Dietary Medium-Chain Fatty Acids for Sustainable Ruminant Nutrition

Paiwan Panyakaew

Dietary Medium-Chain Fatty Acids for Sustainable Ruminant Nutrition

Paiwan Panyakaew

2024

ISBN:

Copyright © 2024 Paiwan Panyakaew
Printed by: Ratchayanyon, Sakon Nakhon, Thailand

The research described in this thesis was performed at the:

Department of Animal Science, Faculty of Natural Resources, Rajamangala University of Technology Isan, Sakon-Nakhon Campus, Sakon Nakhon, Thailand.

Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands.

Department of Animal Sciences, Wageningen University, Wageningen, The Netherlands.

Laboratory for Animal Nutrition and Animal Product Quality (Lanupro), Faculty of Bioscience Engineering, Ghent University, Melle, Belgium.

Laboratory of Microbial Ecology and Technology (LabMET), Faculty of Bioscience Engineering, Ghent University, Gent, Belgium.

Dietary Medium-Chain Fatty Acids for Sustainable Ruminant Nutrition

Middellange Keten Vetzuren in de Voeding van Herkauwers voor een Duurzame Productie

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de
Universiteit Utrecht
op gezag van de
Rector Magnificus, prof. dr. H.R.B.M. Kummeling,
ingevolge het besluit van het College voor Promoties
in het openbaar te verdedigen op

donderdag 30 mei 2024 des middags te 12.15 uur

door

Paiwan Panyakaew

geboren op 22 februari 1979,
te Sakon Nakhon (Thailand)

Promotor:

Prof. dr. W.H. Hendriks

Copromotoren:

Dr. J.T. Schonewille
Dr. Yuangklang

Beoordelingscommissie:

Prof. dr. J.A. Stegeman
Prof. dr. D. Salvatori
Prof. dr. H. Smidt
Dr. S. van Gastelen
Prof. dr. J.A. Wagenaar

This thesis was accomplished with financial support from the European Commission through the Asia-Link programme (Phase III) in cooperation with the Laboratory for Animal Nutrition and Animal Product Quality (Lanupro), the Faculty of Bioscience Engineering, Ghent University, Melle, Belgium and the Department of Animal Science, Faculty of Natural Resources, Rajamangala University of Technology Isan, Sakon-Nakhon Campus, Sakon Nakhon, Thailand. Lotus-Erasmus Mundus Action2 (EU-South-East Asian mobility project) for 10 months.

This thesis is dedicated to my late grandfather and grandmother,

my father and mother, my husband and daughter

with all love and memories

วิทยานิพนธ์นี้อุทิศให้กับปู่และย่าของฉันที่ล่วงลับไปแล้ว

พ่อและแม่ของฉัน สามีและลูกสาวของฉัน

ด้วยความรักและความทรงจำทั้งหมด

Contents

		Page
Chapter 1	General introduction	1
Chapter 2	Medium-chain fatty acids from coconut or krabok oil inhibit *in vitro* rumen methanogenesis and conversion of non-conjugated dienoic biohydrogenation intermediates	11
Chapter 3	Coconut and to a lesser extent krabok oil, depress rumen protozoa in beef cows	25
Chapter 4	Effect of supplementing coconut or krabok oil, rich in medium-chain fatty acids on ruminal fermentation, protozoa and archaeal population of bulls	37
Chapter 5	Replacing of krabok oil by whole krabok seed or krabok residue on the combination levels of fat in feed on *in vitro* methane production	55
Chapter 6	General discussion	71
	Summary	89
	Samenvatting	91
	Acknowledgements	95
	Curriculum vitae	97
	List of publications	99

Chapter 1
General introduction

Introduction

The background of the research described in the current thesis involves the environmental issue of methane (CH_4). Next to carbon dioxide, CH_4 is a well-known greenhouse gas (Figure 1) and this gas has been implicated to affect the temperature of both the earth's surface and the lower atmosphere (Schneider, 1998).

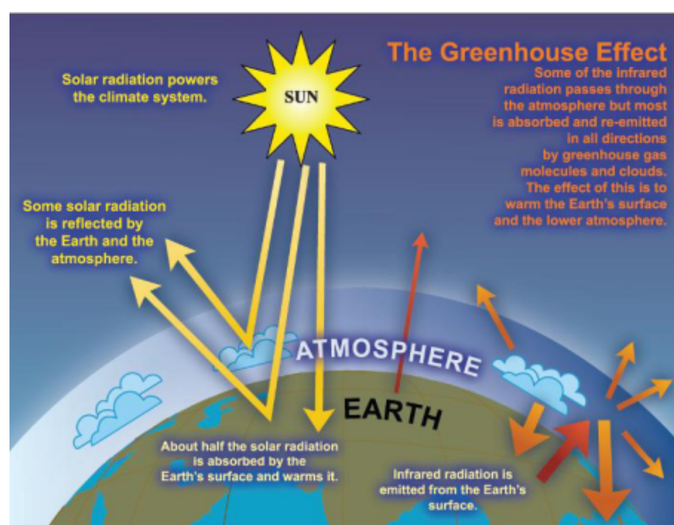


Figure 1. A simplified representation of the greenhouse effect (Le Treut et al., 2007).

Table 1. Overview of natural and anthropogenic sources of methane (Denman et al., 2007; Thorpe, 2009).

Natural sources	Anthropogenic sources
Marshes	Coal mines
Termites	Fossil fuels
Oceans	Oil and industry
CH_4 hydrates	Landfills and waste
Geological sources[1]	Animal production[2]
Wildlife	Rice production
Natural fires	Combustion of biomass

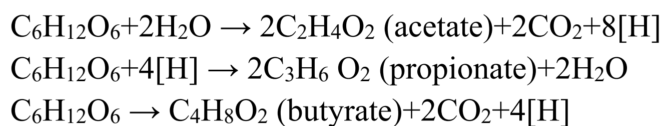
[1]Sources such as fossil methane to the release of natural gas from sedimentary basins and due to geothermal/volcanic activity.
[2]Methane emission by ruminants and manure.

There are both natural and anthropogenic sources of CH_4 (Table 1), and it has been estimated by Milich (1999) that the contribution of anthropogenic sources is two times greater than that of natural sources.
Methane production by ruminants has been estimated to be 86 million metric ton (Tg)/year (McMichael et al., 2007) which is 17 to 37% of the total amount of CH_4 that originates from anthropogenic sources (Lassey, 2008). The global demand of milk is currently increasing and may be doubled in 2050 (FAO, 2008). In view of the ecological footprint of dairy production, it is, therefore, of great interest to reduce CH_4 emission associated with milk (and meat)

production. Reduction of CH_4 formation during the digestion of feed in the rumen coincides also with an improved feed conversion efficiency because enteric CH_4 also constitutes a loss of dietary energy to the animal (Johnson and Johnson, 1995). Thus, the economical relevance of CH_4 mitigation as well, cannot be ignored.

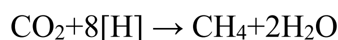
Rumen fermentation

Ruminants host a wide array of micro-organisms in their rumen which enables ruminant species to convert animal feed into human food. These microorganisms (bacteria, protozoa and fungi) hydrolyze proteins, starch and plant cell wall polymers into amino acids and sugars (hexoses and pentoses). These simple products are subsequently fermented to volatile fatty acids (VFA), hydrogen (H_2), and carbon dioxide (CO_2). Acetic-(C2), propionic-(C3), and butyric (C4) acid are the major VFA formed according to following stoichiometry of the reactions (Moss et al., 2000):



$$C_6H_{12}O_6+2H_2O \rightarrow 2C_2H_4O_2\ \text{(acetate)}+2CO_2+8[H]$$
$$C_6H_{12}O_6+4[H] \rightarrow 2C_3H_6O_2\ \text{(propionate)}+2H_2O$$
$$C_6H_{12}O_6 \rightarrow C_4H_8O_2\ \text{(butyrate)}+2CO_2+4[H]$$

The VFA are then absorbed and utilized by the host animal. The microbial fermentation in the rumen takes place anaerobically, thereby implying that in the oxidation of the various substrates can only occur when electrons are transferred to non-O_2 electron acceptors. Initially, the conversion of for instance, hexoses will lead to accumulation of reduced cofactors such as $NADH_2$. Needless to say, that a sustained VFA production is only possible in case $NADH_2$ is oxidized to NAD. However, the regeneration of oxidized NAD cofactors requires a very low partial pressure of H_2 in the rumen (Lingen et al., 2016). Quantitatively, acetic acid is the predominate VFA formed during fermentation and the organisms which produce acetic acid in the fermentation pathway are major producers of H_2 (Van Soest, 1982; Hegarty and Gerdes, 1998). Thus, H_2 has to be removed so as to sustain VFA production. The principal pathway to remove H_2 is the formation of CH_4:



$$CO_2+8[H] \rightarrow CH_4+2H_2O$$

Next to the necessity of H_2 removal to safeguard a sustained VFA production, there is also evidence to suggest that the partial hydrogen pressure in the rumen thermodynamically influences the profile of the synthesized VFA (Ellis et al., 2008; Lingen et al., 2016). A greater partial pressure of H_2 shifts the VFA profile towards propionic acid because, next to CH_4, propionic acid as well can act as a hydrogen sink (see afore mentioned stoichiometry). Thus, secondary to the formation of CH_4, propionate formation can serve as a competitive pathway to remove [H]. A third mechanism involved in the removal of H_2 concerns the process of biohydrogenation. During this process, dietary unsaturated fatty acids such as linoleic acid (C18:2n-6) and α-linolenic acid (C18:3n-3), are converted into ultimately stearic acid (C18:0). Although the process of biohydrogenation is quantitatively of lesser importance to sufficiently remove H_2 to sustain VFA production, biohydrogenation is of utmost

importance to maintain rumen function because long-chain unsaturated fatty acids can be considered toxic to rumen bacteria.

Methanogenic *Archaea*

Methanogenic *Archaea* are the main microorganisms in the rumen that convert hydrogen into CH_4. These methanogenic microorganisms emerged early in evolution and can be distinguished from bacteria (Boadi et al., 2004) due to a differential ribosomal RNA nucleotide sequence. Furthermore, cell membranes of methanogenic *Archaea* contain, unlike bacteria, ether linked lipids but not muramic acid (Demeyer and Fievez, 2000). Furthermore, methanogenic *Archaea* have three unique coenzymes; Coenzyme 420 (involved in electron transfer), Coenzyme M (involved in the transfer of methyl groups) and Factor B which is involved in the enzymatic formation of CH_4 from methyl coenzyme M. Typically, methanogens can only survive in environments with a redox potential <-300 mV while bacteria generally can survive in a much wider array of environmental conditions.
Five species of methanogens are, hitherto, isolated from rumen contents (Boadi et al., 2004) but in view of their role in methanogenesis, *Methanobrevibacter ruminantium* and *Methanosarcina barkeri* are the two most important ones. Methanogenic *Archaea* are known to have symbiotic relationships with rumen protozoa with methanogens residing both intracellularly and extracellularly (Sharp et al., 1998). Protozoa from the genera *Entodinium*, *Polyplastron*, *Epidinium*, and *Ophryoscolexin* commonly serve as host for methanogens mainly from the orders *Methanobacteriales* and *Methanomicrobiales* (Sharp et al., 1998). It has been estimated that the methanogens associated symbiotic with methanogenic *Archaea* are responsible for to 37% of total CH_4 production in the rumen (Machmüller et al., 2003). Thus, nutritional strategies targeting the defaunation of the rumen contents are potentially of interest to mitigate CH_4 production.

Nutritional strategies to reduce CH_4 production by ruminants.

For obvious reasons, the amount of CH_4 produced by ruminants depend on the absolute intake of fermentable organic matter (FOM). In this respect, the importance of factors such as the roughage to concentrate ratio (Suarez et al., 2007), source of roughage (Woolsoncroft et al., 2018) and maturity of roughage (Hatew et al., 2016) cannot be disputed. Overall, ration formulations that aim at maximum FOM intake and, therefore, maximum production of propionic acid without compromising rumen pH to such an extent that rumen acidosis occurs, will result in minimum CH_4 emission per unit of human edible product (*i.e.,* milk or meat). Next to the factors already mentioned, specific feed additives can also be used to reduce CH_4 production. Nitrate for instance, has been proven to be effective in mitigating CH_4 emission (Hristov et al., 2015; Nayak et al., 2015; Baral et al., 2018). Other examples are compounds such as enzymes (Jones et al., 1987; Baker, 1999), plant secondary compounds (Wallace et al., 2002) and dicarboxylic acids (Callaway and Martin, 1996; Asanuma et al., 1999; Lopez et al., 1999). Interestingly, the fatty acid composition of ruminant rations also has been shown to be relevant in the problem of CH_4 emission. Supplementation of ruminant rations with fat (*i.e.,* mainly triacylglycerols (TAGs)) is of particular interest under tropical conditions

because it provides a practical tool to increase the animal's energy intake with a minimal increase in heat increment (Dong et al., 1997). Both long-chain- and medium-chain fatty acids have been implicated on the issue on CH_4 but in this thesis the focus is on medium-chain fatty acids.

Fatty acids: structure, nomenclature, and sources

Quantitatively, TAGs are the most important source of fatty acids in nutrition. Triacylglycerols consist of three fatty which are esterified to a glycerol backbone. These compounds are energy rich and as such provide an attractive tool to increase the energy density of the ration. Moreover, specific fatty acids play an important role in physiology and are, therefore, of utmost importance to maintain the health of the animal.

More than 300 fatty acids have been isolated from plant material but ~10 fatty acids are most common. Generally, fatty acids have an even number of carbon atoms and consist of a carboxyl group and an unbranched carbon chain which can either be saturated or unsaturated (Table 2).

Table 2. Formula and short hand notation of selected fatty acids[1].

Fatty acid	Formula	Short hand notation
Saturated		
Caproic acid	$C_5H_{14}COOH$	C6:0
Caprylic acid	$C_7H_{15}COOH$	C8:0
Capric acid	$C_9H_{19}COOH$	C10:0
Lauric acid	$C_{11}H_{23}COOH$	C12:0
Myristic acid	$C_{13}H_{27}COOH$	C14:0
Palmitic acid	$C_{15}C_{31}COOH$	C16:0
Stearic acid	$C_{17}C_{35}COOH$	C18:0
Unsaturated		
Palmitoleic acid	$C_{15}H_{29}COOH$	C16:1n-7[2]
Oleic acid	$C_{17}H_{33}COOH$	C18:1n-9
Linolic acid	$C_{17}H_{31}COOH$	C18:2n-6
α-Linolenic acid	$C_{17}H_{29}COOH$	C18:3n-3
Archidonic acid	$C_{19}H_{31}COOH$	C20:4n-6
Eicosapentanoic acid	$C_{19}H_{29}COOH$	C20:5n-3
Docosahexanoic acid	$C_{21}H_{36}COOH$	C22:6n-3

[1] Mc Donald et. al., 2011, Animal Nutrition, 7th edition.

[2] The notation "n-9", "n-7", "n-6" or "n-3" indicates that the first double bound in the carbon chain is located after the 9th, 7th, 6th or 3rd C-atom, counted from the terminal methyl group of the carbon chain.

Fatty acids with an aliphatic carbon chain length of 6 to 12 carbon atoms are designated medium-chain fatty acids (MCFAs) and those with ≥14 carbon atoms are classified as long-chain fatty acids (LCFAs) (Marten et al., 2006). However, in most literature sources relevant for the current thesis, myristic acid (C14:0) is indicated as MCFA instead of LCFA and in the

current thesis also, C14:0 is conveniently classified as MCFA. Unsaturated fatty acids have at least one double bound in their carbon chain and those with more than one double bound are commonly referred to as polyunsaturated fatty acids (PUFAs). Both linolic acid and α-linolenic acid are essential nutrients for ruminants because these fatty acids are the parent substrates for the synthesis of eicosanoids, *i.e.,* prostaglandins, leukotrienes and thromboxane's.

Oils derived from seeds of sunflower, soybean, rapeseed, linseed/flaxseed and the palm tree are the dominant sources of fatty acids of plant origin used in ruminant nutrition while tallow is the main fatty acid source from animal origin. The oils are generally rich in unsaturated fatty acids whereas tallow primarily consists of C16:0, C18:0 and C18:1 (Table 3). Typically, coconut and palm kernel oil are rich in MCFAs, especially lauric acid.

Table 3. Selected fatty acid composition (g/100 g fatty acids) of common fat sources in ruminant nutrition[1].

Fatty acid	Sunflower	Soybean	Linseed	Palm	Tallow	Coconut	Palm kernel
C8:0	0.0	0.0	0.0	0.0	0.0	8.0	3.5
C10:0	0.0	0.0	0.0	0.0	0.0	6.4	3.9
C12:0	0.0	0.0	0.0	0.1	1.0	47.4	49.7
C14:0	0.0	0.1	0.0	1.1	4.0	17.9	17.4
C16:0	6.2	10.8	5.5	45.7	26.9	8.7	8.6
C18:0	4.7	4.0	4.3	4.5	20.4	3.0	3.0
C16:1n-7	0.0	0.2	0.0	0.3	4.5	0.0	0.0
C18:1n-9	20.4	23.9	21.1	38.4	38.9	6.2	12.1
C18:2n-6	68.7	53.6	13.3	9.6	3.3	1.9	1.7
C18:3n-3	0.0	7.1	55.8	0.2	0.6	0.0	0.0
Total	100.0	99.8	100.0	99.9	99.7	99.4	99.8

[1]Source: USDA.

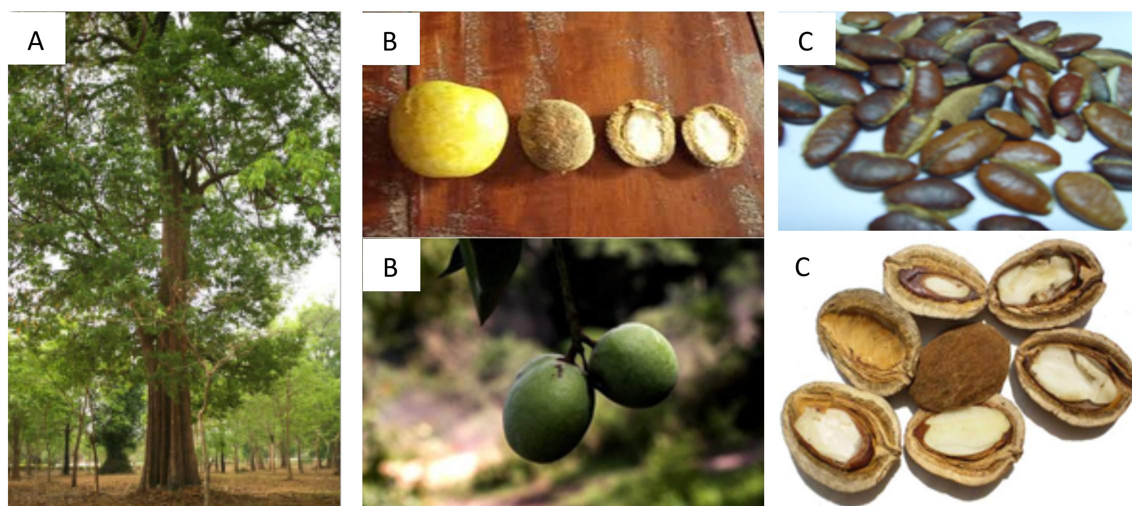


Figure 2. The krabok tree (A), fruit (B) and seeds (C). Source: Wikimedia Commons (2018).

Krabok oil

Extensive evidence exists on the suppressant effect of lauric acid (C12:0) on CH_4 production (Dong et al., 1997; Machmüller et al., 1998, 2000, 2003; Machmüller and Kreuzer, 1999; Dohme et al., 2000, 2001; Lovett et al., 2003; Soliva et al., 2004; Jordan et al., 2006). In all *in vivo* studies, coconut oil was used as a source of lauric acid because coconut oil is particularly rich in this fatty acid, *i.e.,* ~45% of total fatty acids. Unfortunately, the use of coconut oil is not feasible under economically less viable conditions. Thus, investigating an alternative source of lauric acid can be considered opportune. Interestingly, krabok oil has similar amounts of lauric acid but this oil also contains ~44% myristic acid (C14:0) which may have an additional effect in depressing CH_4 formation (Soliva et al., 2004).
Krabok oil is derived from krabok seed which is also named “barking deer's mango” or “Thai almond”. The fruit (Figure 2B) grows on the krabok tree (*Irvingia malayana* Oliv. ex A.W. Benn, Figure 2A) which commonly grows in South-East Asian forests. The seeds (Figure 2C), obtained after dehulling of the fruit, are widely available and as such of interest to be used as a fat source in ruminant rations by local farmers.

Aim of the thesis

The primary objective of the research described in this thesis was to investigate the potential of krabok oil to reduce CH_4 production by ruminants. Four experimental objectives were identified for this research, with the anticipation that the outcome can be applied under practical feeding conditions.

Outline of the thesis

Prior to the studies described in this thesis, the potential of krabok oil to reduce methanogenesis was not demonstrated. Therefore, the first study (Chapter 2) involved an *in vitro* assessment to demonstrate the potential of krabok oil to reduce CH_4 production in rumen fluid. In addition, it was studied whether a reduction of CH_4 production could be explained by an enhanced biohydrogenation. The results from this study confirmed the inhibitory effect of krabok oil on methanogenesis but the process of biohydrogenation was not clearly affected. From a practical perspective, it was not feasible to measure CH_4 production under field conditions and, therefore, only indirect measures of CH_4 production were assessed in the two subsequent *in vivo* studies. Because methanogenic *Archaea* live to a great extent in symbiosis with rumen protozoa, it was tested whether dietary supplementation with krabok oil effectively defaunates the rumen content (Chapter 3). Unfortunately, a defaunating effect of krabok oil could not be demonstrated which was most likely due to the low dose of supplemental fat, *i.e.,* 19.2 g/kg dry matter. It was, therefore, decided to conduct a second *in vivo* experiment using a higher dosage of supplemental fat, *i.e.,* 35 g/kg dry matter (Chapter 4). Next to the numbers of protozoa and methanogenic *Archaea*, an in-depth assessment of the methanogenic community in the rumen was performed so as to potentially gain more insight in the mode of action of krabok oil. It was considered of practical relevance to evaluate the inhibitory effect of krabok oil versus whole krabok seed. Therefore, whole

krabok seed and its derivates were evaluated on their potential to reduce CH_4 production using a fully automated gas production test (Chapter 5). Finally, the results of these studies are summarized and discussed in Chapter 6 of this thesis.

All the research described in this thesis where animals were used adhered to the prevailing national animal ethics laws for the experimentation on animals.

References

Asanuma, N., Iwamoto, M., Hino, T. 1999. Effect of the addition of fumarate on methane production by ruminal microorganisms *in vitro*. J. Dairy Sci. 82, 780-787.

Baker, S. K. 1999. Rumen methanogens, and inhibition of methanogenesis. Aust. J. Agric. Res. 50, 1293-1298.

Baral, K.R., Jego, G., Amon, B., Bol, R., Chantigny, M.H., Olesen, J.E., Petersen, S.O. 2018. Greenhouse gas emissions during storage of manure and digestates: Key role of methane for prediction and mitigation. Agric. Sys. 166, 26-35.

Boadi, D., Benchaar, C., Chiquette, J., Masse, D. 2004. Mitigation strategies to reduce enteric methane emissions from dairy cows: update review. Can. J. Anim. Sci. 84, 319-335.

