chemical composition of the FER of krabok seed used in the current study, that is (values expressed as g/kg fat-free OM): aNDFom, 600; ADFom, 538; ADL, 200; NSC, 81.[1] The ADFom values of FER are in the same range as those from straw of grains such as rice, barley, wheat and oats, but the lignin content is higher (Van Soest, 2006).

[1]NSC = Non-structural carbohydrates, calculated as: Organic matter – Crude protein – Ether extract –Neutral detergent fibre.

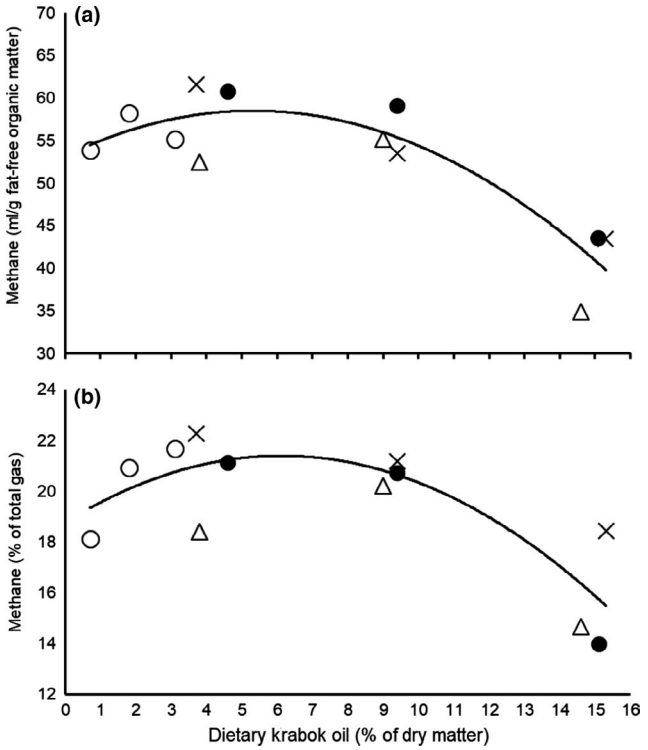

FIGURE 1 Dose–response relationship between dietary krabok oil and methane production either expressed as ml/g fat-free organic matter (Panel A: y = −0.19 x2 + 2.00 x + 53.21, R2adj = 73.6%, p = .001) or expressed as a % of total gas (Panel B: y = −0.07 x2 + 0.86 x + 18.79, R2adj = 58.7%, p = .008). The symbols indicate the four experimental substrates, that is, basal substrate containing either whole krabok seed (WKS, Δ), fat-free residue of WKS (FER, ○) or krabok oil (KO, ●) or FER + KO (×)

5 | CONCLUSION

The inclusion of krabok oil at a levels greater than 9.4% (DM) effectively reduces the formation of CH_4 during in vitro fermentation. The use of whole krabok seed instead of krabok oil, however, must be discouraged due to its high content of lignin and thus low fermentability.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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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