Callaway, T. R., Martin, S. A. 1996. Effects of organic acid and monensin treatment on *in vitro* mixed ruminal micro-organisms fermentation of cracked corn. J. Anim. Sci. 74, 1982-1989.

Demeyer, D., Fievez, V. 2000. Ruminants and environment: Methanogenesis. Annal. de Zootechnie 49, 95-112.

Denman, K.L., Brasseur, G., Chidthaisong, A., Ciais, P., Cox, P.M., Dickinson, R. E., Hauglustaine, D., Heinze, C., Holland, E., Jacob, D., Lohmann, U., Ramachandran, S., da Silva Dias, P.L., Wofsy, S.C., Zhang, X. 2007. Couplings Between Changes in the Climate System and Biogeochemistry. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

FAO. 2008. The state of food and agriculture. Organization of the United Nations Rome. Data accesses on Aug 11, 2018 at http://www.fao.org/3/a-i0100e.pdf

Dohme, F., Machmüller, A., Wasserfallen, A., Kreuzer, M. 2001. Ruminal methanogenesis as influenced by individual fatty acids supplemented to complete ruminant diets. Lett. Appl. Microbiol. 32, 47-51.

Dohme, F., Machmüller, A., Wasserfallen, A., Kreuzer, M. 2000. Comparative efficiency of various fats rich in medium-chain fatty acids to suppress ruminal methanogenesis as measured with Rusitec. Can. J. Anim. Sci. 80, 473-484.

Dong, Y., Bae, H.D., McAllister, T.A., Mathison, G.W., Cheng, K.J. 1997. Lipid-induced depression of methane production and digestibility in the artificial rumen system (Rusitec). Can. J. Anim. Sci. 77, 269-278.

Ellis, J.L., Dijkstra, J., Kebreab, E., Bannink, A., Odongo, N.E., McBride, B.W., France, J. 2008. Aspects of rumen microbiology central to mechanistic modeling of methane production in cattle. J. Agric. Sci. 146, 213-233.

Jordan, E., Lovett, D.K., Hawkins, M., Callan, J.J., O'Mara, F.P. 2006. The effect of varying levels of coconut oil on intake, digestibility, and methane output from continental cross beef Heifers. Anim. Sci. 82, 859-865.

Johnson, K.A., Johnson, D.E. 1995. Methane emissions from cattle. J. Anim. Sci. 73, 2483-2492.

Jones, W.J., Nagle, D.P., Whitman, W.P. 1987. Methanogens and the diversity of archaebacteria. Microbiol. Rev. 53, 135-177.

Hatew, B., Bannink, A., Van Laar, H., de Jonge, L.H., Dijkstra, J. 2016. Increasing harvest maturity of whole-plant corn silage reduces methane emission of lactating dairy cows. J. Dairy Sci. 99, 354-368.

Hegarty, R.S., Gerdes, R. 1998. Hydrogen production and transfer in the rumen. Anim. Nutr. 12, 37-44.

Hristov, A.N., Oh, J., Giallongo, F., Fredericka, T.W., Harpera, M.T., Weeksa, H.L. Brancob, A.F. Moatec, P.J. Deightonc, M H. Williamsc, S.R.O., Kindermann, M., Duval, S. 2015. An inhibitor persistently decreased enteric methane emission from dairy cows with no negative effect on milk production. PNAF. 112, 10663-10668.

Lassey, K.R. 2008. Livestock methane emission and its perspective in the global methane cycle. Aust. J. Exp. Agric. 48, 114-118.

Lingen, H.J., Van Plugge, C.M., Fadel, J. G., Kebreab, E., Bannink, A., Dijkstra, J. 2016. Thermodynamic driving force of hydrogen on rumen microbial metabolism: a theoretical investigation. PLoS ONE 10:e0161362.

Le Treut, H., Somerville, R., Cubasch, U., Ding, Y., Mauritzen, C., Mokssit, A., Peterson T., Prather, M. 2007. Historical overview of climate change science. Data accesses on Aug 12, 2018 at https://www.ipcc.ch/pdf/assessment-report/ar4/wg1/ar4-wg1-chapter1.pdf.

Lopez, S., Valdes, C., Newbold, C.J., Wallace, R.J. 1999. Influence of sodium fumarate on rumen fermentation *in vitro*. Br. J. Nutr. 81, 59-64.

Lovett, D., Lovell, S., Stack, L., Callan, J., Finlay, M., Conolly, J., O'Mara, F.P. 2003. Effect of forage/concentrate ratio and dietary coconut oil level on methane output and performance of finishing beef heifers. Livest. Prod. 84, 135-146.

Marten, B., Pfeuffer, M., Schrezenmeir, J. 2006. Medium-chain triglycerides. Intern. Dairy J. 16, 1374-1382.

Machmüller, A., Ossowski, D.A., Wanner, M., Kreuzer, M. 1998. Potential of various fatty acid feeds to reduce methane release from rumen fermentation *in vitro* (Rusitec). Anim. Feed Sci. Technol. 71, 117-130.

Machmüller, A., Kreuzer, M. 1999. Methane suppression by coconut oil and associated effects on nutrient and energy balance in sheep. Can. J. Anim. Sci. 79, 65-72.

Machmüller, A., Ossowski, D.A., Kreuzer, M. 2000. Comparative evaluation of the effects of coconut oil, oilseeds and crystalline fat on methane release, digestion and energy balance in lambs. Anim. Feed Sci. Technol. 85, 41-60.

Machmüller, A., Soliva, C.R., Kreuzer, M. 2003. Effect of coconut oil and defaunation treatment on methanogenesis in sheep. Reprod. Nutr. Develop. 43, 41-55.

McDonald, P., Edwards, R.A., Greenhalgh, J.F.D., Morgan, C.A., Sinclair, C.A., Wilkinson. 2011. Animal Nutrition 7th Edition.

McMichael, A.J., Powles, J.W., Butler, C.D., Uauy, R. 2007. Food, livestock production, energy, climate change, and health. Lancet 370, 1253-1263.

Milich, L. 1999. The role of methane in global warming: where might mitigation strategies be focused. Global Environ. Change 9, 179-201.

Moss, A.R., Jouany, J.P., Newbold, J. 2000. Methane production by ruminants: its contribution to global warming. Annal. de Zootechnie 49, 231-253.

Nayak, D., Saetnan, E., Cheng, K., Wang, W., Koslowski, F., Cheng, Y.F., Yun Zhu, W., Wang, J.K., Liu, J.X., Moran, D., Yan, X., Cardenas, L., Newbold, J., Pan, G., Lu, Y., Smith, P. 2015. Management opportunities to mitigate greenhouse gas emissions from Chinese agriculture. Agri. Ecosys. Environ. 209, 108-124.

Schneider, S.H. 1998. The greenhouse Effect: Science and Policy. Science 243, 771-781.

Sharp, R.J., McEwan, N.R., McIntosh, F.M., Teferedegne, B., Newbold, C.J. 2002. Natural products as manipulators of rumen fermentation. Asian-Aust. J. Anim. Sci. 15, 1458-468.

Sharp, R., Ziemer, C.J., Stern, M.D., Stahl, D.A. 1998. Taxon specific associations between protozoal and methanogen populations in the rumen and a model rumen system. FEMS Microb. Eco. 26, 71-78.

Soliva, C.R., Meile, L., Hindrichsen, I.K., Kreuzer, M., Machmüller, A. 2004. Myristic acid supports the immediate inhibitory effect of lauric acid on ruminal methanogens and methane release. Anaerobe 10, 269-276.

Soliva, C.R., Hindrichsen, I.K., Meile, L., Kreuzer, M., Machmüller, A. 2003. Effects of mixtures of lauric and myristic acid on rumen methanogens and methanogenesis *in vitro*. Lett. Appl. Microbiol. 37, 35-39.

Suarez, B.J., Van Reenen, C.G., Stockhofe, N., Dijkstra, J., Gerrits, W.J.J. 2007. Effect of roughage source and roughage to concentrate ratio on animal performance and rumen development in veal calves. J. Dairy Sci. 90, 2390-2403.

Thorpe, A. 2009. Enteric fermentation and ruminant eructation: the role (and control?) Or methane in the climate change debate. Climatic Change 93, 407-431.

US National Nutrient Database, Release 28, United States Department of Agriculture. May 2016. Retrieved 6 September 2020.

Van Soest, P.J. 1982. Nutritional ecology of the ruminant. Cornell University Press, Ithaca, NY, USA.

Wikimedia Commons, 2018. *Irvingia malayana*. Data accesses on Aug 12, 2018 at https://en.wikipedia.org/wiki/Irvingia_malayana.

Woolsoncroft, M.A., Youngers, M.E., McPhillips, L.J., Lockard, C.G., DeSocio, E.S., Ryan, W.R., Richards, C.J., Wilson, B.K. 2018. Effects of exercise and roughage source on the health and performance of receiving beef calves. Profess. Anim. Scient. 34, 183-191.

Chapter 2

Medium-chain fatty acids from coconut or krabok oil inhibit *in vitro* rumen methanogenesis and conversion of non-conjugated dienoic biohydrogenation intermediates

Paiwan Panyakaew[1,2], Gunjan Goel[1], Marta Lourenço[1],
Chalermpon Yuangklang[2], Veerle Fievez[1,]

[1]*Laboratory for Animal Nutrition and Animal Product Quality (Lanupro), Faculty of Bioscience Engineering, Ghent University, Proefhoevestraat 10, 9090 Melle, Belgium;*
[2]*Department of Animal Science, Faculty of Natural Resources, Rajamangala University of Technology Isan, Sakon-Nakhon Campus, Sakon Nakhon 47160 Thailand.*

Published in Animal Feed Science and Technology 180, 18-25 (2013).

Abstract

Myristic (C14:0) and lauric (C12:0) acid have been suggested to synergistically influence rumen methanogenesis. This experiment compared the effect of krabok and coconut oil on rumen fermentation, in an attempt to assess this synergism using two natural oil sources which contain similar amounts of C12:0 but with krabok oil containing greater proportions of C14:0 than coconut oil. As a simultaneous action on both rumen methanogenesis and biohydrogenation has been reported for another medium-chain fatty acid (C10:0), rumen biohydrogenation also was monitored during the current *in vitro* study. Five treatments were used: one control (CON), without supplementation of coconut or krabok oil, two coconut oil and two krabok oil supplemented incubations. Coconut and krabok oil were supplemented in two doses, providing either 80 (C80 and K80) or 120 mg (C120 and K120) of C12:0+C14:0 per 100 ml of incubation fluid. A standard concentrate typically fed to ruminant livestock in Thailand (200 mg), buffer (20 ml) and rumen fluid (5 ml) were added to each incubation flask, with or without an external PUFA source (20 mg of a mixture of sunflower and linseed oil). All flasks were incubated at 39 °C for 24 h. Both krabok and coconut oil reduced methane production (P<0.05) and increased propionate production (P<0.05) at the expense of acetate (P<0.05) and butyrate production (P<0.05). Krabok and coconut oil induced similar changes and effects were stronger in combination with linseed and sunflower oil, whereas the latter, in the amounts supplemented here, did not change methane production nor induced shifts in the production of any of the VFA. A trend for lower amounts of C18:2n-6 and C18:3n-3 after 24 h incubation was observed indicating a higher rate of lipolysis and isomerisation of C18:2n-6 and C18:3n-3, as the inclusion levels of krabok oil increased. Overall, the effect of krabok and coconut oil on rumen biohydrogenation was limited.

Key words: krabok oil, coconut oil, rumen methanogenesis, medium-chain fatty acids

Introduction

Lauric (C12:0) and myristic (C14:0) acid show potential *in vitro* to reduce rumen methanogenesis and significantly reduce the number of methanogens (Dohme et al., 2001), although the mitigation potential of C14:0 was suggested to be limited when not combined with C12:0 (Soliva et al., 2003). Further, Dohme et al. (1999) reported coconut oil, which is particularly rich in C12:0 (470 g/kg) and C14:0 (180 g/kg) to reduce methane by half when 756 mg/d was fed in a Rusitec fermentation study. *In vivo* methane (CH_4) emissions in sheep have been reduced by 26% up to 73% through the incorporation of coconut oil (70 g/d) (Machmüller and Kreuzer, 1998; Machmüller et al., 2000). Coconut oil (250 g/d) reduced (CH_4) output of beef heifers by 39% (Jordan et al., 2006).
Krabok oil is another source of C12:0 and C14:0, with a similar C12:0 (444 g/kg) but much greater C14:0 (437 g/kg) content than coconut oil (Wongsuthavas et al., 2007). This oil which is derived from krabok seeds or barking deer's mango (*Irvingia malayana* Oliv. ex A.W. Benn) are widely available in South-East Asian forests. As these seeds contain large amounts of fat, they are of interest to be used as a fat source in animal diets by local farmers. Moreover, due to their specific fatty acid profile and given the synergistic effect of C14:0 when combined with C12:0 (Soliva et al., 2003), we hypothesized that krabok oil would reduce rumen methanogenesis to a larger extent than coconut oil. Moreover, simultaneous action on both methanogenesis and rumen biohydrogenation of poly-unsaturated fatty acids (PUFA) has been reported through *in vitro* supplementation of another medium-chain fatty acid (MCFA), i*.e.,* capric acid (C10:0) (Goel et al., 2009). The effect of C12:0 and C14:0 on rumen biohydrogenation is unknown.
Therefore, the aim of this *in vitro* study was to evaluate the replacement of tallow by either krabok or coconut oil at two inclusion levels. Rumen fermentation parameters, with respect to methane and volatile fatty acid (VFA) production were assessed after 24 h incubations in combination or not with sunflower and linseed oil. In the PUFA supplemented incubation, rumen biohydrogenation from these external PUFA sources was also monitored.

Materials and methods

Treatments

The treatments in this study were: 1) control (CON) without supplementation of krabok or coconut oil; 2) 20 mg/incubation flask of C12:0+C14:0 from coconut oil (C80); 3) 20 mg/incubation flask of C12:0+C14:0 from krabok oil (K80); 4) 30 mg/incubation flask of C12:0+C14:0 from coconut oil (C120) and 5) 30 mg/incubation flask of C12:0+C14:0 from krabok oil (K120). Given the amount of incubation fluid in the current experimental set-up (25 ml), this corresponded to the addition of 80 and 120 mg C12:0+C14:0 per 100 ml of incubation fluid. This concentration reference was used throughout the paper to better allow comparison with other *in vitro* studies. Coconut and krabok oil were derived from four different batches and their fatty acid composition is given in Table 1. These treatments were incubated with and without an external PUFA source: a mixture of sunflower oil (10 mg) and linseed oil (10 mg). Both oils were added as an oil-hexane solution, with hexane being

evaporated under N_2 flow before incubation. Fat added through coconut or krabok oil supplementation was compensated by tallow, which was added to the incubation flasks as an ethanol solution. Tallow is a largely saturated fatty acid source (Table 1) which is considered a rumen inert fat, just as lard, which had been used for this purpose earlier (Fievez et al., 2007). An overview of fat and oil added to the incubation flasks is given in Table 2.

Table 1. Fatty acid composition (g/100 g FAME) of coconut oil, krabok oil, tallow, sunflower oil, linseed oil and concentrate (n=4).

Fatty acid	Coconut oil	Krabok oil	Tallow	Sunflower oil	Linseed oil	Concentrate
C8:0	1.86 ± 1.40	0.04 ± 0.04	0.01	0.00	0.00	0.00
C10:0	4.38 ± 2.31	2.26 ± 0.67	0.05	0.00	0.00	0.28
C12:0	44.1 ± 1.52	46.1 ± 3.66	0.12	0.00	0.00	0.12
C14:0	18.5 ± 1.79	43.3 ± 2.55	3.97	0.11	0.05	0.38
C16:0	9.16 ± 2.01	3.95 ± 0.66	26.8	3.08	5.19	15.7
C18:0	4.54 ± 1.77	0.36 ± 0.06	25.7	3.79	3.47	6.42
C18:1c9	8.64 ± 3.26	2.53 ± 0.78	23.5	32.7	13.9	25.9
C18:2n-6	1.96 ± 0.53	0.36 ± 0.10	0.53	54.5	16.4	42.2
C18:3n-3	0.04 ± 0.06	0.00 ± 0.00	0.39	1.13	60.6	0.00

Table 2. Amount of fat or oil added to each of the incubation flasks (mg/100 ml of incubation fluid) (n=4).

		Tallow	Coconut oil	Krabok oil	Linseed + sunflower oil
-PUFA	CON	195.0 ± 2.84	0	0	0
	C80	64.8 ± 1.00	130.0 ± 1.84	0	0
	C120	0	195.0 ± 2.84	0	0
	K80	105.0 ± 2.72	0	89.6 ± 1.04	0
	K120	60.8 ± 2.80	0	134.0 ± 1.48	0
+PUFA	CON	195.0 ± 2.84	0	0	80.0
	C80	64.8 ± 1.00	130.0 ± 1.84	0	80.0
	C120	0	195.0 ± 2.84	0	80.0
	K80	105.0 ± 2.72	0	89.6 ± 1.04	80.0
	K120	60.8 ± 2.80	0	134.0 ± 1.48	80.0

PUFA: polyunsaturated fatty acid supply through a combination of sunflower and linseed oil; CON: control, supplemented with tallow only or tallow + PUFA; C80, K80, C120 and K120: supplementation of 80 or 120 mg/100 ml of C12:0+C14:0 from either coconut (C) or krabok (K) oil.

Substrate

The substrate used was a standard concentrate typically fed to ruminant livestock in Thailand (g/kg product) (cassava chips, 421; rice straw, 211; dry tomato pomace, 158; molasses, 73.7; rice bran, 52.6; soybean meal, 31.6; urea, 21.0; salt, 10.5; di-calcium phosphate, 7.4; oysters meal, 5.3; mineral premix, 5.3; sulfur, 3.2), of which 200 mg was added as basal substrate in all the treatments. The proximate chemical analysis of the substrate was (g/kg DM, except for

DM: g/kg FM): dry matter (DM) 925, organic matter (OM) 896, crude protein (N×6.25; CP) 124, neutral-detergent fibre (NDF) 437, acid-detergent fibre (ADF) 272, crude ash 110.

In vitro *incubation*

An *in vitro* incubation was carried out according to Boeckaert et al. (2007). Rumen fluid was collected from three rumen fistulated Holstein dairy cows before the morning feeding (08:00 h) and immediately transferred into pre-warmed thermos flasks. Donor animals were fed (on DM basis): 8.83 kg corn silage, 0.2 kg soybean meal, 0.05 kg mineral mix (AVEVE, Belgium) and grass silage *ad libitum*. The rumen fluid of the three cows were mixed and filtered through four layers of cheese cloth under continuous flushing with CO_2. Rumen fluid and phosphate buffer (per litre distilled water: 28.8 g $Na_2HPO_4 \cdot 12H_2O$; 6.1 g $NaH_2PO_4 \cdot H_2O$; 1.4 g NH_4Cl, and adjusted to pH 6.8) were mixed (1/4, v/v) and added to each incubation flask (25 ml of the mixture). The flasks were incubated in a shaking water bath at 39 °C for 24 h. Gases were accumulating in the flasks, allowing gas pressure to build up over the 24 h-period of the incubation. All incubations were completed in quadruplicate in three runs on separate days. In each of the three runs, all four krabok or coconut oil batches were considered. After 24 h incubation, the flasks were placed in an ice bath to stop fermentation and the gas phase was analysed for CH_4 and hydrogen (H_2) by gas chromatography (Micro GC 3000A, Agilent, USA). Flasks were then opened, the pH measured, and 0.5 ml of the incubation contents were sampled for volatile fatty acid (VFA) analysis. The remainder was kept for extraction and methylation of the extracted fatty acids.

Sampling

Samples for VFA analysis (5 ml) were acidified with 0.1 ml of phosphoric/formic acid (10/1, v/v) and centrifuged for 15 min at 22000×*g* at 4 °C (MSE, Amsterdam, The Netherlands). The supernatant was recovered, transferred to vials and analyzed by gas chromatography (Schimadzu GC-14A, Belgium), according to Van Ranst et al. (2010). The net amount of VFA produced during the 24 h incubation period was calculated by deducting the amount of VFA in the corresponding non-incubated sample. The latter was determined as the VFA concentration in a directly acidified (50/1, v/v, rumen fluid/phosphoric-formic acid (10/1, v/v) mixture), non-incubated rumen fluid sample. Hydrogen recovery (2Hr) was calculated as (2 Propionate +2 Butyrate + 4 CH_4) / (2 Acetate + Propionate + 4 Butyrate), with acetate, propionate, butyrate and CH_4 expressed as net molar productions (Marty and Demeyer, 1973).

Laboratory analysis

The medium- and long-chain fatty acids of incubation contents and concentrate were extracted in duplicate with chloroform/methanol (C/M, 2/1, v/v), based on the method described by Folch et al. (1957), with modifications as described by Boeckaert et al. (2007). Briefly, 1.0 g of sample was weighed in an extraction tube and 34 ml C/M (2/1, v/v) and 10 mg of nonadecanoic acid (C19:0), used as internal standard, were added. Concentrate in C/M

samples were homogenized for 1 min by an ultra-turrax mixer (Ultra-Turrax T25, IKA-Labortechnik, Belgium) and were extracted overnight. The next morning, all extracts were centrifuged for 10 min at 1820×g. The lower layer was recovered with a syringe and brought into a volumetric flask of 100 ml. Afterwards, two consecutive centrifugations were performed after the addition of 20 and 15 ml of C/M (2/1, v/v), respectively and the chloroform fraction was again added to the volumetric flask. The combined extract was then transferred from the volumetric flasks to an extraction tube and washed once with distilled water. After centrifugation, the combined extract was transferred into a separation funnel and the non-aqueous fraction was recovered in a volumetric flask of 100 ml. The final volume was brought to 100 ml with C/M (2/1, v/v).
Methylation of FA in oil or concentrate extracts and extracts of incubation contents was performed as described by Raes et al. (2001) with NaOH/MeOH (0.5 M) followed by HCl/MeOH (1:1, v/v) at 50 °C. The fatty acid methyl esters (FAME) were extracted twice with 3 and 2 ml of hexane respectively and pooled extracts were evaporated to dryness under N_2 stream. The residue was re-dissolved in 1 ml of hexane and analyzed by gas chromatography (HP 6890, Brussels, Belgium) on a CP-Sil88 column for FAME (100 m×250 μm×0.2 μm, Chrompack, Middelburg, The Netherlands; Raes et al., 2001). Fatty acid methyl ester peaks were identified based on their retention times, compared to external standards [Supelco external standard mix (Supelco Inc. Park Bellefonte, USA) to which pure odd- and branched chain and trans C18:1 FA standards were added (Larodan Fine Chem. Malmo, Sweden)] and to our laboratory internal library. The quantification of FAME was based on the internal standard technique, using tri-decanoic acid (C13:0) as internal standard, and on the conversion of chromatogram peak areas to weight of fatty acids using a theoretical relative response factor (RRF) of each FA (Ackman and Sipos, 1964; Wolff et al., 1995). Unknown chromatogram peaks were quantified similarly as for known chromatogram peaks, and the RRF used for the conversion of each unknown chromatogram peak into weight of fatty acids was an average of the RRF of the two closest known chromatogram peaks. On average, the percentage of unknown chromatogram peaks was 2.6%. Total fatty acids are expressed gravimetrically (mg/incubation) and include both known and unknown chromatogram peaks. The FA composition of all oils is presented in Table 2.

Calculations

The efficiency of disappearance of C18:2n-6 and C18:3n-3 in the various steps, including lipolysis + isomerisation and two (C18:2n-6) or three (C18:3n-3) hydrogenation steps were calculated as detailed by Boeckaert et al. (2007). Efficiency of disappearance is the proportion of precursor which is converted into product in each step of the biohydrogenation pathway. The predominant biohydrogenation pathways for the conversion of C18:2n-6 to C18:0 and of C18:3n-3 to C18:0 were considered, *i.e.,* C18:2n-6 to C18:2c9t11 to C18:1t11 to C18:0 and C18:3n-3 to C18:3c9t11c15 to C18:2t11c15 to C18:1t11 to C18:0. *E.g.* the calculation of the efficiency of disappearance of C18:2t11c15 to C18:1t11 is based on the amount of C18:2t11c15 which accumulates and does not disappear, *i.e.,* (100-100×x/y) with x=the amount of C18:2t11c15 accumulating and y=the amount of substrate available for the process, *i.e.,* C18:3n-3 added at the beginning of the incubation minus C18:3c9t11c15

accumulating. Exact details on the calculation are presented in the paper by Boeckaert et al. (2007). As no distinction was made between esterified and non-esterified fatty acids in the current experiment, lipolysis + isomerisation were assessed together and corresponded to the the proportional loss of C18:2n-6 and C18:3n-3 after 24 h of *in vitro* incubation, *i.e.,* (FA_{0h}-FA_{24h})/FA_{0h} with FA_{0h} and FA_{24h} the amount of C18:2n-6 and C18:3n-3 in the incubation flask (mg/incubation) at the start of the incubation and after 24 h incubation, respectively.

Statistical analysis

Runs were considered as analytical replicates and batches as statistical replicates. Hence, before statistical analysis, batch replicates were averaged (*i.e.,* averages were calculated across runs for every batch and per dose).

The data obtained for rumen fermentation parameters were statistically evaluated using the general linear model procedure (univariate) according to $Y_{ijk}=\mu+T_{i=1\text{-}5}+P_{j=1\text{-}2}+T_i\times P_j+\varepsilon_{ijk}$, where Y_{ij} is the response; μ the overall mean; $T_{i=1\text{-}5}$ the effect of supplementation or not with a MCFA source at one of the two concentrations (fixed factor) (CON vs. C80 vs. K80 vs. C120 vs. K120); $P_{j=1\text{-}2}$ the effect of PUFA supplementation or not (fixed factor) and $T_i\times P_j$ the incubation of both effects; ε_{ijk} the residual error. In the results, the P-value of the fixed factor P_j was presented to assess the effect of PUFA supplementation. The effect of supplementation of MCFA source was assessed against a non-MCFA-supplemented control using a 2-sided Dunnett's test as post-hoc test: all coconut or krabok oil supplemented incubations were compared with the non-supplemented incubation (CON). All statistical analyses were performed using SPPS (SPPS software for Windows, release 16.0. SPSS Inc. USA).

The data of biohydrogenation products were statistically evaluated based on PUFA supplemented incubations only using the general linear model procedure (univariate) according to $Y_i=\mu+T_{i=1\text{-}5}+\varepsilon_i$, with effects as defined before.

Results

Rumen fermentation characteristics

The effects of krabok and coconut oil on volatile fatty acid and methane production after 24 h incubation are presented in Table 3. The methane production was reduced *(P*<0.05*)* in all treatments as compared with the non-supplemented control although at the higher coconut oil dose (C120) this was only a trend. The reduction in CH_4 was stronger in incubations with PUFA compared to flasks without PUFA (28-38% vs. 13-14%), with K120 in combination with PUFA provoking the greatest effect on CH_4 (-38%) with shifts towards propionate (+37%) at the expense of acetate (-15%). Besides acetate, butyrate proportions were generally decreased (*P*<0.05). The lowest values for butyrate were observed at the lower krabok oil dose (-47%). As expected from rumen stoichiometry propionate proportions increased (*P*<0.05) in all treatments (25% to 37%). Hydrogen did not accumulate and shifts in the fermentation pattern were according to rumen stoichiometry as indicated by the high 2Hr. Supplementation of krabok or coconut oil did not affect total rumen VFA production. Hence,

changes in the production of individual VFA were reflected by changes in the VFA proportions as reported in Table 3, with the same statistical significances (data not shown). Shifts in VFA proportion are reported here as they were considered more meaningful to assess shifts in fermentation patterns and hence, functionality of a microbial community.

C18 isomer profile

The effect of coconut oil and krabok oil on the individual C18 fatty acid profile (mg/incubation) is shown in Table 4. The greater amount of C18:0 in the control is due to the greater supplementation in this incubation flask of tallow. All treatments, including the control, led to 0.45-0.58 recovery of C18:2n-6 and 0.24-0.39 C18:3n-3 recovery when compared to the amount of incubated C18:2n-6 and C18:3n-3, respectively. The lowest recoveries were observed when supplementing the higher krabok oil dose, which - accordingly - increased the efficiency of lipolysis + isomerisation (Table 5). However, no trienoic hydrogenation intermediate of C18:3n-3 (*i.e.,* C18:3c9t11c15) accumulated, indicating its complete conversion during the 24 h *in vitro* incubation. The conversion efficiency of the hydrogenation step of C18:2c9t11 to C18:1t11 at the higher coconut oil dose (C120) was slightly reduced which increased the amount of C18:2c9t11 accumulating in the incubation flask (Table 4). Additionally, the conversion efficiency of the second hydrogenation step (C18:2t11c15 to C18:1t11) was reduced through krabok or coconut oil supplementation irrespective of the type of MCFA source and its dose. Nevertheless, the effect of krabok oil was more pronounced, resulting in lower hydrogenation rates of C18:2t11c15 (Table 5). Finally, krabok oil decreased the efficiency of the last hydrogenation step of C18:1t11 to C18:0 (K80 vs. C80 and K120 vs. C120), but no differences with the non-supplemented control were observed.

Discussion

Effect of coconut and krabok oil on rumen fermentation

Compared to the control, the addition of coconut oil (C80 and C120) or krabok oil (K80 and K120) resulted in a decreased methane production. The inhibitory effect of coconut oil has been reported by Dohme et al. (1999) and Jordan et al. (2006) both *in vitro* and *in vivo*, respectively. In addition, Soliva et al. (2004) reported a decrease in CH_4 production by 74% when incubating C12:0 alone and a 90% reduction in CH_4 production when C12:0 was incubated in combination with C14:0 (3:1). However, no inhibitory effect on methanogenesis was reported when C14:0 was incubated alone. Hence, a synergistic effect of C14:0 on the inhibitory activity of C12:0 was suggested (Soliva et al., 2004). In this study, the anti-methanogenic effect of both coconut and krabok oil was similar, despite the higher proportions of C14:0 in the krabok oil supplemented flasks compared with the coconut oil (205 vs. 464 g/kg for coconut and krabok oil, respectively). Hence, the results of this study could suggest that both C12:0 and C14:0 have a similar inhibitory activity on methanogenesis although a synergistic effect between both FA might not be excluded (Soliva et al., 2004). Moreover, the methane reducing effect of krabok and coconut oil was stronger in the

presence of a PUFA source (additional decrease of 10.4 to 24.9%), whereas PUFA, in the amounts supplemented here, did not provoke a reduction in rumen methanogenesis.

Table 3. Effect of two levels of krabok oil (K80 and K120) and coconut oil (C80 and C120) with (+) or without (-) polyunsaturated fatty acids (PUFA on volatile fatty acid and methane produced during 24 h *in vitro* incubations.

Parameter	PUFA	CON	C80	K80	C120	K120	SEM	P[a] (n=20)	P×T[b] (n=4)	T[c] (n=8)
Total VFA (μmol/incubation)	-	875	801	810	807	835	34.3	0.03	0.37	0.57
	+	898	928	879	804	929				
Dunnett's test (n=8)			Ns	Ns	Ns	Ns				
		(mmol/mol of total VFA)								
CH_4	-	235	205	203	201	205	18.4	0.22	0.51	0.07
	+	249	172	170	180	154				
Dunnett's test (n=8)			*	*	(*)	*				
Acetate	-	646	575	580	538	553	14.5	0.31	0.25	0.03
	+	598	556	544	550	506				
Dunnett's test (n=8)			*	*	*	*				
Propionate	-	237	351	321	344	352	18.4	0.23	0.38	<0.001
	+	256	342	363	341	407				
Dunnett's test (n=8)			*	*	*	*				
Butyrate	-	180	124	96	139	110	10.2	0.18	0.58	0.18
	+	160	121	88	110	111				
Dunnett's test (n=8)			*	*	*	*				
2Hr (mol/mol)[5]	-	0.80	0.89	0.89	0.91	0.94	0.03	0.86	0.43	0.34
	+	0.87	0.83	0.89	0.88	0.90				
Dunnett's test (n=8)			Ns	Ns	Ns	Ns				

PUFA: polyunsaturated fatty acid supply through a combination of sunflower and linseed oil; VFA: volatile fatty acids; CON: control, supplemented with tallow only or tallow + PUFA; C80, K80, C120 and K120: supplementation of 80 or 120 mg/100 ml of C12:0+C14:0 from either coconut (C) or krabok (K) oil; SEM: standard error of mean; 2Hr=Hydrogen recovery, calculated based on molar productions of acetate, propionate, butyrate and methane as outlined in Materials and methods.

*P<0.05, (*)0.05≤P<0.1: Dunnett's test was used to compare each level of krabok and coconut oil with control (CON).

[a] P=P-value representing effect of PUFA supplementation.

[b] P×T=P-value representing effect of interaction of PUFA with either coconut or krabok oil.

[c] T=P-value representing effect of treatment (no or one of the two levels of either coconut or krabok oil).

Table 4. Effect of two levels of krabok oil (K80, K120) and coconut oil (C80, C120) on C18 fatty acids (mg/100 ml incubation fluid) in incubations supplemented with sunflower and linseed oil as PUFA source (n=4).

Parameter	CON	C80	K80	C120	K120	SEM
C18:0	49.6	27.0[a]	28.2[a]	14.0[ab]	20.0[ab]	0.84
C18:1t6-t8	1.64	1.08[a]	1.08[a]	0.76[ab]	1.06[ab]	0.08
C18:1t9	1.44	1.16[a]	0.92[a(c)]	0.92[ab]	0.84[ab(c)]	0.04
C18:1t10	2.16	1.48[a]	1.4[a]	1.12[ab]	1.16[ab]	0.12
C18:1t11	12.6	11.5	13.5	10.9	15.8	1.04
C18:1t12-t14	1.08	1.04	1.08	0.84[b]	1.12[b]	0.08
C18:1c9	41.6	39.7	33.6[ac]	31.8[ab]	26.9[abc]	1.06
C18:1c11	1.28	1.32	1.20	0.80[ab]	1.24[b]	0.08
C18:1c12	1.04	0.72	0.68	0.68	0.64[(a)]	0.12
C18:1c13	1.20	0.88	1.24[c]	0.84	1.00[c]	0.12
C18:1c14	0.16	0.12	0.16	0.04[a(b)]	0.12[a(b)]	0.00
C18:1c15	0.56	0.20[a]	0.28[ac]	0.12[ab]	0.20[abc]	0.00
C18:2c9t11	0.36	0.48	0.48	0.60[a]	0.44	0.04
C18:2t11c15	2.04	3.96[a]	5.32[ac]	4.84[a]	6.68[ac]	0.40
C18:2n-6	15.6	19.6[a]	16.0[(c)]	18.2	14.0[(c)]	1.06
C18:3n-3	7.32	7.96	5.96	6.52	4.92[(a)]	0.64

CON: control, supplemented with tallow + PUFA; C80, K80, C120 and K120: supplementation of 80 or 120 mg/100 ml of C12:0+C14:0 from either coconut (C) or krabok (K) oil; SEM: standard error of mean.

[a] $P<0.05$, [(a)] $0.05 \le P<0.1$: Dunnett's test was used to compare each level of krabok and coconut oil with control (CON).

[b] $P<0.05$, [(b)] $0.05 \le P<0.1$: T-test comparing both doses within the same oil, *i.e.,* C80 vs. C120, K80 vs. K120.

[c] $P<0.05$, [(c)] $0.05 \le P<0.1$: T-test comparing krabok vs. coconut oil within the same dose, *i.e.,* C80 vs. K80, C120 vs. K120.

Table 5. Effect of two levels of krabok oil (K80, K120) and coconut oil (C80, C120) on the efficiency of lipolysis+isomerisation[1] (C18:2n-6 → C18:2c9t11) and hydrogenation processes of C18:2n-6 and C18:3n-3 in incubations supplemented with sunflower and linseed oil as PUFA source (n=4).

Parameter			CON	C80	K80	C120	K120	SEM
C18:2n-6	→	C18:2c9t11	0.45	0.43	0.47	0.45	0.55[a]	0.208
C18:3n-3	→	C18:2t11c15	0.61	0.63	0.70	0.67	0.76[a]	0.269
C18:2t11c15	→	C18:1t11	0.82	0.69[a]	0.61[ac]	0.62[a]	0.56[ac]	0.168
C18:2c9 t11	→	C18:1t11	0.97	0.97	0.97	0.96[a]	0.97	0.036
C18:1t11	→	C18:0	0.44	0.50	0.39[c]	0.51	0.38[c]	0.329

CON: control, supplemented with tallow + PUFA; C80, K80, C120 and K120: supplementation of 80 or 120 mg of C12:0+C14:0 from either coconut (C) or krabok (K) oil; SEM: standard error of mean.

[1]As C18:3c9t11c15 did not accumulate, the extent of lipolysis + isomerization was not calculated for C18:3n-3.

[a] $P<0.05$: Dunnett's test was used to compare each level of krabok and coconut oil with control (CON).

[c] $P<0.05$: T-test comparing krabok vs. coconut oil within the same dose, *i.e.,* K80 vs. C80 and K120 vs. C120.

The anti-methanogenic activity of MCFA has been attributed to their antimicrobial activity. Pure C12:0 and C14:0 have been reported to decrease CH_4 production by 12% when incubated at 0.6 mg/ml of incubation fluid (Dohme et al., 2001) whereas others reported 74% up to 96% reduction in CH_4 when incubating C12:0 alone or in combination with C14:0 (C12:0:C14:0, 2:1) at 1 mg/ml of incubation fluid (Soliva et al., 2003). Although C12:0+C14:0 concentrations in the current study were in line with the former study (0.8 to 1.2 mg/ml), methane inhibition was lower. This might be related to the MCFA source (pure MCFA studied by Soliva et al. (2003) vs. esterified MCFA in coconut or krabok oil in the current study) as well as the incubation circumstances, *e.g.,* incubation gases consisted of CO_2+H_2 without additional fermentation substrate in the incubations performed by Soliva et al. (2003) as they focussed on the activity of methanogens only. Methane inhibition was associated with a dose-dependent shift in VFA towards propionate at the expense of acetate and butyrate.

Eventually, changes through coconut or krabok oil supplementation might have been underestimated due to confounding variation in the amount of tallow and MCFA source. In our experimental set up this was assumed to be negligible as tallow was considered an inert fat source to keep the fat content in the incubation constant. However, Getachew et al. (2001) reported a decrease of 0.38% in total acetate per percent increase in tallow per g of DM feed and an increase of 0.48% in total propionate per percent increase in tallow per g of DM feed. Applying these results to our observations, might have provoked acetate proportions to be 9% lower in the current control (with tallow) as compared to a control without tallow. Inversely, propionate proportions in a control without tallow could have been 11% lower as compared

to the current tallow supplemented control. However, others supported inertness of tallow towards the rumen fermentation pattern (*e.g.,* Onetti et al., 2002) justifying the current experimental set up.
Regarding the shifts in VFA proportion (decrease in acetate and increase in propionate): somewhat other shifts were observed in the current study as compared with the study of Dohme et al. (2001) who reported more butyrate and reduced propionate proportions. However, pure C12:0 or C14:0 at lower concentrations were used in the latter study.

Effect of coconut or krabok oil on rumen biohydrogenation.

Effect of coconut or krabok oil on rumen biohydrogenation (efficiency) is relatively limited. Both MCFA sources reduce the second step in the hydrogenation of C18:3n-3, *i.e.,* the conversion of C18:2t11C15 to C18:1t11 which resulted in higher accumulation of C18:2t11c15. Similar effects on accumulation of C18:2t11c15, when incubating capric acid (C10:0) at 0.8 and 1.2 mg/ml (Goel et al., 2009). Other fatty acids, such as docosahexanoic acid (DHA, C22:6n-3) also resulted in an accumulation of C18:2t11c15 (Vlaeminck et al., 2008). However, in the latter studies the last hydrogenation step converting C18:1t11 to C18:0 was completely inhibited as reflected by the accumulation of trans octamonoenes and the lack of increase in C18:0, after incubation as compared with C18:0 at the start. Such extensive inhibition was not observed with the current MCFA sources. Moreover, in the current study C12:0+C14:0 from coconut and krabok oil in contrast to pure C10:0 (Goel et al., 2009) do not reduce lipolysis and/or isomerisation of C18:2n-6 and C18:3n-3. Much stronger effects on the accumulation of biohydrogenation intermediates were observed with PUFA sources as fish oil or microalgae (Chow et al., 2004; Boeckaert et al., 2007). Before it has been suggested that CH_4 inhibitors also affect rumen biohydrogenation (Boeckaert et al., 2007; Fievez et al., 2007). However, our results indicate no complete correspondence between the effect of C12:0+C14:0 sources on rumen methanogenesis and biohydrogenation.

Conclusions

The *in vitro* supplementation of C12:0 and C14:0 from krabok oil can decrease CH_4 output to a similar extent as coconut oil, whereas the effect on rumen biohydrogenation is limited to the specific accumulation of C18:2t11c15.

Acknowledgements

This experiment was funded by the EU-community (project TH/Asia-Link/014-141-176). Post-doctoral grant of M. Lourenço funded by the Foundation for Science and Technology - Portugal. Gunjan Goel was funded by the EU-community (project Food-CT-23006-36241, Prosafebeef).

References

Ackman, R.G., Sipos, J.C. 1964. Application of specific response in the gas chromatographic analysis of methyl esters of fatty acids with flame ionization detectors. J. Am. Oil Chem. Soc. 41, 377-378.

Boeckaert, C., Vlaeminck, B., Mestdagh, J., Fievez, V. 2007. *In vitro* examination of DHA-edible micro algae: 1. Effect on rumen lipolysis and biohydrogenation of linoleic and linolenic acid. Anim. Feed Sci. Technol. 136, 63-79.

Chow, T.T., Fievez, V., Moloney, A.P., Raes, K., Demeyer, D., De Smet, S. 2004. Effect of fish oil on *in vitro* rumen lipolysis, apparent biohydrogenation of linoleic and linolenic acid and accumulation of biohydrogenation intermediates. Anim. Feed Sci. Technol. 117, 1-12.

Dohme, F., Machmüller, A., Estermann, B.L., Pfister, P., Wasserfallen, A., Kreuzer, M. 1999. The role of the rumen ciliate protozoa for methane suppression caused by coconut oil. Lett. Appl. Microbiol. 29, 187-192.

Dohme, F., Machmüller, A., Wasserfallen, A., Kreuzer, M. 2001. Ruminal methanogenesis as influenced by individual fatty acids supplemented to complete ruminant diets. Lett. Appl. Microbiol. 32, 47-51.

Fievez, V., Boeckaert, C., Vlaeminck, B., Mestdagh, J., Demeyer, D. 2007. *In vitro* examination of DHA-edible micro algae: 2. Effect on rumen methane production and apparent degradability of hay. Anim. Feed Sci. Technol. 136, 80-95.

Folch, J., Lee, M., Stanley, S.G.H. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497-509.

Getachew, G., Depeters, E.J., Robinson, P.H., Taylor, S.J. 2001. *In vitro* rumen fermentation and gas production: influence of yellow grease, tallow, corn oil and their potassium soaps. Anim. Feed Sci. Technol. 93, 1-15.

Goel, G., Arvidsson, K., Vlaeminck, B., Bruggeman, G., Deschepper, K., Fievez, V. 2009. Effects of capric acid on rumen methanogenesis and biohydrogenation of linoleic and alpha-linolenic acid. Anim. 3, 810-816.

Jordan, E., Lovett, D.K., Monahan, F.J., Callan, J., Flynn, B., O'Mara, F.P. 2006. Effect of refined coconut oil or copra meal on methane output and on intake and performance of beef heifers. J. Anim. Sci. 84, 162-170.

Machmüller, A., Kreuzer, M. 1998. Methane suppression by coconut oil and associated effects on nutrient and energy balance in sheep. Can. J. Anim. Sci. 6, 65-72.

Machmüller, A., Ossowski, D.A., Kreuzer, M. 2000. Comparative evaluation of the effects of coconut oil, oilseeds and crystalline fat on methane release, digestion and energy balance in lambs. Anim. Feed Sci. Technol. 85, 41-60.

Marty, R., Demeyer, D. 1973. The effect of inhibitors of methane production on fermentation pattern and stoichiometry *in vitro* using rumen contents from sheep given molasses. Brit. J. Nutr. 30, 369-376.

Onetti, S.G., Shaver, R.D., McGuire, M.A., Palmquist, D.L., Grummer, R.R. 2002. Effect of supplemental tallow on performance of dairy cows fed diets with different corn silage: alfalfa silage ratios. J. Dairy Sci. 85, 632-641.

Raes, K., De Smet, S., Demeyer, D. 2001. Effect of double-muscling in Belgian Blue young bulls on the intramuscular fatty acid composition with emphasis on conjugated linoleic acid and polyunsaturated fatty acids. Anim. Sci. 73, 253-260.

Soliva, C.R., Hindrichsen, I.K., Meile, L., Kreuzer, M., Machmüller, A. 2003. Effects of mixtures of lauric and myristic acid on rumen methanogens and methanogenesis *in vitro*. Lett. Appl. Microbiol. 37, 35-39.

Soliva, C.R., Meile, L., Hindrichsen, I.K., Kreuzer, M., Machmüller, A. 2004. Myristic acid supports the immediate inhibitory effect of lauric acid on ruminal methanogens and methane release. Anaerobe 10, 269-276.

Van Ranst, G., Fievez, V., Vandewalle, M., Van Waes, C., De Riek, J., Van Bockstaele, E. 2010. Influence of damaging and wilting red clover on lipid metabolism during ensiling and *in vitro* rumen incubation. Anim. 4, 1528-1540.

Vlaeminck, B., Mengistu, G., Fievez, V., de Jonge, L., Dijkstra, J. 2008. Effect of *in vitro* docosahexanoic acid supplementation to marine algae adapted and unadapted rumen inoculum on the biohydrogenation of unsaturated fatty acids in freeze-dried grass. J. Dairy Sci. 91, 1122-1132.

Wolff, R.L., Bayard, C.C., Fabien, R.J. 1995. Evaluation of sequential methods for the determination of butterfat fatty acid composition with emphasis on *trans*-18:1 acids. Application to the study of seasonal variations in French butters. J. Am. Oil Chem. Soc. 72, 1471-1483.

Wongsuthavas, S., Yuangklang, C., Wittayakun, S., Vasupen, K., Mitchaothai, J., Srenanul, P., Beynen, A.C. 2007. Dietary soybean oil, but not krabok oil, diminishes abdominal fat deposition in broiler chickens. Poultry Sci. 6, 792-795.

Chapter 3

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

Paiwan Panyakaew[1,2], J. Thomas Schonewille[3], Veerle Fievez[4], Gunjan Goel[4,5], Nico Boon[6], Chalermpon Yuangklang[7], Wouter H. Hendriks[1,3]

[1]*Department of Animal Sciences, Wageningen University, Wageningen, The Netherlands;*
[2]*Department of Animal Science, Faculty of Natural Resources, Rajamangala University of Technology Isan, Sakon-Nakhon Campus, Sakon Nakhon 47160 Thailand;*
[3]*Department of Farm Animal health, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands;*
[4]*Laboratory for Animal Nutrition and Animal Product Quality (Lanupro), Faculty of Bioscience Engineering, Ghent University, Proefhoevestraat 10, 9090 Melle, Belgium;*
[5]*Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Solan, India;*
[6]*Laboratory of Microbial Ecology and Technology (LabMET), Faculty of Bioscience Engineering, Ghent University, Gent, Belgium;*
[7]*Department of Agricultural Technology and Environment, Faculty of Science and Liberal Arts, Rajamangala University of Technology-Isan, Nakhon Ratchasima, 3000 Thailand.*

Published in Journal of Science and Agricultural Technology 1, 26-32 (2020).

Abstract

Krabok and coconut oil were assessed for their ability to affect rumen protozoa via a 3×3 Latin square design experiment with three rumen cannulated beef cows. The diets consisted of a concentrate 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, 3, 6, 9 and 12 h 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 oil 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.

Key words: 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 (Asanuma et al., 1999; Lila et al., 2003). 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 A.W. Benn) and contains roughly equal amounts of C12:0 and C14:0, *i.e.,* 45% of total fatty acids (Panyakaew et al., 2013a; Wongsuthavas et al., 2007). 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 interpretated 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 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; Machmüller and Kreuzer, 1999; Jordan et al., 2006; Panyakaew et al., 2013b).

Materials and methods

Animals and experimental design

Three beef cows (Brahman × 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 the department of Animal Science, Faculty of Natural Resources, Rajamangala University of Technology Isan, Sakon-Nakhon campus. Sakon Nakhon, is located 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.

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 rations 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 constant intake of non-variable nutrients. The rations were offered daily in two equal portions at 07:30 and 16:00 h, and feed refusals, if any, were recorded.

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.

Rumen sample collection

Rumen contents (~200 ml) were collected on day 23 and 27 of each period at 0, 3, 6, 9 and 12 h 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). A 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 12 h) 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 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 were analyzed by gas chromatography (Schimadzu GC-14A, Belgium) as described by Van Ranst et al. (2010).

Microbiota counts

Total protozoa were counted by means of a haemacytometer (Boeco, Hamburg, Germany) of 0.1 mm depth and a microscope (Model Olympus B×50). 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).

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
C18:1c9	23.5	13.1	2.57	25.9
C18:1c11	1.16	0.42	0.43	0.96
C18:2n-6	0.53	2.63	0.32	42.2
C18:3n-3	0.39	0.02	0.04	0.00
C18:2c9t11	0.37	-	-	0.23

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 0.4 g rumen sample following the protocol for 'Isolation of DNA from 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. By using this specific combination of primers, most of the degeneracies present in the ciliate specific primers 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 $MgCl_2$ (1×), 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 16 h 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 analyzed with the BioNumerics software version 3.5 (Applied Maths, Kortrijk, Belgium). Similarities were calculated by the Pearson correlation, which takes into account the band intensity and band position. The clustering algorithm of Ward was used to calculate dendrograms (Boeckaert et al., 2007).

Statistical analysis

Parameters were statistically analyzed 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 analyzed 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 on the basis of 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 $0.05 \leq P<0.10$.

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 body weights of the animals for experimental periods 1, 2 and 3 were 429, 415 and 422 kg, respectively.

Selected indices of rumen fermentation and protozoa count

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 ratio comparable to the T diet.
Total protozoa count per unit rumen fluid were significantly ($P=0.010$) 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 ($y=1.869\times-3.038$) 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).

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 ($\times 10^5$ 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 ($\times 10^7$ cell/ml)	5.74	6.37	6.49	0.42	0.472
Cellulolytic bacteria ($\times 10^9$ cell/ml)	6.73	7.28	7.69	0.27	0.152
Proteolytic bacteria ($\times 10^7$ 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 ($0.05 \leq P < 0.10$). VFA=volatile fatty acids.

DGGE analysis

The DGGE profile of one sample of a cow fed the KO diet show 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.

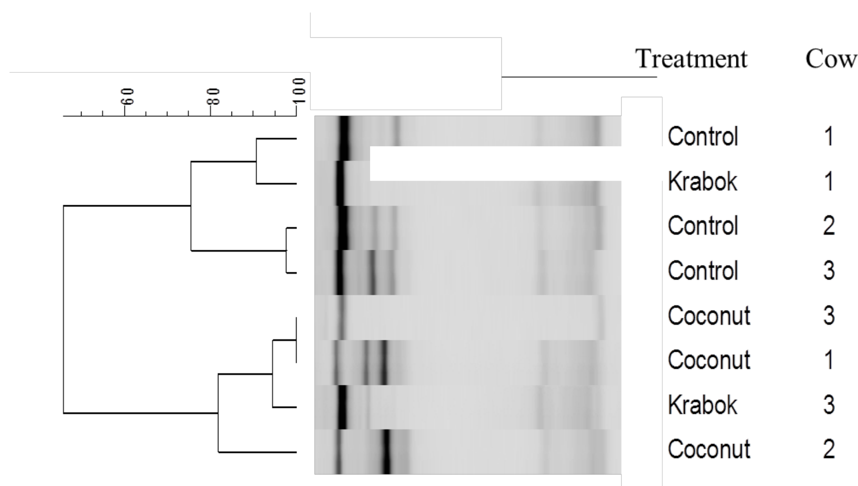


Figure 1. Cluster analysis of the denaturing gel electrophoresis profile of ciliate protozoa present in the rumen of beef cows fed a total mixed ration supplemented with either tallow (control), coconut oil or krabok oil + tallow.

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% were reported by the latter author when 3.5 and 7.0% of coconut oil was 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 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 values 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 was more than two time greater when the CO (1:2.23) instead of KO (1:0.9) diet was fed in the current study (calculated from Table 2), thereby, causing a minor effect of KO on ciliate numbers and DGGE profile (Dohme et al., 2001; Soliva et al., 2003). 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 reduce 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 compared 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 oil 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.

Acknowledgements

This experiment was funded by the EU-community (project TH/Asia-Link/014-141-176) and the Laboratory of Microbial Ecology and Technology (LabMET), Faculty of Bioscience Engineering, Ghent University, Gent, Belgium.

References

Asanuma, N., Iwamoto, M., Hino, T. 1999. Effect of the addition of fumarate on methane production by ruminal microorganisms *in vitro*. J. Dairy Sci. 82, 780-787.

Boeckaert, C., Fievez, V., Vanhecke, D., Verstraete, W., Boon, N. 2007. Changes in rumen biohydrogenation intermediates and ciliate protozoa diversity after algae supplementation to dairy cattle. Eur. J. Lipid Sci. Technol. 109, 767-777.

Boeckaert, C., Jouany, J.P., Lassalas, B., Morgavi, D.P., Vlaeminck, B., Boon, N., Fievez, V. 2008. The rumen protozoon *Isotricha prostoma* and its associated bacteria play no major role in the biohydrogenation of linoleic acid. In *Gut Microbiome: Functionality, Interaction with the Host and Impact on the Environment*. 6th INRA-RRI symposium, Clermont-Ferrand, France, p. 44.

Boon, N., Top, E.M., Verstraete, W., Siciliano, S.D. 2003. Bioaugmentation as a tool to protect the structure and function of an activated-sludge microbial community against a 3-chloroaniline shock load. Appl. Environm. Microbiol. 69, 1511-1520.

Dohme, F., Machmüller, A., Estermann, B.L., Pfister, P., Wasserfallen, A., Kreuzer, M., 1999. The role of the rumen ciliate protozoa for methane suppression caused by coconut oil. Lett. Appl. Microbiol. 29, 187-192.

Dohme, F., Machmüller, A., Wasserfallen, A., Kreuzer, M. 2001. Ruminal methanogenesis as influenced by individual fatty acids supplemented to complete ruminant diets. Lett. Appl. Microbiol. 32, 47-51.

Finlay, B.J., Esteban, G., Clarke, K.J., Williams, A.G., Embley, T.M., Hirt, R.P. 1994. Some rumen ciliates have endosymbiotic methanogens. FEMS Microbiol. Lett. 117, 157-162.

Galyean, M., 1989. Laboratory Procedure in Animal Nutrition Research. 1st edn., Department of Animal and Life Science, New Mexico States University, USA., pp. 162-167.

Goel, G., Arvidsson, K., Vlaeminck, B., Bruggeman, G., Deschepper, K., Fievez, V. 2009. Effects of capric acid on rumen methanogenesis and biohydrogenation of linoleic and alpha-linolenic acid. Anim. 3, 810-816.

Guyader, J., Eugene, M., Noziere, P., Morgavi, D.P., Doreau, M., Martin, C. 2014. Influence of rumen protozoa on methane emission in ruminants: a meta-analysis approach. Anim. 8, 1816-1825.

Hobson, P.N. 1969. Rumen bacteria. In Methods in Microbiology, Vol. 3B, eds. J.R. Norris and D.W. Ribbons. Academic Press, London, pp. 133-149.

Hook, S.E., Wright, A.D.G., Mcbride, B.W. 2010. Methanogens: methane producers of the rumen and mitigation strategies. Archaea 945785.

Hungate, R.E., 1969. A roll tube method for cultivation of strict anaerobes. Meth. Microbiol. 3, 117-132.

Jordan, E., Lovett, D.K., Monahan, F.J., Callan, J., Flynn, B., O'Mara, F.P. 2006. Effect of refined coconut oil or copra meal on methane output and on intake and performance of beef heifers. J. Anim. Sci. 84, 162-170.

Kreuzer, M., 1986. Methodik und Anwendung der Defaunierung beim Wachsenden Wiederkäuer. J. Vet. Med. 33A, 721-745.

Lila, Z.A., Mohammed, N., Ksanda, S., Kamada, T., Itabashi, H., 2003. Effect of sarsaponin on ruminal fermentation with particular reference to methane production *in vitro*. J. Dairy Sci. 86, 3330-3336.

Machmüller, A., Kreuzer, M. 1999. Methane suppression by coconut oil and associated effects on nutrient and energy balance in sheep. Can. J. Anim. Sci. 79, 65-72.

Machmüller, A., Soliva, C.R., Kreuzer, M. 2003. Effect of coconut oil and defaunation treatment on methanogenesis in sheep. Reprod. Nutr. Develop. 43(1), 41-55.

Machmüller, A. (2006). Medium-chain fatty acids and their potential to reduce methanogenesis in domestic ruminants. Agric. Ecosys. Environm. 112, 107-114.

Panyakaew, P., Goel, G., Lourengo, M., Yuangklang, C., Fievez, V. 2013a. Medium-chain fatty acids from coconut or krabok oil inhibit *in vitro* rumen methanogenesis and conversion of non-conjugated dienoic biohydrogenation intermediates. Anim. Feed Sci. Technol. 180, 18-25.

Panyakaew, P., Goel, G., Yuangklang, C., Boon, N., Schonewille, J.Th., Hendriks, W.H., Fievez, V. 2013b. Effect of supplementing coconut or krabok oil, rich in medium-chain fatty acids on ruminal fermentation, protozoa and archaeal population of bulls. Anim. 7(11), 1950-1958.

Van Ranst, G., Fievez, V., Vandewalle, M., Van Waes, C., De Riek, J., Van Bockstaele, E. 2010. Influence of damaging and wilting red clover on lipid metabolism during ensiling and *in vitro* rumen incubation. Anim. Sci. 4, 1528-1540.

Soliva, C.R., Hindrichsen, I.K., Meile, L., Kreuzer, M., Machmüller, A. 2003. Effects of mixtures of lauric and myristic acid on rumen methanogens and methanogenesis *in vitro*. Lett. Appl. Microbiol. 37, 35-39.

Wongsuthavas, S., Yuangklang, C., Wittayakun, S., Vasupen, K., Mitchaothai, J., Srenanul, P., Beynen, A.C. 2007. Dietary soybean oil, but not krabok oil, diminishes abdominal fat deposition in broiler chickens. Poultry Sci. 6, 792-795.

Chapter 4

Effect of supplementing coconut or krabok oil, rich in medium-chain fatty acids on ruminal fermentation, protozoa and archaeal population of bulls

Paiwan Panyakaew[1,2,3], Nico Boon[2], Gunjan Goel[3,5], Chalermpon Yuangklang[2], J. Thomas Schonewille [6], Wouter Hendriks[1,6], Veerle Fievez[1,a]

[1]*Department of Animal Sciences, Wageningen University, Wageningen, The Netherlands;*
[2]*Department of Animal Science, Faculty of Natural Resources, Rajamangala University of Technology Isan, Sakon-Nakhon Campus, Sakon Nakhon 47160 Thailand;*
[3]*Department of Animal Production, Laboratory for Animal Nutrition and Animal Product Quality (Lanupro), Faculty of Bioscience Engineering, Ghent University, Proefhoevestraat 10, 9090 Melle, Belgium;*
[4]*Department of Biochemical and Microbial Technology, Laboratory of Microbial Ecology and Technology (LabMET), Faculty of Bioscience Engineering, Ghent University, Gent, Belgium;*
[5]*Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Solan, India;*
[6]*Department of Farm Animal Health, Animal Nutrition Division, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands*

Published in Animal 7:12, 1950-1958 (2013).

Abstract

Medium-chain fatty acids (MCFA), for example, capric acid (C10:0), myristic (C14:0) and lauric (C12:0) acid, have been suggested to decrease rumen archaeal abundance and protozoal numbers. This study aimed to compare the effect of MCFA, either supplied through krabok (KO) or coconut (CO) oil, on rumen fermentation, protozoal counts and archaeal abundance, as well as their diversity and functional organization. KO contains similar amounts of C12:0 as CO (420 and 458 g/kg FA, respectively), but has a higher proportion of C14:0 (464 v. 205 g/kg FA, respectively). Treatments contained 35 g supplemental fat per kg DM: a control diet with tallow (T); a diet with supplemental CO; and a diet with supplemental KO. A 4th treatment consisted of a diet with similar amounts of MCFA (*i.e.,* C10:0+C12:0+C14:0) from CO and KO. To ensure isolipidic diets, extra tallow was supplied in the latter treatment (KO+T). Eight fistulated bulls (two bulls per treatment), fed a total mixed ration predominantly based on cassava chips, rice straw, tomato pomace, rice bran and soybean meal (1.5% of BW), were used. Both KO and CO increased the rumen volatile fatty acids, in particular propionate and decreased acetate proportions. Protozoal numbers were reduced through the supplementation of an MCFA source (CO, KO, and KO + T), with the strongest reduction by KO. Quantitative real-time polymerase chain reaction assays based on archaeal primers showed a decrease in abundance of Archaea when supplementing with KO and KO + T compared with T and CO. The denaturing gradient gel electrophoresis profiles of the rumen archaeal population did not result in a grouping of treatments. Richness indices were calculated from the number of DGGE bands, whereas community organization was assessed from the Pareto–Lorenz eveness curves on the basis of DGGE band intensities. KO supplementation (KO and KO + treatments) increased richness and evenness within the archaeal community. Further research including methane measurements and productive animals should elucidate whether KO could be used as a dietary methane mitigation strategy.

Key words: Krabok oil, coconut oil, rumen protozoa, *Archaea*, microbial community organization

Implication

Krabok seeds are widely available in South-East Asian forests, contain large amounts of fat and as such are of interest to be used as a fat source in animal diets by local farmers. As krabok oil (KO) almost exclusively consists of lauric and myristic acid, it not only should be considered an energy supplier and its potential as modifier of the rumen microbial community was assessed here. KO showed potential to reduce rumen protozoal and archaeal numbers and shift rumen fermentation towards more propionate at smaller quantities than coconut oil (CO), another more common source of lauric and myristic acid.

Introduction

Lauric (C12:0) and myristic (C14:0) acid show potential to reduce the number of rumen *Archaea* and protozoa (*e.g.,* Dohme et al., 1999; Machmüller et al., 2003b; Soliva et al., 2003; Klevenhusen et al., 2011), which make these medium-chain fatty acids (MCFA) important candidates for dietary methane mitigation in ruminants. The reduction in archaeal numbers might be partially because of the defaunating effect of MCFA (Dohme et al., 2001), as some methanogens are endosymbionts or ectosymbionts of protozoa (Finlay et al., 1994), although independent action of MCFA sources on rumen *Archaea* has also been reported (Dohme et al., 1999). From batch *in vitro* incubations, C12:0 has been identified as the most powerful of the two MCFAs (Soliva et al., 2003, 2004). In contrast, Rusitec and *in vivo* data (*e.g.,* Dohme et al., 2001; Machmüller et al., 2003b; Klevenhusen et al., 2011) indicated a similar effect of C12:0 and C14:0 on rumen *Archaea*, although protozoal numbers were suppressed less by C14:0 than by C12:0 (Dohme et al., 2001). *In vivo* changes in archaeal numbers through the addition of coconut oil (CO), which is particularly rich in C12:0 (470 g/kg FA) and C14:0 (180 g/kg FA), are equivocal (Machmüller et al., 2003a; Pilajun and Wanapat, 2011). Furthermore, not only the total number of *Archaea*, but also the composition of the methanogenic population might be altered by MCFA supplementation, for example, studies using order-specific probes revealed *Methanococcales* to be less sensitive to C14:0 (Machmüller et al., 2003b), whereas C12:0 seemed to have a greater effect on the free-living *Methanomicrobiales* than on the *Methanobacteriales*, which are frequently associated with protozoa (Klevenhusen et al., 2011). Similarly, CO changed the diversity, dominance, and functional organization of *Archaea* during an *in vitro* batch study, as evidenced from DGGE profiles (Patra and Yu, 2013), although Hristov et al. (2009), using the same molecular approach, could not attribute specific changes in abundance of methanogens to CO supplementation to dairy cows. Although former observations indicate a different mode of action of C12:0 and C14:0, most comparative studies were conducted *in vitro*, whereas *in vivo* studies mainly used either C12:0 (*e.g.,* Klevenhusen et al., 2011), C14:0 (*e.g.,* Machmüller et al., 2003b) or CO (*e.g.,* Hristov et al., 2009), with a fixed C12:0/C14:0 ratio. However, in practice, dietary fats with significant amounts of C14:0 in addition to C12:0 might be of major importance because of the lower palatability of C12:0 (Soliva et al., 2004). Krabok oil (KO), an oil from krabok seeds (*Irvingia malayana* Oliv. ex A.W. Benn), is such a dietary oil source that contains similar amounts of C12:0, but is particularly richer in C14:0 (464 g/kg FA) as compared with CO. Hence, the comparison of two natural oil sources with similar amounts of C12:0 but varying in C14:0 content is considered of practical relevance.

The objective of this *in vivo* study was to compare the effect of CO and KO on rumen fermentation pattern, number of protozoa and *Archaea*, as well as their diversity and functional organization. Unfortunately, the experimental infrastructure did not allow for concomitant monitoring of methane emissions.

Materials and methods

Animals

Eight rumen-fistulated bulls were used in a 4×4 Latin square design (Table 1). The experimental diets were fed as a total mixed ration (TMR) and consisted of (g/kg TMR): 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; oyster meal, 5.3; mineral premix, 5.3; sulfur, 3.2. Water was provided *ad libitum*. The animals were adjusted to the TMR for 14 days followed by 4 experimental periods of 21 days (Table 1), which were separated from each other by a wash-out period of 21 days during which the basal diet was fed without external fat. At the beginning of each experimental period, the bulls were weighed to adjust the feed supply to 1.5% of BW, which was kept constant during the experimental period and supplied in two daily meals. Rumen sampling took place between day 15 and 21 of the experimental period.

Table 1. Overview of the sequence of experimental treatments in a 4×4 Latin square design experiment with four treatments (supplementation (35 g/kg DM) with either beef T, CO, KO or KO+T) and eight bulls (B) in each of the four periods (P), the amounts of oil added to each treatment and the supply of medium-chain fatty acids (C10:0, C12:0 and C14:0) from CO or KO (average ± sd of four batches of CO and KO).

Period	T	CO	KO	KO+T
P1	B1, B2	B3, B4	B5, B6	B7, B8
P2	B5, B6	B1, B2	B7, B8	B3, B4
P3	B3, B4	B7, B8	B1, B2	B5, B6
P4	B7, B8	B5, B6	B3, B4	B1, B2
Tallow (g/kg DM)	35	0	0	6
Coconut oil (g/kg DM)	0	35	0	0
Krabok oil (g/kg DM)	0	0	35	29
Total C10:0+C12:0+C14:0 (g/kg DM)	0	23.9±0.76	31.5±0.67	26.1±0.56

T=tallow; CO=coconut oil; KO=krabok oil.

Treatments

The four isolipidic diets in this study were: (1) TMR with 35 g/kg DM of beef tallow (T); (2) TMR with 35 g/kg DM of coconut oil (CO); (3) TMR with 35 g/kg DM of krabok oil (KO); and (4) TMR with 29 g/kg DM of krabok oil+6 g/kg DM tallow (KO+T). Treatment KO+T was designed in an attempt to provide similar amounts of MCFA as by CO (Table 1). Besides C12:0 and C14:0, C10:0 was also included in this calculation as the latter has been shown to effectively reduce methane production and/or the number of methanogens and protozoa (Dohme et al., 2000, 2001; Goel et al., 2009). Dietary inclusion of oil supplements in the KO+T treatment was based on fatty-acid compositions of the oils used in a concomitant *in vitro* experiment (Panyakaew et al., 2013). Although oils were from a similar source for the

current *in vivo* experiment, the composition of the four oil batches used in the current study deviated slightly (Table 2). Nevertheless, differences were small, allowing the original objectives to be assessed, as MCFA supplies of the treatments CO and KO+T did not differ significantly (P>0.05) (Table 1).

Rumen sample collection

Rumen contents were collected on day 15, 18, 21 of each period at 4 and 8 h after the morning feeding (0700 h) by sampling in different directions of the rumen. Samples were divided into three portions: the first portion of rumen contents (10 ml) that was stored at -80 °C until DNA extraction, the second portion rumen aliquot (10 ml) was acidified with 0.2 ml of phosphoric:formic acid (10:1) and stored at -20 °C until VFA analysis, and the third portion of rumen contents (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.

Table 2. Fatty-acid composition (g/100 g fatty acids) of the CO, KO, T and TMR.

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

CO=coconut oil; KO=krabok oil; T=tallow; TMR=total mixed ration.

VFAs

Samples for VFA analysis were pooled per day and cow, centrifuged at 15000×g (4 °C) for 10 min (HKFI Ltd. Mainland, China). The supernatant was filtered through a nylon membrane (0.2 µm) and transferred to vials. The VFA analysis was performed using gas chromatography (Schimadzu GC-14A, ’s-Hertogenbosch, The Netherlands) (Van Ranst et al., 2010).

Protozoal counts

Microscopical counting was done using a haemacytometer (Boeco, Hamburg, Germany) of 0.1 mm depth. Twenty aliquots per sample were counted (Galyean, 1989).

Archaea *analysis*

DNA extraction

The rumen samples were pooled per day and bull before DNA extraction. Total DNA was extracted from 0.4 g rumen contents using the QIAamp Stool Kit DNA extraction method following the protocol for 'Isolation of DNA from stool for Pathogen Detection' in the handbook supplied by Qiagen Ltd. (Crawley, UK), according to the manufacturer's recommendation.

PCR for denaturing gradient gel electrophoresis (DGGE) analysis

Amplification of a fragment of the 16S rDNA gene of *Archaea* for DGGE was performed using a single PCR with a forward GC-ARCH915 and reverse UNI-b-rev primer as described by Yu et al. (2008). The PCR mixture was based on the one described by Yu and Morrison (2004), with adjustments according to the manufacturer's instructions (Fermentas, St. Leon-Rot, Germany). PCR amplifications were performed in 25-µl volumes containing KCl (50 mM), MgCl2 (1.5 mM), dNTP mix (200 µM), both primers (200 nM each), 0.6 U Taq DNA polymerase, 480 ng bovine serum albumin/µl, 1 µl DNA extract and 2.5 µl 10X Taq buffer. Adjustments to 25 µl were made with DNase-, RNase-free filter-sterilized water (Sigma, Bornem, Belgium). Amplification conditions were according to Yu and Morrison (2004) with small adjustments: initial denaturation at 94 °C for 5 min; 10 cycles of touch-down PCR (denaturation at 94 °C for 0.5 min, annealing for 0.5 min with an 0.5 °C/cycle decrement at a temperature of 5 °C above the annealing temperature of 70 °C and DNA synthesis at 72 °C for 1 min); 25 cycles of regular PCR (denaturation at 94 °C for 0.5 min, annealing at 65 °C for 0.5 min, DNA-synthesis at 72 °C for 1 min); and a final extension step at 72 °C for 10 min.

DGGE analysis

Archaea-specific PCR fragments were loaded onto a 7.5% (w/v) polyacrylamide gel with denaturing gradients ranging from 45% to 60%. The electrophoresis was run for 16 h at 60 °C and 50 V. DGGE patterns were visualized by staining with SYBR Green I nucleic acid gel stain (Molecular Probes, Eugene OR, USA). Each gel included all treatments (n=4) and sampling days (n=3) per bull. To normalize the differences among gels, *Archaea*-specific fragments of one rumen reference sample were loaded in triplicate on each gel that was used as a standard for comparison of gels. The obtained DGGE patterns were analyzed with the BioNumerics software version 3.5 (Applied Maths, Kortrijk, Belgium). Similarities were calculated by the Pearson correlation. The clustering algorithm of Ward was used to calculate dendrograms (Boeckaert et al., 2007). The DGGE profiles allow to gain insight into changes in diversity and functional organization of rumen microbial communities, which are assessed here through various indices and approaches: (i) the range-weighted richness (Rr), calculated according to Boon et al. (2011) based on the total number of bands (N), and the denaturing gradient comprised between the first and the last band of the pattern (Dg) as: $Rr=N^2\times Dg$; (ii) the Shannon (H), (iii) dominance (c) and (iv) evenness index (e), calculated as described by

Patra et al. (2012) based on the peak surface of each band (n_i), the sum of all peak surfaces of all bands (N) and the total number of bands (S): $H=-\Sigma[(n_i/N)\times\ln(n_i/N)]$; $c=\Sigma(n_i/N)^2$; $e=H/\ln(S)$; (v) the Pareto-Lorenz evenness curves (PL curves), based on the DGGE profile using the Lorenz-Gini method (Mertens et al., 2005; Marzorati et al., 2008; Wittebolle et al., 2009) and constructed by ranking the respective DGGE bands for each sample from high to low intensity and using the cumulative proportion of bands and their respective cumulative proportions of intensities as X- and Y-axis; and (vi) the community organization, which describes the evenness (equality) of the archaeal population and is the normalized area between a given PL-curve and the perfect evenness line. Community organization ranges from 0 to 100 and represents a single value that describes a specific degree of evenness. The higher the community organization coefficient, the more uneven a community (Boon et al., 2011).

Quantitative PCR (qPCR)

Archaea rRNA gene copies present in the DNA extract of ruminal digesta samples were quantified as described by Boeckaert et al. (2007) and Boon et al. (2003), using an ABI Prism SDS 7000 instrument (Applied Biosystems, Lennik, Belgium). Amplification reactions were carried out in 25 µl volumes containing 12.5 µl SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK), 6 µl RNA-free water and 5 µl diluted (1:20) DNA extract, as well as the ARCH915 and UNI-b-rev primers at a final concentration of 300 nM each. Measurements were taken in triplicate for each run. A standard curve for qPCR was constructed using six different DNA concentrations (n=3), ranging from 1.13 copies to 1.13×10^8 copies of DNA per µl. An *Archaea* 492 bp PCR fragment inserted in a TOPO vector was used as a template for the standard curve. Average amplification efficiency, slope and R^2 of qPCR assays were 102% (calculated as $E=10^{(-1/slope)}-1$), -3.27 and 0.99, respectively.

Statistical analysis

Before statistical analysis, protozoa numbers were averaged over a day (*i.e.*, average of 3, 6, 9 and 12 h after feeding) and log transformed. Daily average protozoal numbers, VFA concentrations and proportions, the effective degradability, archaeal log copies from qPCR and the indices as calculated from the DGGE profiles were subjected to a linear mixed model using SPSS 21 (SPSS Inc. IBM, Chicago, USA), according to the model:

$$Y_{ijk}=\mu+A_i+B_j+C_k+\varepsilon_{ijk}$$

were Y_{ijk}=the overall response; μ=the overall of mean; A_i=the mean effect of treatment (i=1-4) (fixed factor); B_j=the mean effect of the period (j=1-4) (random factor); C_k=the mean effect of animal (k=1-8) (random factor) and ε_{ijk}=the residual error. Differences between means were considered significant at the $P<0.05$ level, tendencies at $P<0.01$. Treatment differences were distinguished using a two-sided Dunnett's test.

Results

Rumen fermentation characteristics

Treatments with MCFA resulted in a higher concentration of total VFA (P<0.05), a lower proportion of acetate (P<0.01) and higher proportion of propionate (P<0.01) in comparison with the control treatment T (Table 3). Feeding CO and KO+T diets, resulted in a similar proportion of acetate and propionate. The KO diet was associated with the lowest proportion of acetate (P<0.05) and the highest proportion of propionate (P<0.001) and minor VFA (iso-butyrate, valerate and iso-valerate) (P<0.05). CO and KO+T also tended to increase proportions of minor VFA (P<0.10) as compared with T.

Table 3. Total VFA concentrations and proportions of individual VFA in the rumen samples of bulls on a total mixed ration supplemented (35 g/kg DM) with either beef T, CO, KO or a combination of KO+T.

Fatty acid	T	CO	KO	KO+T	SEM
Total VFA (mmol/l)	73.7^{a}	80.3^{b}	79.6^{b}	80.7^{b}	7.16
Acetate (mmol/mol total VFA)	650^{a}	617^{b}	597^{b}	609^{b}	17.8
Propionate (mmol/mol total VFA)	179^{a}	220^{b}	252^{c}	231^{bc}	24.6
Butyrate (mmol/mol total VFA)	140^{a}	131^{ab}	117^{c}	128^{b}	12.9
Other VFA (mmol/mol total VFA)*	30^{a}	32^{+b}	35^{b}	32^{+b}	3.3

VFA=volatile fatty acid; CO=coconut oil; KO=krabok oil.
$^{a-c}$ Means within a row with different superscripts differ (P<0.05).
$^{+b}$ Means within a row with different superscripts tend to differ (P<0.10).
* Include isobutyrate, isovalerate and valerate.

Protozoa

All treatments supplemented with a MCFA source (CO, KO and KO+T) showed a lower number of protozoa than the control treatment (T). Treatments with the same level of MCFA reduced the protozoa numbers to a similar extent (0.17 and 0.15 log units for CO and KO+T, respectively) (Table 4), whereas KO (with the highest levels of MCFA) had the lowest number of protozoa, which represented a decrease by 0.30 log unit compared with the control (T).

Molecular characterization of Archaea

Quantitative real-time PCR assays showed a decrease in numbers of *Archaea* (P<0.05 and P=0.06) when supplementing krabok oil (KO and KO+T) compared with the CO and control treatment (T) (Table 4). The DGGE profile of the archaeal population in the rumen did not result in a grouping of treatments, nor of animals (Figure 1). This could be caused by inter-animal variation in the methanogenic population. To check this, the DGGE profile of the *Archaea* present in the rumen when animals received the control treatment (T treatment) is presented (Figure 2). Three main groups of archaeal profiles with differences in predominant

bands were observed. This clustering was not determined by the prior treatment (Table 1) and did not represent bull pairs receiving the same treatment sequence (Table 1), suggesting both the washout and the adaptation period, guaranteeing an interval of at least 35 days before rumen sampling, were enough to remove the effect of the prior treatment in the current study. To evaluate whether stress, such as the supplementation of a MCFA source provoked shifts in diversity and organization of the archaeal population, irrespective of the inter-animal variation, several indices were calculated from the DGGE profiles (Table 4).
Greater differences were observed between KO (KO and KO+T treatments) and CO supplementation than between any of the MCFA treatments and T. The rumen of bulls receiving KO (both KO and KO+T treatments) seemed colonized by a more diverse (based on the range-weighted richness, Rr and Shannon index, H) and less-dominated (based on the dominance index and the community organization) archaeal community (Table 4). The latter was also illustrated in the PL curves (Figure 3): in case of CO supplementation, 30% of all bands of the archaeal community accounted for 80% of their cumulative abundance, whereas 40% of all bands were required to cover this level of abundance when supplementing with KO+T.

Table 4. Protozoa counts, qPCR of *Archaea* and diversity and community structure indices of rumen *Archaea*, on the basis of archaeal DGGE profiles (range-weighted richness, Shannon H-index, evenness, dominance, and community organization) of the rumen samples of bulls on a TMR supplemented (35 g/kg DM) with either beef T, CO, KO or KO+T.

Item	T	CO	KO	KO+T	SEM
Protozoa ($\times 10^5$/ml)	9.2^{a}	6.3^{b}	4.5^{c}	6.5^{b}	2.41
Archaea (log copy/g rumen sample)	5.31^{a}	5.33^{a}	4.75^{b}	5.09^{+b}	0.231
Range-weighted richness	4.96^{a}	4.92^{a}	5.69^{b}	5.75^{b}	0.270
Shannon H-index	1.32^{ab}	1.25^{b}	1.39^{+b}	1.45^{a}	0.635
Evenness	0.80	0.79	0.78	0.84	0.036
Dominance	0.32^{ab}	0.37^{a}	0.31^{ab}	0.29^{b}	0.028
Community organization	61.2^{ab}	63.6^{a}	57.5^{bc}	53.2^{c}	2.18

DGGE=denaturing gradient gel electrophoresis; TMR=total mixed ration; T=tallow; CO=coconut oil; KO=krabok oil.
$^{a-c}$ Means within a row with different superscripts differ (P<0.05).
$^{+b}$ Means within a row with different superscripts tend to differ (P<0.10).

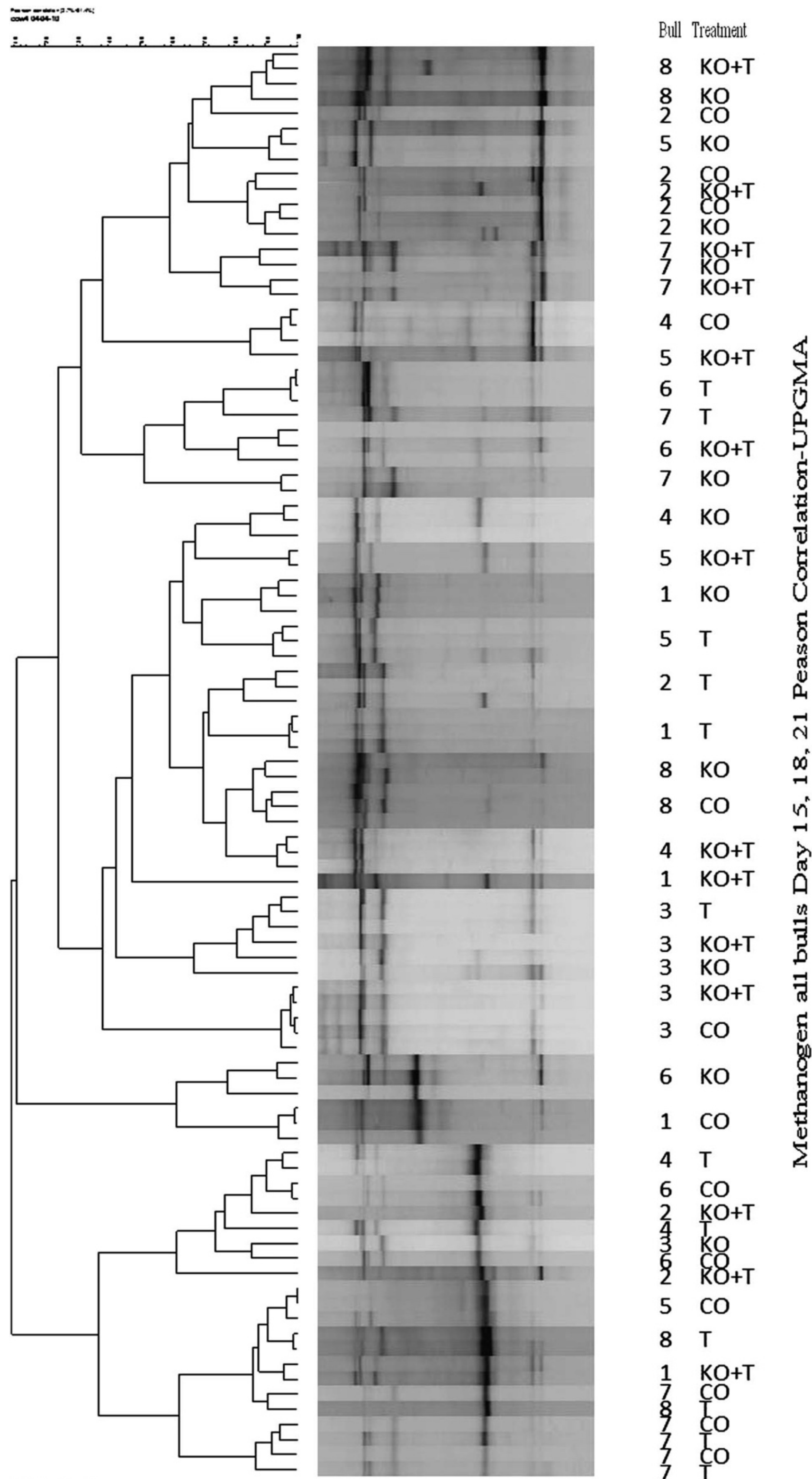


Figure 1. Cluster analysis of the denaturing gradient gel electrophoresis (DGGE) profile of *Archaea* present in the rumen of bulls on a total mixed ration supplemented (35 g/kg DM) with either beef tallow (T), coconut oil (CO), krabok oil (KO) or KO+T. To guarantee readability, the reference numbers of 'bull' and 'treatment' codes were combined when replicates clustered, as indicated by vertical lines next to the profile.

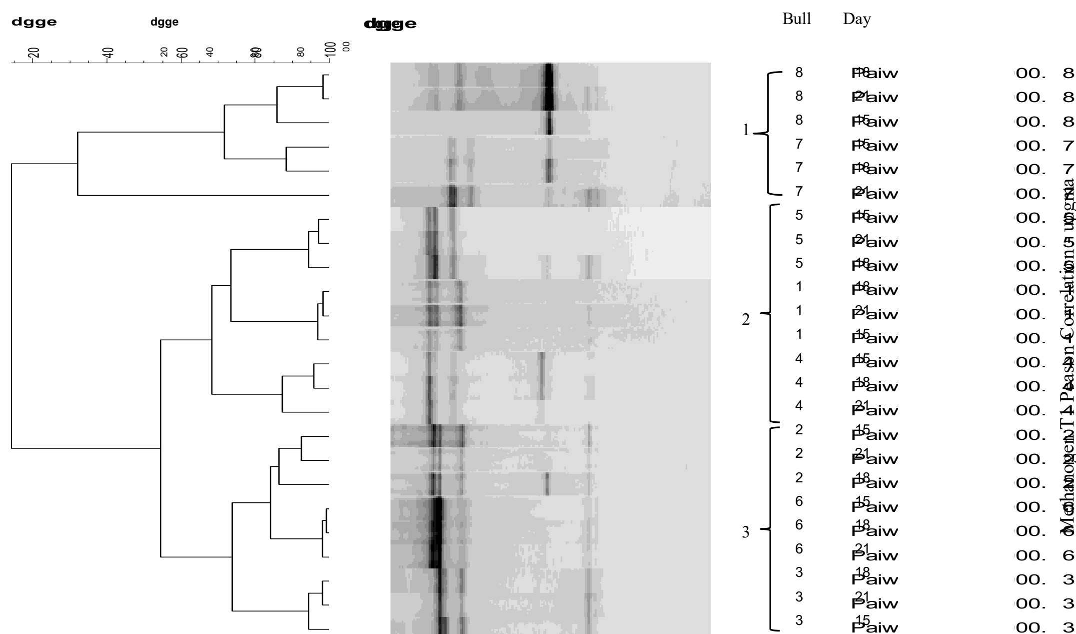


Figure 2. Denaturing gradient gel electrophoresis (DGGE) profile indicates diversity in the rumen archaeal community, despite uniform diet fed to all bulls (total mixed ration supplemented with 35 g/kg DM of beef tallow). Three major clusters can be distinguished, indicated as 1 (grouping bull 7 and 8), 2 (grouping bulls 1, 4 and 5) and 3 (grouping bulls 2, 3 and 6).

Discussion

Coconut oil and KO markedly increased propionate proportions (23 to 41%) at the expense of acetate and decreased the numbers of protozoa in the rumen. This is in line with *in vitro* studies by Panyakaew et al. (2013), who reported that MCFA supply by CO and KO (0.8 or 1.2 mg/ml of incubation fluid) increased the proportion of propionate by 35 to 49%, which was accompanied by an inhibition in methane production of 13 to 38%. Propionate formation is an alternative to H_2 formation, which is typically favored when concentrations of dissolved H_2 increase as hydrogen gas formation under these circumstances becomes thermodynamically unfavorable (Janssen, 2010). As the latter is the main energy source of methanogenic *Archaea*, greater propionate proportions might be indicative of less CH_4 formation per unit of rumen-fermented feed (Janssen, 2010).

The number of protozoa decreased by 0.15 to 0.31 log units in the CO and KO supplemented diets compared with the control diet. Defaunating effects of CO have been reported before: for example, a reduction in ciliate protozoa counts of 0.91 and 1.46 log units were reported in

sheep when supplementing 35 and 70 g/kg DM of CO (Machmüller and Kreuzer, 1999). Another study from the same group (Machmüller et al., 2000) reported a 0.59 log unit decrease in protozoal numbers when sheep were fed CO at a concentration of 56 g/kg DM, which is 1.5 times more than the amount used in our study. A similar supplementation rate (50 g/kg DM) to dairy cows, provoked a decrease of 1.05 log units in total protozoa, with a shift in the protozoal community towards *Epidinium* and *Isotricha*, whereas *Entodinium* and *Dasytricha* seemed more sensitive to CO (Reveneau et al., 2012).

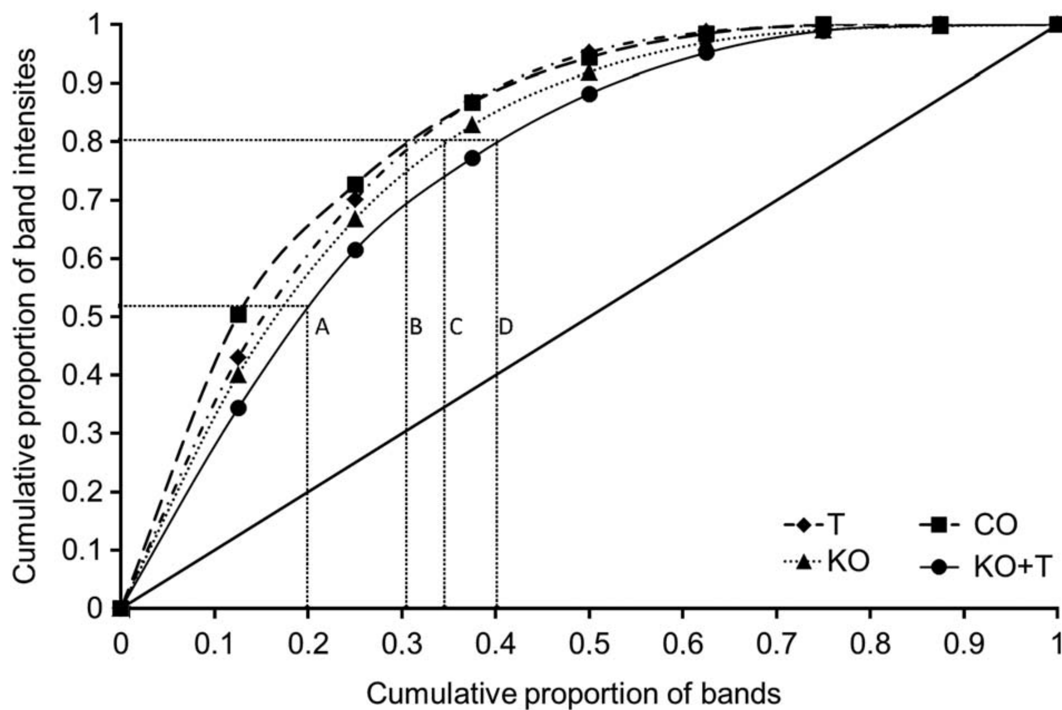


Figure 3. Pareto-Lorenz distribution curves based on denaturing gradient gel electrophoresis (DGGE) profiles of archaeal communities in the rumen of bulls supplemented (35 g/kg DM) with either beef tallow (T), coconut oil (CO), krabok oil (K) or KO+T. Line A indicates the cumulative band intensity corresponding to a combination of 20% of all the bands for the KO+T treatment (52%). Lines B, C and D indicate the proportion of bands required to cover 80% of the cumulative band intensities for T and CO (30.2%), KO (33.3%) and KO+T (40.0%) treatments, respectively.

In their study on the effect of individual fatty acids, Dohme et al. (2001) suggested C10:0 and C12:0 to provoke an equally effective defaunating effect, whereas C14:0 did not reduce protozoal numbers. However, the supplementation rates of C10:0+C12:0 in the CO treatment were highest (16.8 g/kg DM), whereas the KO and KO+T treatments supplied lower amounts of these MCFA (15.3 and 12.6 g/kg DM, respectively). Nevertheless, the KO treatment resulted in the strongest effect on protozoal numbers, whereas reductions by the CO and KO+T treatments were similar, which is in line with the total amount of C10:0+C12:0+C14:0 supplemented (Table 1). The latter might suggest C14:0 to be equally effective in reducing protozoal numbers as C10:0 and C12:0 or a supportive effect of C14:0 when combined with

C12:0 and C10:0, as has been suggested for ruminal methanogens and methane production (Soliva et al., 2004).

Cluster analysis on the DGGE profiles of rumen *Archaea* from bulls monitored in the current study (supplementation of maximum 35 g/kg CO or KO) showed no treatment grouping. The result was similar to that of Kongmun et al. (2011), supplying a somewhat lower dose (26 g/kg of CO) to rumen fistulated swamp buffalo bulls. The latter supplementation rate also did not change the archaeal numbers as assessed by real-time PCR (qPCR). Similarly, in the current study, no change in the archaeal numbers were induced by CO supplementation. Nevertheless, supplementing these levels of KO (35 g/kg DM) reduced the archaeal numbers (-0.56 log copies/g rumen contents). Even when reducing the level of krabok oil (KO+T) to supply similar amounts of MCFA as through CO, archaeal numbers tended to be lower (-0.22 log copies/g rumen contents). This would suggest C14:0 to be more powerful in reducing methanogenic numbers than C12:0, as the main difference between KO and CO is its greater C14:0 content. This is in contrast with what has been suggested by Soliva et al. (2003 and 2004), from their *in vitro* studies using the Hohenheim gas test. They reported a stronger decrease in the number of rumen *Archaea* with an increasing proportion of C12:0. A 2/1 proportion of C12:0/C14:0 (a ratio which is similar to what is observed for CO) decreased the total archaeal counts by 1.54 log units, whereas a 1/1 ratio (similar to that found in KO) decreased total *Archaea* by 0.44 log units (Soliva et al., 2003). In a follow-up study (Soliva et al., 2004), this was confirmed, although a synergistic effect of C12:0 in combination with C14:0 was suggested, with archaeal numbers being lowest for a C12:0/C14:0 of 4/1 and 3/2. Differences between the two *in vitro* studies and our study might be related to the nature of the experiment (*i.e., in vitro* vs. *in vivo*), where *in vitro* methodology eventually reflects a more acute action, whereas *in vivo* measurements were taken after at least 2 weeks of supplementation of the MCFA source. Lower (acute) toxicity of C14:0 has been proposed to potentially induce a lag-time in response, which might result in an underestimation of its effectiveness when assessed through batch *in vitro* incubations (Soliva et al., 2004).

In the current study, there was no noticeable relationship between the protozoal population, which was affected by both CO and KO, and the archaeal population. Similarly, in the Rusitec study on CO (54 g/kg DM) (Dohme et al., 1999), the decrease in archaeal numbers occurred, irrespective of the faunating status (defaunated or faunated) of the rumen inoculum. This could suggest MCFA to act independently on protozoa and methanogens.

As the cluster analysis of DGGE data of the archaeal community did not allow distinction across treatments, we assessed whether treatment affected the diversity of the archaeal community on the basis of measures such as richness (Rr and H) and community organization (evenness, dominance and community organization) (Table 4). For all parameters, contrasts were largest between the CO and KO+T treatments, whereas the KO and KO+T treatments did not differ. Richness (Rr and H), as assessed from the number of DGGE bands, was increased by KO supplementation, whereas CO supplementation did not change diversity indices. This is in contrast with Pilajun and Wanapat (2011), who suggested an increased diversity of methanogens, from their *in vivo* study on CO (36 g/kg DM) supplemented to swamp buffaloes, although no indices were calculated from the DGGE profiles presented in their paper, or at least these were not shown. Furthermore, the archaeal community showed a more dominated structure (greater dominance and community organization) when

supplementing CO as compared with KO or KO+T. Increases in dominance and community organization were also shown when supplementing CO *in vitro* (Patra and Yu, 2013). Nevertheless, a recent *in vivo* study on dairy cattle fed lauric acid, showed a decrease in the prevalence of *Methanobrevibacter*, the most predominant *Archaea*, whereas the prevalence of the less-abundant *Methanosphaera* increased (Hristov et al., 2012). This reduction in dominance within the archaeal community was hypothesized to be linked to strong decreases (about 2 log-units) in protozoal numbers with which *Methanobrevibacter* might be associated. However, in our study, protozoal numbers were reduced both by CO, KO and KO+T treatments, whereas treatments containing krabok oil (KO and KO+T treatments) showed greater diversity and reduced dominance as compared with the CO treatment. Hence, differences in archaeal diversity and dominance seemed independent of changes in protozoal numbers in our study. Morgavi et al. (2012) showed that the DGGE profiles of the rumen methanogenic community only differed between faunated and defaunated animals when protozoa had been removed for more than 2 years, whereas short-term defaunation had not yet provoked changes in the DGGE profiles generated from rumen methanogens. In our study, changes in organization and diversity of the archaeal community seemed to be more linked to reductions in archaeal numbers (provoked only by KO and KO+T treatments) rather than reductions in protozoal numbers (provoked both by CO, KO and KO+T treatments). Obviously, it would be of utmost interest to link changes in microbial community diversity and organization to function (*i.e.,* methane production in this case) in the future, as reduced dominance and larger diversity were suggested to allow methanogens to adapt more easily to different niches (*e.g.,* free-living, particulate associated, ectosymbionts or endosymbionts of protozoa) and fluctuating substrate concentrations (in particular H_2) (Attwood et al., 2011), which might be induced by methane mitigation strategies such as oil supplementation (Janssen, 2010).
Although both *in vivo* and *in vitro* research suggested C14:0 to be less effective in modifying rumen metabolism (*e.g.,* Hristov et al., 2012), KO, which is particularly enriched in C14:0, seemed to induce shifts in rumen fermentation patterns, protozoal numbers and archaeal abundance, diversity and community structure at lower doses than CO. Further research should elucidate whether KO could be used as a methane mitigation strategy as rumen methanogen numbers do not always seem a key factor determining CH_4 production (*e.g.,* Popova et al., 2011).

Conclusions

Both krabok oil and coconut oil supplementation shifted rumen fermentation towards propionate at the expense of acetate, without changing rumen degradability. Both MCFA sources reduced protozoal numbers. However, decrease in archaeal numbers and evenness in the organization of the archaeal community as well as increase in the diversity of the archaeal population were limited to the krabok oil supplemented treatments.

Acknowledgements

This experiment was funded by the EU-community (project TH/Asia-Link/014-141-176 and Food-CT-23006-36241, Prosafebeef), Paiwan Panyakaew further received grants from the Rajamangala University of Technology Isan, Thailand and Lotus-Erasmus Mundus Action2 (EU-South-East Asian mobility project).

References

Boeckaert, C., Fievez, V., Van Hecke, D., Verstraete, W., Boon, N. 2007. Changes in rumen biohydrogenation intermediates and ciliate protozoa diversity after algae supplementation to dairy cattle. Eur. J. Lipid Sci. Technol. 109, 767-777.

De Boer, G., Murphy, J.J., Kennelly, J.J. 1987. A modified method for determination of *in situ* rumen degradation of feedstuffs. Can. J. Anim. Sci. 67, 93-102.

Boon, N., Top, E.M., Verstraete, W., Siciliano, S.D. 2003. Bioaugmentation as a tool to protect the structure and function of an activated-sludge microbial community against a 3-chloroaniline shock load. Appl. Environ. Microbiol. 69, 1511-1520.

Boon, N., Pycke, B.F.G., Marzorati, M., Hammes, F. 2011. Nutrient gradients in a granular activated carbon biofilter drives bacterial community organization and dynamics. Water Res. 45, 6355-6361.

Dohme, F., Machmüller, A., Estermann, B.L., Pfister, P., Wasserfallen, A., Kreuzer, M. 1999. The role of the rumen ciliate protozoa for methane suppression caused by coconut oil. Lett. Appl. Microbiol. 29, 187-192.

Dohme, F., Machmüller, A., Wasserfallen, A., Kreuzer, M. 2000. Comparative efficiency of various fats rich in medium-chain fatty acids to suppress ruminal methanogenesis as measured with Rusitec. Can. J. Anim. Sci.80,473-484.

Dohme, F., Machmüller, A., Wasserfallen, A., Kreuzer, M. 2001. Ruminal methanogenesis as influenced by individual fatty acid supplemented to complete ruminal diets. Lett. Appl. Microbiol. 32,47-51.

Finlay, B.J., Esteban, G., Clarke, K.J., Williams, A.G., Embley, T.M., Hirt, R.P. 1994. Some rumen ciliates have endosymbiotic methanogens. FEMS Microbiol. Lett. 117, 157-162.

Galyean, M. 1989. Laboratory Procedure in Animal Nutrition Research. Department of Animal and range Sciences, New Mexico State University, U.S.A.

Janssen, P.H. 2010. Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. Anim. Feed Sci. Technol. 160, 1-22.

Jordan, E., Lovett, D.K., Hawkins, M., Callan, J.J., O'Mara, F.P. 2006. The effect of varying levels of coconut oil on intake, digestibility and methane output from continental cross beef Heifers. Anim. Sci. 82, 859-865.

Kreuzer, M. 1986. Methods and application of defaunation in the growing ruminant. J. Vet. Med. 33, 721-745.

Kongmun, P., Wanapat, M., Pakdee, P., Navanukraw, C., Yu, Z. 2011. Manipulation of rumen fermentation and ecology of swamp buffalo by coconut oil and garlic powder supplementation. Livest. Sci. 135, 84-92.

Koster, I.W., Cramer, A. 1987. Inhibition of methanogenesis from acetate in granular sludge bylong-chain fatty acids. Appl. Environ. Microbiol. 53, 403-409.

Lovett, D., Lovell, S., Stack, L., Callan, J., Finlay, M., Conolly, J., O'Mara, F.P. 2003. Effect of forage/concentrate ratio and dietary coconut oil level on methane output and performance of finishing beef heifers. Livest. Product. 84, 135-146.

Machmüller, A., Ossowski, D.A., Wanner, M., Kreuzer, M. 1998. Potential of various fatty acid feeds to reduce methane release from rumen fermentation *in vitro* (Rusitec). Anim. Feed Sci. Technol. 71, 117-130.

Machmüller, A., Kreuzer, M. 1999. Methane suppression by coconut oil and associated effects on nutrient and energy balance in sheep. Can. J. Anim. Sci. 79, 65-72.

Machmüller, A., Ossowski, D.A., Kreuzer, M. 2000. Comparative evaluation of the effects of coconut oil, oilseeds and crystalline fat on methane release, digestion and energy balance in lambs. Anim. Feed Sci.Technol. 85, 41-60.

Machmüller, A., Soliva, C.R., Kreuzer, M. 2003. Effect of coconut oil and defaunation treatment on methanogenesis in sheep. Reprod. Nutr. Develop. 43, 41-55.

Machmüller, A. 2006. Medium-chain fatty acids and their potential to reduce methanogenesis in domestic ruminants. Agri. Ecosys. Environm. 112, 107-114.

Marzorati, M., Wittebolle, L., Boon, N., Daffonchio, D., Verstraete, W. 2008. How to get more out of molecular fingerprints: practical tools for microbial ecology. Environm. Microbiol. 10, 1571-1581.

Mertens, B., Boon, N., Verstraete, W. 2005. Stereo specific effect of hexachlorocyclohexane on activity and structure of soil methanotrophic communities. Environm. Microbiol. 7, 660-669.

Ohajuruka, 0.A., Wu, Z., Palmquist, D.L. 1991. Ruminal metabolism, fiber, and protein digestion by lactating cows fed calcium soap or animal-vegetable fat. J. Dairy Sci. 74, 2601.

Panyakaew, P., Goel, G., Lourengo, M., Yuangklang, C., Fievez, V. (2013). Medium-chain fatty acids from coconut or krabok oil inhibit *in vitro* rumen methanogenesis and conversion of non-conjugated dienoic biohydrogenation intermediates. Anim. Feed Sci. Technol. 180, 18-25.

Reveneau, C., Karnati, S.K.R., Oelker, E.R., Firkins, J.L. 2012. Interaction of unsaturated fat or coconut oil with monensin in lactating dairy cows fed 12 times daily. I. Protozoal abundance, nutrient digestibility, and microbial protein flow to the omasum. J. Dairy Sci. 95, 2046-2060.

Soliva, C.R., Hindrichsen, I.K., Meile, L., Kreuzer, M., Machmüller, A. 2003. Effects of mixtures of lauric and myristic acid on rumen methanogens and methanogenesis *in vitro*. Lett. Appl. Microbiol. 37, 35-39.

Soliva, C.R., Meile, L., Hindrichsen, I.K., Kreuzer, M., Machmüller, A. 2004. Myristic acid supports the immediate inhibitory effect of lauric acid on ruminal methanogens and methane release. Anaerobe 10, 269-276.

Van Ranst, G., Fievez, V., Vandewalle, M., Van Waes, C., De Riek, J., Van Bockstaele, E. 2010. Influence of damaging and wilting red clover on lipid metabolism during ensiling and *in vitro* rumen incubation. Anim. 4, 1528-1540.

Wittebolle, L., Marzorati, M., Clement, L., Balloi, A., Daffonchio, D., Heylen, K., De Vos, P., Verstraete, W., Boon, N. 2009. Initial community evenness favours functionality under selective stress. Nature 458, 623-626.

Yu, Z., Morrison, M. 2004. Comparisons of different hypervariable regions of rrs genes for use in fingerprinting of microbial communities by PCR-denaturing gradient gel electrophoresis. Appl. Environm. Microbiol. 70, 4800-4806.

Yu, Z., García-González, R., Schanbacher, F.L., Morrison, M. 2008. Evaluations of different hypervariable regions of archaeal 16S rRNA genes in profiling of methanogens by Archaea-Specific PCR and denaturing gradient gel electrophoresis. Appl. Environm. Microbiol. 74, 889-893.

Chapter 5

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

Paiwan Panyakaew[1,*], J. Thomas Schonewille[2], John W. Cone[3], Wilbert F. Pellikaan[3], Veerle Fievez[4], Chalermpon Yuangklang[5] and Wouter H. Hendriks[2,3]

[1]*Department of Animal Science, Faculty of Natural Resources, Rajamangala University of Technology Isan, Sakon-Nakhon Campus, Sakon Nakhon 47160 Thailand;*
[2]*Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands;*
[3]*Animal Nutrition Group, Department of Animal Sciences, Wageningen University & Research, Wageningen, The Netherlands;*
[4]*Laboratory for Animal Nutrition and Animal Product Quality (Lanupro), Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium;*
[5]*Department of Agricultural Technology and Environment, Faculty of Science and Liberal Arts, Rajamangala University of Technology-Isan, Nakhon Ratchasima, 30000 Thailand.*

Published in Journal of Animal Physiology and Animal Nutrition 104, 453-461 (2020).

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 fiber and acid detergent fiber 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 ($P \geq 0.115$), 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 ($P \leq 0.019$). 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=0.019). Volatile fatty acids were likewise affected ($P \leq 0.001$). 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

Introduction

Ruminants are considered important contributors to the emission of methane (CH_4) into the atmosphere (Moss et al., 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 and Sun, 2008). Various strategies have been proposed to reduce enteric CH_4 production by ruminants (Boadi et al., 2004; Morgavi et al., 2010), including the supplementation of medium-chain fatty acids in diets (MCFA, Dohme et al., 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 et al., 2013a). Previous research has shown the potential of krabok oil (KO) to inhibit methanogenesis (Panyakaew 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 et al., 2013b) to practice may not be warranted because of economic reasons. The potential of KO, however, to reduce CH_4 production at lower levels of supplementation is hitherto unknown, but Panyakaew et al. (2013b) reported a KO induced reduction of *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 et al. (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.

Materials and methods

Preparation of experimental substrates

Whole krabok seed was purchased from a local market (PuPan mountain, Sakon Nakhon, Thailand) and ground at 0 °C to pass a 1-mm screen, using a Willey mill (Peppink 100 AN, Olst, The Netherlands). 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 Soxhlet 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).

Table 1. Analyzed 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	1000
	----------g/kg DM----------			
Crude ash	107	21	38	-
Crude protein	183	133	246	-
Ether extract	54	564	190	1000
Neutral detergent fiber	358	270	464	-
Acid detergent fiber	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
C18:2n-6 *(cis, cis)*	39.89	0.42	0.62	0.37
C18:3n-3 *(cis, 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; sulfur, 3.2.
[c] Residue after fat extraction (hexane) from whole krabok seed.

The experimental substrates were formulated to keep the amounts of incubated fat-free organic matter (OM), crude protein (CP), neutral detergent fiber 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.

Table 2. Ingredient and analyzed 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	FER	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 fiber; CP, crude protein; DM, dry matter; EE, ether extract; NDF, neutral detergent fiber; 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; sulfur, 3.2.

The ingredient composition of the basal substrate (Panyakaew 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; sulfur, 3.2.

In vitro *gas and CH_4 production*

Gas production profiles of the experimental substrates were determined using fully automated GP equipment (Cone et al., 1996) with GP being measured over 48 h. 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, Mainz, Germany). 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 h after the morning feeding at 8:00 h. 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 cheese cloth 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 h) and directly injected into a gas chromatograph to determine the CH_4 concentration (Pellikaan et al., 2011).

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 et al. (1996). The non-linear least squares regression procedure was used (SAS Institute Inc., 2010) and the data were fitted according to the following equation:

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

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 et al. (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 phase 1, 2 and 3 (A1, A2 and A3, respectively) were set at 3 h for A1, 17 h later for A2 and 28 h 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 et al., 2005):

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

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

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

Chemical analysis

The DM content was determined after drying at 103 °C overnight (ISO 6496; ISO, 1999) and ash content after incineration for 3 h at 550 °C (ISO 5984; 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 fiber (NDF) and acid detergent fiber (ADF) were analyzed as per the standard methods; Van Soest et al. (1991) and Van Soest et al. (1973), respectively. Neutral detergent fiber was analyzed after a pre-treatment with a heat-stable amylase. Acid detergent fiber (ADF) was analyzed 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 analyzed as described by Panyakaew et al. (2013a). Volatile fatty acids (VFA) were analyzed using gas chromatography (Thermo, Milan, Italy) equipped with a flame ionization detector with an HP-FFAP column (30 m×0.53 mm i.d. 1.00 µm film thickness) (Agilent, Jew, USA) using hydrogen as carrier gas (Dieho et al., 2016).

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:

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

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 $P<0.05$.

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 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.

Gas production

Except for the cumulative GP measured after 48 h and its associated asymptote GP of the non-soluble fraction (A2), all other relevant kinetic parameters were not affected by S×IL ($P\geq0.259$). 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-h but all other relevant kinetic parameters were found to be not significantly affected ($P\geq0.122$).
Gas production at 48h, expressed as ml gas/g fat free OM, was affected by both type ($P<0.001$) and inclusion level ($P=0.004$) 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 ($P=0.019$) with the average GP 48-h of 244 ml/g fat free OM for the three substrates (*i.e.,* FER, FER+KO, WKS; $P=0.150$). This value was found to be 22.4% lower ($P\leq0.037$) 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 ($P=0.329$) to the GP 48-h values measured at the lowest inclusion level of the experimental substrates, irrespective ($P=0.149$) 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-h, that is, the dose of supplemental KO explained only ~1% of the variation in GP-48h ($P=0.315$).

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.

Parameter	Type of supplemental substrate (S)				Inclusion level (IL)			SEM	*P*-values		
	FER	KO	FER+KO	WKS	Low	Medium	High		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

[a-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 h of incubation; A1and A2, asymptote of gas production in phase 1 and 2, respectively; OM, Organic matter; R_{max1} and R_{max2}, maximum gas production rate in phase 1 and 2, respectively; TR_{max1} and TR_{max2}, time occurrence of R_{max} in phase 1 and 2, respectively.

The asymptote of GP of the soluble fraction (A1) was different between the experimental substrates (*P*=0.010), 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*=0.027) 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 (R_{max1}), but its associated time occurrence was found to be not different (*P*=0.529) between treatments.
The asymptote GP of the non-soluble fraction (A2) differed significantly by the type of supplemental substrate (*P*<0.001) 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*=0.005) 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 (TR_{max2}) were influenced by any treatment (*P*≥0.255).

Methane production

Methane production and associated kinetic parameters (Table 4) were not affected by S×IL (*P*≥0.079). Irrespective of the type of supplemental substrate (*P*≥0.115), both absolute (ml/g fat-free OM) and relative (% of total GP) CH_4 production was found to be reduced at the highest inclusion level of the supplemented substrates (*P*≤0.019). The asymptotic CH_4 production (A) was found to be lowest on the highest inclusion level of any experimental substrate (*P*<0.001). The incubation of supplemental WKS versus FER resulted in a 16.4% lower A value (*P*=0.050). Values for the halftime of the asymptotic CH_4 production (B) were affected in a similar manner as the asymptotic CH_4 production (*P*≤0.006). 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*=0.001). 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.

Volatile fatty acids

The concentration of total VFA, as well as total VFA expressed as μmol/mg fat-free OM, were influenced (*P*<0.001) by inclusion level×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*<0.001). The proportion of HAc was neither affected by inclusion level×type of substrate (*P*=0.383) nor by type of supplemental substrate (*P*=0.158). In contrast, the lowest proportions of HAc were found at the highest inclusion level of the experimental substrates (*P*<0.001) while group mean values of the HP proportions were found to be reciprocal of HAc (R^2=0.937, *P*<0.001).

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.

Parameter	Type of supplemental substrate (S)				Inclusion level (IL)			SEM	P-values		
	FER	KO	FER+KO	WKS	Low	Medium	High		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-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}.

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.

Inclusion level (IL)	Substrate (S)	Total VFA		Individual VFA (mol/100 mol)				AP	NGR
		mM	umol/mg fat-free OM	Acetic acid	Propionic acid	Butyric acid	BCVFA		
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

^{a,b,c,d} means within a column with different superscripts differ (P<0.05) significantly.

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

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=0.017). The proportions of BCFA and NGR were likewise affected ($P \leq 0.001$). 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.

DISCUSSION

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 ($P \leq 0.008$). The response of KO on CH_4 production is in line with Panyakaew 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 et al. (2013b) who found a KO induced reduction of *Archaea* numbers when the ration of bulls contained 3.5% supplemental KO. The results of Panyakaew 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 cannot be easily explained by the type of basal substrate because the ingredient composition of the ration provided to the bulls (Panyakaew 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 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 dependent shift in VFA from HAc to HP (Panyakaew 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 and Newbold, 2008).

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 unambiguous explanation for the discrepancy in results is difficult to provide, but it may be related to the physical form of the oilseeds itself.

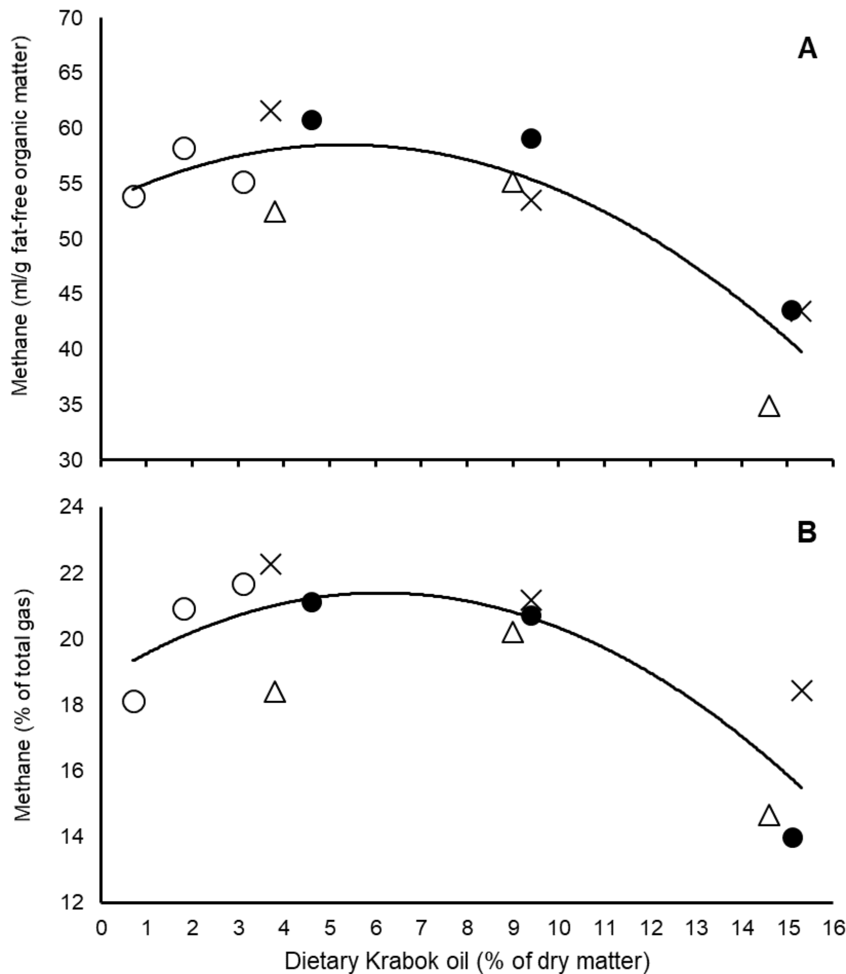


Figure. 1. Dose-response relationship between dietary krabok oil and methane production either expressed as ml/g fat-free organic matter (Panel A: $y=-0.19x^2+2.00x+53.21$, $R^2_{adj}=73.6\%$, $P=0.001$) or expressed as a % of total gas (Panel B: $y=-0.07x^2+0.86x+18.79$, $R^2_{adj}=58.7\%$, $P=0.008$). 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 (×).

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-h (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, *i.e.,* (values expressed as g/kg fat-free OM): aNDFom, 600; ADFom, 538; ADL, 200; NSC, 81. 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).

Conclusions

The inclusion of krabok oil at a level 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.

Acknowledgements

The study was financially supported by the EU-community through the Asia-Link Programme (Phase III) (project TH/Asia-Link/014-141-176) in cooperation with Wageningen University & Research and the Laboratory for Animal Nutrition and Animal Product Quality (Lanupro) of Ghent University.

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.

References

AOAC. 1990. Official Methods of Analysis, 15th edn. Assoc. Off. Anal. Chem., Arlington, VA, USA.

Boadi, D., Benchaar, C., Chiquette, J., Masse, D. 2004. Mitigation strategies to reduce enteric methane emissions from dairy cows: Update review. Can. J. Anim. Sci. 84, 319-342.

Cone, J.W., Van Gelder, A.H., Visscher, G.J.W., Oudshoorn, L. 1996. Influence of rumen fluid and substrate concentration on fermentation kinetics measured with a fully automated time related gas production apparatus. Anim. Feed Sci. Technol. 61, 113-128.

Cone, J.W., Van Gelder, A.H., Driehuis, F. 1997. Description of gas production profiles with a three-phasic model. Anim. Feed Sci. Technol. 66, 31-45.

Dieho, K., Dijkstra, J. Schonewille, J.T., Bannink, A. 2016. Changes in ruminal volatile fatty acid production and absorption rate during the dry period and early lactation as affected by rate of increase of concentrate allowance. J. Dairy Sci. 99, 5370-5384.

Dohme, F., Machmüller, A., Wasserfallen, A., Krezer, M. 2000. Comparative efficiency of various fats rich in medium-chain fatty acid to suppress ruminal methanogenesis as measured with Rusitec. Can. J. Anim. Sci. 80, 473-483.

Groot, J.C.J., Cone, J.W., Williams, B.A., Debersaque, F., Lantinga, E.A. 1996. Multiphasic analysis of gas production kinetics for *in vitro* fermentation of ruminant feeds. Anim. Feed Sci. Technol. 64, 77-89.

ISO 6496. 1999. Animal Feeding Stuffs. Determination of moisture and other volatile matter content. International Organization for Standardization, Geneva, Switzerland.

ISO 5984. 2002. Animal Feeding Stuffs. Determination of Crude Ash. International Organization for Standardization, Geneva, Switzerland.

Machmüller, A. 2006. Medium-chain fatty acid and their potential to reduce methanogenesis in domestic ruminants. Agric., Ecosys. Environm. 112, 107-114.

Martin, C., Rouel, J., Jouany, J. P., Doreau, M., Chilliard, Y. 2008. Methane output and diet digestibility in response to feeding dairy cows crude linseed, extruded linseed, or linseed oil. J. Anim. Sci. 86, 2642-2650.

McAllister, T.A., Newbold, C.J. 2008. Redirecting rumen fermentation to methanogens. Aust. J. Exp. Agri. 248, 7-13.

Mitsumori, M., Sun, W. 2008. Control of rumen microbial fermentation for mitigating methane emissions from the rumen. Asian-Austr. Assoc. Anim. Prod. Sci., 21, 144-154.

Morgavi, D.P., Forano, E., Martin, C., Newbold, C.J. 2010. Microbial ecosystem and methanogenesis in ruminants. Anim. 4(7), 1024-1036.

Moss, A.R., Jouany, J.P., Newbold, J. 2000. Methane production by ruminants: its contribution to the global warming. Annal. de Zootechnie, 49, 231-253.

Ørskov, E.R. 1977. Capacity for digestion and effects of composition of absorbed nutrients on animal metabolism. J. Anim. Sci., 45, 600-608.

Panyakaew, P., Goel, G., Lourengo, M., Yuangklang, C., Fievez, V., 2013a. Medium-chain fatty acids from coconut or krabok oil inhibit *in vitro* rumen methanogenesis and conversion of non-conjugated dienoic biohydrogenation intermediates. Anim. Feed Sci. Technol. 180, 18-25.

Panyakaew, P., Goel G., Yuangklang C., Boon N., Schonewille. J.Th., Hendriks W.H., Fievez, V. 2013b. Effect of supplementing coconut or krabok oil, rich in medium chain fatty acids on ruminal fermentation, protozoa and archaeal population of bulls. Anim. 7(11), 1950-1958.

Pellikaan, W.F., Hendriks, W.H., Bongers, L.J.G.M., Becker, P.M., Cone, J.W. 2011. A novel method to determine methane production simultaneously during *in vitro* gas production measurements. Anim. Feed Sci. Technol. 168, 196-205.

SAS. 2010. Statistical Analysis Software. 9.3 User's Guide. SAS Institute Inc., Cary, NC.

Van Gelder, A.H., Hetta, M., Rodrigues, M.A.M., De Boever, J.L., Den Hartigh, H., Rymer, C., Van Oostrum, M., Van Kaathoven, R., Cone, J.W. 2005. Ranking of *in vitro* fermentability of twenty feedstuffs with an automated gas production technique. Results of a ring test. Anim. Feed Sci. Technol. 123, 243-253.

Van Soest, P.J. 1973. Collaborative study of acid detergent fibre and lignin. J. Assoc. Off. Anal. Chem. 56, 781-784.

Van Soest, P.J. 2006. Rice straw, the role of silica and treatments to improve quality. Anim. Feed Sci. Technol. 130, 137-171.

Van Soest, P.J., Robertson, J.B., Lewis, B.A. 1991. Methods for dietary fibre, neutral detergent fibre, and non-starch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74, 3583-3597.

Yang, H.J., Tamminga, S., Williams, B.A., Dijkstra, J., Boer, H. 2005. *In vitro* gas and volatile fatty acids production profiles of barley and maize and their soluble and washout fractions after feed processing. Anim. Feed Sci. Technol. 120, 125-140.

Chapter 6

General discussion

The background of the current thesis concerns the environmental issues related to the current worldwide changes in climate concept. The production of so-called greenhouse gasses, including methane (CH_4), are commonly believed to be related with the global climatological changes. Methane production is inherently associated with the production of meat and milk by ruminants and there are currently extensive reviews available (Asanuma et al., 1999; Lila et al., 2003; Hook et al., 2010; Hristov et al., 2010; Duval et al., 2016; FAO, 2017; Black et al., 2021) on nutritional strategies to mitigate CH_4 emission originating from ruminants. One of the nutritional strategies in question involves the supplementation of fat/oil to ruminant rations.

In contrast to saturated fatty acids, unsaturated fatty acids are subject to the process of biohydrogenation in the rumen. The process of biohydrogenation involves *cis-trans* isomerization of dietary unsaturated fatty acids to *trans* fatty acid intermediates which are subsequently hydrogenated to saturate double bounds (Dewanckele et al., 2018). Although the rumen biohydrogenation of unsaturated is extensive, a wide array of C18:2 and C18:3 intermediates escape from complete hydrogenation of their double bounds and these intermediates are known to affect the synthesis of milk fat in dairy cows (Giinari et al., 2000; Bauman and Griinary, 2003; Bauman et al., 2011; Jenkins and Harvatine, 2014). Extensive experimental work is already published on the mitigating effects of long-chain fatty acids on CH_4 emission by ruminants (Machmüller et al., 1998; Dohme et al., 2001; Martin et al., 2008; Yang et al., 2009; Patra and Yu, 2013; Abubakr et al., 2014).

Fully hydrogenated medium-chain fatty acids (MCFAs), such as lauric- and myristic acid, are in practice referred to as rumen inert fatty acids. That is, lauric- and myristic acid are inert with respect to the process of biohydrogenation but these fatty acids do affect rumen microbiota and CH_4 emission (this thesis). It was already mentioned in the general introduction of the current thesis that fatty acids with an aliphatic carbon chain length of 6 to 12 carbon atoms are generally classified as MCFAs but in this section, myristic acid (C14:0) also, is conveniently considered as MCFA.

Inhibitory action of krabok oil on CH_4 production

The two *in vitro* studies presented in this thesis are the only studies providing data to evaluate the direct effect of krabok oil on CH_4 production. For the two studies combined, an overall depressant effect of krabok oil on CH_4 production can be demonstrated (Fig. 1). Both, linear and quadratic models were attempted to fit the data and it appeared that both approaches were equally suitable to describe the dose-response relationship between supplemental krabok oil and CH_4 production.

This result is not entirely in line in case only the results presented in Chapter 2 are taken into account. In Chapter 3 it was shown that a quadratic model versus a linear explained the variation in CH_4 production to a greater extent. The lack in clarity between the results is mainly caused by the two observations originating from Chapter 4 with relatively high CH_4

production at a high level of dietary krabok oil (~13.5%). Nevertheless, the outcome of both experiments is consistent in that supplemental krabok oil inhibits CH_4 production.

Putative mode of action

Methanogenic *Archaea* are the sole producers of CH_4 in the rumen environment (Dohme et al., 2001; Machmüller et al., 2003a,b; Soliva et al., 2003; Pilajun and Wanapat, 2011). Thus, dietary measures targeting either a reduction in the number of methanogenic *Archaea* or interfering with the biochemical pathways leading to a reduced CH_4 synthesis are likely to reduce CH_4 production. To the best of the author's knowledge, a generally accepted mode of action is, however, not yet established. It is interesting to note that there is a considerable body of evidence on the suppressant effect of coconut oil on CH_4 production (Dong et al., 1997; Machmüller et al., 1998, 2000, 2003; Machmüller and Kreuzer, 1999; Dohme et al., 2000, 2001; Lovett et al., 2003; Soliva et al., 2004; Jordan et al., 2006).

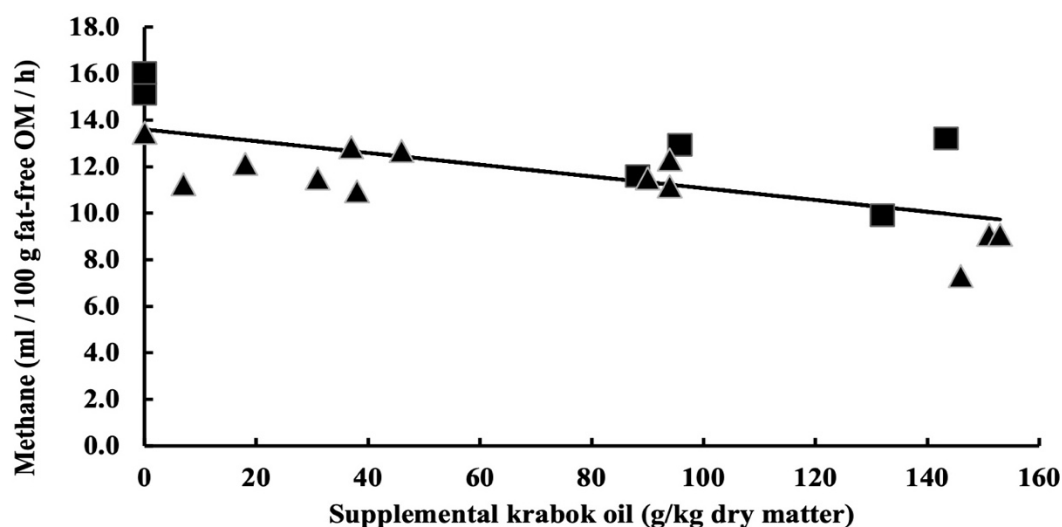


Figure 1. Dose-response relationship between supplemental krabok oil and *in vitro* methane production expressed as ml/100 g fat-free organic matter (OM)/h. The data originate from Chapters 2 and 5 of the current thesis. The original methane production values presented in Chapter 2 (*i.e.,* expressed as μmol) are converted to ml/100 g fat-free OM assuming that 1 mol of gas=25.6 L (Malcome et al., 2017). Thereafter, methane values were standardized by time in an attempt to account for the difference in incubation times between the two *in vitro* studies; *i.e.,* 24 h (Chapter 2) and 48 h (Chapter 5). Symbols: ■, data originating from Chapter 2;▲, data originating from Chapter 5. Trendline: Y=13.59-0.025×X (R^2=46.4%, P=0.001).

Next, to coconut oil, palm kernel oil as well has been shown to suppress ruminal CH_4 production (Dohme et al., 2000). It is well known that the fatty acid profiles of coconut-, palm- and krabok oil predominantly contain MCFAs, *i.e.,* fatty acids with a chain length ranging from 6 to 14 carbon atoms. Typically, coconut-, palm kernel- and krabok oil are rich in lauric acid (C12:0) while krabok oil also contains a considerable amount of myristic acid

(C14:0), *i.e.,* ~44% of total fatty acids. It can, therefore, be suggested that the inhibitory action of krabok oil on methanogenesis is related to its high content of MCFAs.

Interestingly, Freese et al. presented in 1973 a model on the mechanism of the antimicrobial effect of MCFA. According to this model, MCFA enter the intracellular space of microbes and then, at least to a certain extent, dissociate, thereby, rendering H^+ in the cytosol and subsequently lower the intracellular pH. This condition ultimately leads to the inhibition of ATP synthesis and subsequent cell death. Following the reasoning of Freese et al. (1973) it can be hypothesized that the antimicrobial effect of caprylic acid (C8:0, pKa 4.89) is greater compared to that of lauric acid (C12:0, pKa 5.30). This hypothesis, however, is not confirmed by the outcome of experimental work reported by Dohme et al. (2001). These researchers evaluated the effect of pure, saturated fatty acids, with an aliphatic carbon chain length ranging from 8 to 18 carbon atoms, on *in vitro* ruminal methanogenesis. The fatty acids were supplemented to a basal substrate at a level of 50 g/kg dry matter and it was shown that supplemental caprylic acid (C8:0, pKa 4.89) caused a 1.5 times numerical increase in methanogenic *Archaea* numbers while supplemental lauric acid (C12:0, pKa 5.30) caused an almost 50% numerical reduction in methanogenic *Archaea* numbers. Moreover, on a molar base the amount of supplemental lauric acid versus caprylic acid was almost 30% lower. Furthermore, it is difficult to see that the mode of inhibitory action, if any, of fatty acids ≥14 carbon atoms is related to intracellular acidification of microbes because it is well known that the water-solubility of C14:0, C16:0 and 18:0 is very low (<20 mg/l). In view of the latter, it seems unlikely that the model proposed by Freese et al. (1973) can be applied to krabok oil so as to explain its inhibitory action on CH_4 production. Obviously, the current thesis does not provide further insights into this matter, but it may be suggested that MCFAs interfere with membrane integrity ultimately leading to cell death of the methanogens.

The latter idea is fueled by the outcome of experimental work of Zhou et al. (2013) who reported that the inhibition of CH_4 formation by C12:0 and C14:0 is coincided with potassium (K) leakage and cell viability of *Methanobrevibacter ruminantium*. It can be speculated that the leakage of K interferes with the potential difference across the outer membrane (interior negative) of methanogens causing a decrease in the protonmotive force (Butsch and Bachofen, 1984) and subsequent decrease in ATP production (Sauer et al., 1979) which in turn may lead to a diminished growth rate and/or death of the methanogen in question. The aforementioned sequence of events appears to be triggered by the fact that MCFAs can adsorb onto the outer membrane of bacteria (Galbraith and Miller, 1973) and subsequently interfere with membrane integrity leading to the leakage of K (Zhou et al., 2013) and subsequent cell death (Galbraith and Miller, 1973; Zhou et al., 2013). It can be speculated that especially methanogens are sensitive to K leakage from their cytosol because methanogens typically have very high intracellular K concentrations, with values reported to range from 200 to 1225 mM (Jarrel et al., 1984; Schönheit et al., 1984). Needless to say, that the latter notions are highly speculative and further research is warranted to elucidate the underlying inhibitory mechanism of MCFAs in general, and myristic acid in particular, on methanogenesis.

Comparison between lauric and myristic acid

It was already mentioned that next to krabok oil, coconut oil is a potent inhibitor of CH_4 production (Machmüller and Kreuzer, 1999; Machmüller et al., 2000, 2003). Coconut oil is particularly rich in lauric acid (C12:0), *i.e.,* ~45% of total fatty acids while krabok oil contains a similar amount of C12:0 but it also contains ~44% myristic acid (C14:0). Thus, in view of the inhibitory effect of krabok oil on methanogenesis, it is of scientific interest to gain more insight into the efficacy of the two individual fatty acids to mitigate CH_4 production.
Currently, there are five published reports on the inhibitory effect of lauric- and myristic acid on *in vitro* CH_4 production when they are supplemented as pure, individual fatty acids (Table 1). Overall, the data clearly indicate that C12:0 inhibits CH_4 production although the magnitude of the effect depends on the applied dose and the substrate offered to the methanogenic *Archaea* to synthesize CH_4, *i.e.,* either a mixture of H_2 and CO_2 or practical feed ingredients such as roughages and concentrates. In contrast, the issue with respect to the inhibitory action of C14:0 on CH_4 formation, if any, is not straightforward. In all three studies conducted by Soliva et al. (2003, 2004a,b), the supplementation of C14:0 only did not affect CH_4 production while in the study of Zhou et al. (2013), supplemental C14:0 almost completely inhibited CH_4 production. The inhibitory effect of C14:0 on CH_4 production is corroborated by Dohme et al. (2001) although the magnitude of the depressant effect on CH_4 production is lower; ~12% lower compared to the control values (Table 1). The discrepancy in results with respect to myristic acid between the studies (Table 1) is not easy to explain but the specific experimental conditions in each study may have played a role. Zhou et al. (2013) for instance used pure cultures of *Methanobrevibacter ruminantium* and these *Archaea* were fueled with H_2 and CO_2 in combination with centrifuged, filtered and autoclaved rumen fluid to supply the bacteria with minerals, vitamins etc. Under these heavily controlled conditions *Methanobrevibacter ruminantium* did not survive when challenged with myristic- (and lauric) acid. Thus, in case a mixed population of methanogens was challenged with C14:0, and fueled with H_2 and CO_2, CH_4 production was not altered. Perhaps *Methanobrevibacter ruminantium* is highly sensitive to MCFAs. The observations of Dohme et al. (2001) indicate that myristic- and lauric acid are equally effective in depressing CH_4 production (Table 1) while Soliva et al. (2004a) did not observe an inhibitory action of C14:0 on CH_4 production. In both studies, an identical rumen simulation technique (Rusitec) was used and in both cases the mixed rumen microbes were offered practical feed ingredients such as roughages and concentrates. Perhaps the difference in dietary background of the donor cows and, therefore, a difference in microbial flora may have played a role. Alternatively, it can be suggested that the difference in outcome between the two studies is related to the difference in the roughage to concentrate ratio, *i.e.,* 39 to 61 (Soliva et al., 2004a) and 57 to 43 (Dohme et al., 2001). Following this notion, it can be speculated that myristic acid inhibits CH_4 formation only in roughage rich rations. This speculation is not in line with Machmüller et al. (2006) who concluded that the greatest CH_4-suppressing effect is found when MCFAs are supplemented to rations with low contents of structural carbohydrates, *i.e.,* concentrate rich rations. The conclusion of Machmüller et al. (2006) is in line with the outcome of the study reported in Chapter 4 of this thesis where the inhibitory effect of krabok oil was demonstrated using

substrate with a roughage to concentrate ratio of 21 to 79. Clearly, the data in the current thesis do not provide a clear answer and future research is warranted to shed more light on this issue.

The data reported by Soliva et al. (2003, 2004a,b) can be interpretated in that myristic acid synergistically affects the lauric acid induced suppression of methanogenesis (Table 1). In view of this notion, it is of interest to note that Dohme et al. (2000) reported that palm kernel oil, at least numerically, inhibited methanogenesis to a greater extent than coconut oil. Palm kernel oil and coconut oil have similar C12:0 contents but palm kernel oil contains almost 2.4 times more oleic acid (C18:1). Moreover, canola oil type "B"[1] (Dohme et al., 2000) versus coconut oil was equally effective in depressing CH_4 production while the C12:0 content was 18% lower. However, the canola oil in question had a 5 times greater content of C18:1. It can thus be speculated that also oleic acid potentiates the action of C12:0 on CH_4 production. Finally, the data reported in Chapter 4 of this thesis also show that the depressant effect of krabok oil on methanogenesis was more pronounced when the incubation medium also contained polyunsaturated fatty acids (*i.e.,* a mixture of linseed- and sunflower oil). It can thus be suggested that unsaturated fatty acids also act in concert, either or not in a synergistic fashion, with C12:0 to mitigate CH_4.

***Methanogenic Archaea*, protozoa and CH_4**

The current thesis includes two *in vivo* experiments (Chapters 3 and 4) investigating the use of krabok oil to mitigate CH_4 emission under feeding conditions. However, under the practical conditions at the experimental farm of Rjamangala University of Technology Isan, Sakhon Nakohn campus, it was not feasible to directly measure CH_4 production. Instead, indices related to CH_4 production were measured, i*.e.,* methanogenic *Archaea* - and protozoa counts amongst others.

It was already mentioned that methanogenic *Archaea* are the sole producers of CH_4 in the rumen environment, and it is, therefore, not surprising that the number of methanogenic *Archaea* positively correlates with CH_4 production (Table 2). Caution, however, is warranted to depend solely on methanogenic *Archaea* counts as an indicator of methanogenesis because methanogenic *Archaea* are known to have symbiotic relationships with rumen protozoa (Dohme et al., 2000, 2001; Machmüller et al., 2003a,b; Soliva et al., 2004a; Patra and Yu, 2012). This notion is line with the data reported in Chapter 4 showing a positive correlation between methanogenic *Archaea* - and protozoa counts (Table 2, Panyakaew et al., 2013b). Finlay et al. (1994) classified methanogens as ectosymbionts or endosymbionts of protozoa. In other words, methanogenic *Archaea* can attach to the protozoa's outer membrane or protozoa can be inhabited by methanogenic *Archaea* and in that case the methanogens reside intracellularly (Dohme et al., 2000, 2001; Soliva et al., 2004a; Patra and Yu, 2012). In case of traditional counting of microbial cells, for instance by means of microscopic counting using haemacytometer, the number of methanogenic *Archaea* may be underestimated. From this viewpoint, the counting of rRNA gene copies from the 16S rDNA region by means of

[1]Dohme et al. (2000) used two types of canola oil in their study, i*.e.,* type A and B. The two types contained similar amounts of C12:0 but type B versus A contained 4.6 times lower C18:0 and 2.7 times greater contents of oleic acid (C18:1).

quantitative polymerase chain reaction (PCR) yields more accurate numbers. In the current thesis,

Table 1. Overview on the inhibitory effect of lauric- and myristic acid on *in vitro* methane (CH_4) production when supplemented as pure, individual medium-chain fatty acids (MCFA). Values between brackets refer to the amount of supplemented fatty acid in that specific treatment.

Reference		Soliva et al., 2004b		Soliva et al., 2003	Zhou et al., 2013			Soliva et al., 2004a	Dohme et al., 2001
		Exp 1	Exp 2		Exp 1	Exp 2	Exp 3		
Substrate for CH_4 synthesis		H_2 and CO_2		H_2 and CO_2	H_2 and CO_2			R/C (39/61)	R/C (57/43)
Initial CH_4 production (unsupplemented control treatment)		0.71 (mmol/24 h)	0.72	0.70 (mmol/24 h)	0.02 (umol/mg/min)	not shown	not shown	8.7 (mmol/d)	7.3 (mmol/d)
Amount of total MCFA		30 mg	20-60 mg	30 mg	30 ug/ml	30 ug/ml	30 ug/ml	47.6 g/kg DM	46.4 g/kg DM
Lauric acid	Myristic acid								
(% of total supplemental MCFA)		(CH_4 production as a % relative to unsupplemented control values)							
33.3	-	99	100 (30 mg)	-	10	10	0	-	-
50.0	-	80	100 (30 mg)	-	-	-	-	-	-
66.7	-	63	48 (30 mg)	-	-	-	-	-	-
100.0	-	17	28 (30 mg)	7	0	0	-	58	88
-	33.3	101	-	-	31	-	0	-	-
	50.0	102	-	-	-	-	-	-	-
-	66.7	103	-	-	-	-	-	-	-
-	100.0	106	-	107	4	-	-	96	88
33.3	66.7	-	85 (30 mg)	43	-	-	-	-	-
40.0	60.0	-	-	31	-	-	-	68	-
50.0	50.0	-	-	29	-	-	-	52	-
60.0	40.0	-	-	21	-	-	-	40	-
66.7	33.3	-	46 (30 mg)	9	-	-	-	-	-
		-	100 (20 mg)	-	-	-	-	-	-
50.0	50.0	-	21 (40 mg	-	-	-	-	-	-
		-	8 (60 mg)	-	-	-	-	-	-

R/C=Roughage and concentrate and their ratio between brackets.

methanogenic *Archaea* counts are only supported in Chapter 3 and 4 in that qPCR was used to estimate the methanogenic *Archaea* counts. However, it must be noted that genetic material is counted in case of qPCR which implicates that the counting method in question cannot distinguish between living and dead cells. Needless to say, that the latter is important to explain the inhibitory actions of dietary measures, such as MCFA supplementation on CH_4 mitigation. Culture and real-time PCR are the methods most used for quantitation of microorganisms in different conditions or environments (Sontakke et al., 2009). On the other hand, qPCR detects all cells in a sample, including the dead cells or the DNA of some of them that can be found in the environment (Pathak et al., 2012). However, a shortcoming of this method is that DNA is typically not rapidly degraded in intact cells and, therefore, standard PCR and quantitative PCR approaches are not able to distinguish between living and dead bacteria (Lauri and Mariani, 2009). One approach used to discriminate between viable and dead cells is the inclusion of propidium monoazide (PMA) prior to DNA extraction and PCR amplification of pathogen-specific target genes (Nocker et al., 2007).Due to the interrelationships between methanogenic *Archaea*, protozoa and CH_4, defaunation of the rumen contents is clearly of potential interest to mitigate CH_4 (Table 2). The defaunating effect of MCFAs reported in Chapter 3 of the current thesis is corroborated by various reports (Dohme et al., 2000, 2001; Soliva et al., 2003, 2004a; Patra and Yu, 2012) and it was found that coconut- and krabok oil were equally effective in reducing the protozoa counts. In contrast to the experiment described in Chapter 4, supplemental MCFA did not affected protozoa counts in a follow up study with beef cattle (Panyakaew et al., 2013b) although methanogenic *Archaea* counts were, at least numerically, lowered after the feeding of rations with supplemental MCFA. The differential effect of MCFA on the two different micro-organisms is most likely related to the difference in the composition of their outer membrane (Machmüller et al., 2003a,b; Liu et al., 2011). It is of interest to note that protozoa- and methanogenic *Archaea* counts were inversely related in the study reported by Machmüller et al. (2003a). In the latter study, however, the investigators aimed to study the depressant effect of coconut oil on methanogenesis in sheep which were either or not treated with nonyl phenol ethoxylate. The latter chemical is known to be a highly effective compound to defaunate the rumen content (Machmüller et al., 2003b) and in this study methanogenic *Archaea* counts were, on average, 3.6 times greater in defaunated sheep. Furthermore, supplemental coconut oil caused a significant reduction in CH_4 emission. Clearly, these data are quite puzzling, and the interpretation is unclear but it suggests an uncoupling between the number of methanogenic *Archaea* and the amount of CH_4 produced. Perhaps, coconut oil depressed the efficiency of CH_4 synthesis in individual cells. However, such conditions would lead to an increased partial pressure of H_2 and, therefore, increased proportions of propionic acid (see next paragraph). The latter was, however, not confirmed by Machmüller et al. (2003a). Perhaps, the data are not repeatable and are based on coincidence. Future studies are needed to generalize the outcome of the study reported by Machmüller et al. (2003a).

Table 2. Overview of the correlations between either methanogenic *Archaea* – or protozoa counts and methane production and methanogenic *Archaea*– and protozoa counts. The number of observations (N) refers to the number of independent pairs of values (*i.e.,* treatment means) that was used to calculate Pearson's correlation coefficient (R^2). In all studies cited, at least one of treatments involved the use of medium-chain fatty acids to potentially manipulate rumen fermentation.

Research type Reference	*Archaea* and methane		Protozoa and methane		Protozoa and *Archaea*		N
	R^2	*P*-value	R^2	*P*-value	R^2	*P*-value	
In vitro							
Soliva et al., 2004a	0.964	0.000	0.873	0.005	0.736	0.038	8
Soliva et al., 2003	0.813	0.000	NA		-	-	17
Dohme et al., 2001	0.897	0.002	-0.073	0.863	-0.039	0.927	8
Dohme et al., 2000	0.818	0.013	0.848	0.008	0.926	0.001	8
Machmüller et al., 2002	NA		0.953	0.047	-	-	4
Patra and Yu, 2012	0.760	0.136	0.517	0.373	0.099	0.874	5
Yabuuchi et al., 2006	NA		0.815	0.026	-	-	7
Cieslak et al., 2006	NA		0.967	0.033	-	-	4
In vivo							
Machmüller et al., 2003a	-0.342	0.658	0.130	0.870	-0.627	0.373	4
Machmüller et al., 2003b	0.667	0.148	0.572	0.236	0.576	0.231	6
Machmüller and Kreuzer, 1999	NA		0.813	0.396	-	-	3
Jordan et al., 2006	NA		0.956	0.190	-	-	3
Ding et al., 2012	NA		0.072	0.928	-	-	4
Liu et al., 2011	0.905	0.013	0.815	0.048	0.939	0.005	6
Panyakaew et al., 2013b	NA		NA		0.773	0.227	4

NA=Not analyzed.

Relationship between MCFA, volatile fatty acids and CH_4

The data reported in Chapters 2, 3, 4, and 5 indicate an inverse relationship between CH_4 production and propionate concentrations in rumen fluid. This inverse relationship is commonly explained by the fact that propionic acid can act as a hydrogen sink (General introduction). The conversion of hexoses and pentoses into volatile fatty acids by rumen bacteria will potentially lead to accumulation of $NADH_2$ (Bryant, 1979) and its regeneration to NAD requires a very low partial pressure of H_2 in the rumen (Lingen et al., 2016). Methanogens play a very important role in this matter because these microbes use H_2 to reduce CO_2 into CH_4, thereby, reducing the partial pressure of H_2. In principle, H_2 can also be used to synthesize acetate but this process is thermodynamically much less favorable

compared to methanogenesis (Liu, 2010). Thus, H_2 mediated acetogenesis is generally considered unimportant to reduce partial pressure of H_2 in the rumen environment. In case methanogens are killed by MCFA (Zhou et al., 2013), the partial pressure of hydrogen will increase and consequently complicate the oxidation of $NADH_2$. This condition subsequently leads to the stimulation of propionate synthesis because this process requires $NADH_2$. Following this reasoning, the production of propionate is secondary to the inhibition of CH_4 synthesis.

In view of the relative low growth rates of methanogens (Rother et al., 2010; Patra et al., 2017) and, therefore, the relative slow removal of H_2 from the rumen contents, rumen bacteria opportunistically shift the fermentation of carbohydrates from acetic acid to the synthesis of propionic acid when the amount- and/or the fermentation rate of the substrate increases (Lingen et al., 2016). Taking this notion into account it is of interest to consider the experimental work of Machmüller et al. (2001) indicating that the suppressing effect of coconut oil on methanogenesis is greatest when bacteria ferment substrates with low contents of structural carbohydrates. This observation is difficult to explain but it can be interpreted in that at low fermentation rates, a MCFA induced reduction in viable methanogenic *Archaea* does only marginally alter partial H_2 pressure and thus CH_4 formation and, therefore, a minor change in acetate to propionate ratio of the volatile fatty acids. In contrast, at high fermentation rates, supplemental MCFA cause a rapid increase in the partial H_2 pressure and pushes the end-product of fermentation towards propionate and consequently a more pronounced decrease in CH_4 synthesis. Needless to mention that these notions are highly speculative and need to be confirmed by experimental data. The outcome of the experimental work published by Machmüller et al. (2001) is subject to debate because the results related to the fermentation rate of carbohydrates are confounded by 1) the amount of carbohydrates in the concentrates, 2) the source of protein and 3) the protein content of the experimental substrates. It appears that the microbial N supply per unit of time coincides with the difference in fermentation rate of the carbohydrates (Machmüller et al., 2001). Future studies are, therefore, warranted to elucidate the importance of the ingredient composition of the experimental substrate/ration on the efficacy of MCFA to inhibit methanogenesis.

MCFA-rich oil and *in vivo* fiber digestion

Unfortunately, there are no published data available in peer-reviewed scientific journals on supplemental krabok oil and *in vivo* fiber (*i.e.,* NDF) digestibility. There are, however, two publications reporting on the effect of supplemental myristic acid versus supplemental lauric- or stearic acid on fiber digestibility. Dohme et al. (2004) fed early lactating Brown Swiss cows, roughage-based rations (60% DM basis) which were supplemented with 40 g/kg DM of either C12:0, C14:0 or C18:0. Hristov et al. (2011) administrated 240 g/d of either stearic acid, lauric acid or myristic acids directly into the rumen (*i.e.,* 8.9, 12.0 and 9.3 g/kg DMI, respectively) of Holstein cows (95 days in milk) fed a ration containing 57.8% (DM basis) roughage. In both studies (Dohme et al., 2004; Hristov et al., 2011), the NDF digestibility's

were found to be similar between the experimental treatments. Thus, myristic- versus lauric acid does not seem to differentially affect fiber digestion.

Since krabok oil essentially only differs from coconut oil in its C14:0 content, it was, therefore, considered opportune to use data originating from digestibility trials with ruminants fed rations containing either or not supplemental coconut oil. The current data (Fig. 2) indicate an overall depressant effect of supplemental coconut oil on cell wall digestibility, *i.e.,* a linear decrease of ~5.5% in NDF digestibility for each 20 g/kg dry matter of supplemental coconut oil.

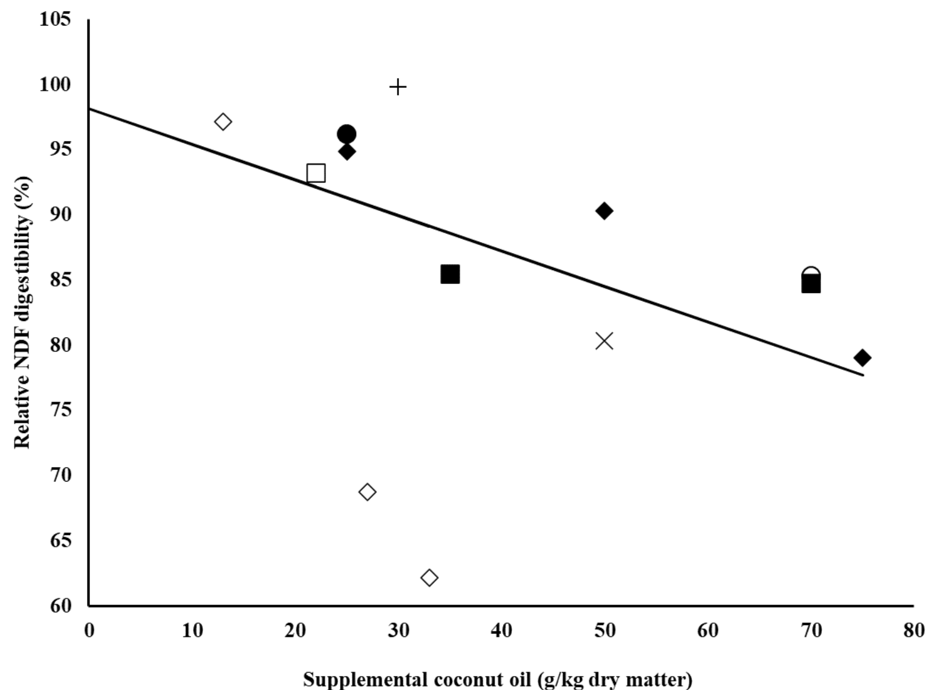


Figure 2. Dose-response relationship between the level of supplemental coconut oil and the digestibility of neutral detergent fiber (NDF) in ruminants. The data originate from eight independent experiments and the digestibility values from each individual study are expressed as a percentage of the unsupplemented (fat) control rations. Symbols: ■, Machmüller and Kreuzer (1998); ●, Machmüller et al. (2000); ♦, Bhatt et al. (2011); □, Hristov et al. (2009); ○, Kongmun et al. (2010); ◊, Hollmann et al. (2012); ×, Reveneau et al. (2012); +, Faciola and Broderick (2014). The solid line represents the trendline, *i.e.,* Y=98.2-0.27×X (R^2_{adj}=36.0%, P=0.002).

NB: The experiments represented by a solid marker are conducted in ovine with the exception of the study reported by Kongmun et al. (2010) who used swap buffalo. The remaining studies were conducted using lactating dairy cows.

The depressant effect of coconut oil on fiber digestibility may be somewhat overestimated due to the two observations with relative NDF digestibility <70% (Fig. 2) but in case these two datapoints are omitted from regression analysis, the trendline remains highly significant

but the slope slightly decreases from 0.27 to 0.25%/g supplemental coconut oil per kg dry matter (R^2_{adj}=81.6%, P<0.001). Under the assumption that the depressant effect of supplemental coconut oil on fiber digestibility can be extrapolated to krabok oil, it appears that caution is warranted to use krabok oil as a CH_4 mitigating tool.

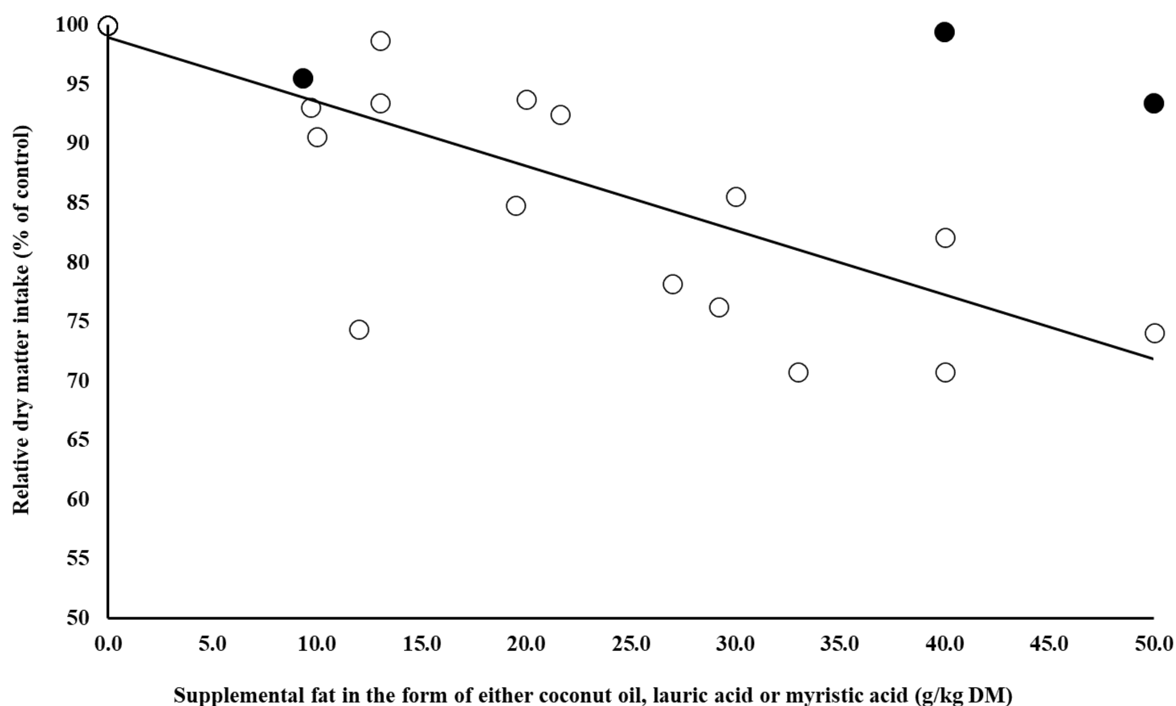


Figure 3. Dose-response relationship between the level of supplemental coconut oil, lauric acid or myristic acid and the dry matter intake (DMI) in dairy cows. The data originate from eight independent experiments and the DMI values from each individual study are expressed as a percentage of either the unsupplemented (fat) control rations or rations supplemented with stearic acid. Symbols: ○, data related to supplementation of either coconut oil or lauric acid and the data were obtained from: Dohme et al. (2004), Hollman and Beede (2012), Hollman et al. (2012), Faciola and Broderick (2013, 2014), and Hristov et al. (2009, 2011); ● data related to supplementation of myristic acid and the data were obtained from: Dohme et al. (2004), Hristov et al. (2011), and Odingo et al. (2007). The solid line represents the trendline calculated with the use of data obtained from studies where either coconut oil or lauric acid, but not myristic acid was used as a source of supplemental fat, *i.e,* Y=99.0-0.54×X (R^2_{adj}=56.2%, P<0.001).

MCFA and dry matter intake

During the *in vivo* experiments described in Chapters 3 and 4 of the current thesis, neither supplemental coconut oil nor krabok oil affected dry matter intake (DMI) of the experimental animals. However, during the aforementioned studies the animals were fed restrictively so as to ensure a constant intake of non-variable nutrients. In *ad libitum* fed animals, however,

MCFAs depress DMI (Fig. 3) but compared to coconut oil/lauric acid, the depressant effect of myristic acid, if any, can be typified as minor.

The depressant effect of coconut oil/lauric acid on DMI is most easily explained by the low palatability of lauric acid, the taste can be characterized as "soapy" (Dohme et al., 2004). This notion is in line with the observation that supplemental palm kernel oil, which is almost identical to coconut oil in terms of fatty acid profile (Chapter 1), also reduced DMI by 39.5% and 47.5% in finishing lambs and Nellore bulls, respectively (Castro et al., 2022; dos Santos et al., 2022). Unfortunately, the effect of krabok oil, or a combination of lauric acid and myristic acid that resembles krabok oil, on DMI in *ad libitum* fed cows is not yet reported and is difficult to speculate on the dose-response relationship, if any, between krabok oil and DMI. In view of previous notions in this section, however, a lack of effect of krabok oil on DMI can only be expected in case myristic acid counteracts the low palatability of lauric acid. Next to the issue of palatability, it cannot be excluded that supplemental MCFA (oils) reduce DMI due to an increase in rumen fill (Allen, 2000) caused by a reduced NDF digestibility (see previous section). The current data on DMI point to some potential practical limitations of krabok oil as a tool to mitigate CH_4 production.

Conclusion

Krabok oil has the ability to mitigate CH_4 emission from ruminant animals. In view of the potential issues related to fiber digestibility and dry matter intake, future studies should be conducted to determine the optimum dose of krabok oil to mitigate CH_4.

References:

Abubakr A., Alimon A.R., Yaakub H., Abdullah N., Ivan, M. 2014. Effect of feeding palm oil by-products based diets on total bacteria, cellulolytic bacteria and methanogenic archaea in the rumen of goats. PloS One, 9(4), e95713.

Allen, M.S. 2000. Effects of diet on short-term regulation of feed intake by lactating dairy cattle. J. Dairy Sci. 83, 1598-1624.

Asanuma, N., Iwamoto, M., Hino, T. 1999. Effect of the addition of fumarate on methane production by ruminal microorganisms *in vitro*. J. Dairy Sci. 82, 780-787.

Bryant, M.P. 1979. Microbial methane production-Theoretical aspects. J. Anim. Sci. 48, 193-201.

Black, J.L., Davison, T.M., Box, I. 2021. Methane emissions from ruminants in Australia: Mitigation potential and applicability of mitigation strategies. Anim. 11, 951.

Castro, D.P.V., Pimentel, P.R.S., De Silva Junior, J.M., Junior, G.F.V., De Andrade, E.A., Barbosa, A.M., Pereisa, E.S., Di Mambro Riberiro, C.V., Bezerra, L.R., Oliverira, R.L. 2022. Dietary effect of palm kernel oil inclusion in feeding finishing lambs on meat quality. Anim. 12, 3242.

Jarrell K.F., Sprott, G.D. Matheson, A.T. 1984. lntracellular potassium concentration and relative acidity of the ribosomal proteins of methanogenic bacteria. Can. J. Microbiol. 30, 663-668.

Galbraith, H., Miller, T.B. 1973. Effect of metal cations and pH on the antibacterial activity and uptake of long chain fatty acids. J. Appl. Bact. 36, 635-640.

Bauman, D.E., Griinari, J.M. 2003. Nutritional regulation of milk fat synthesis. Annu. Rev. Nutr. 23, 203-227.

Bauman, D.E., Harvatine, K.J., Lock, A.L. 2011. Nutrigenomics, rumen-derived bioactive fatty acids, and the regulation of milk fat synthesis. Annu. Rev Nutr. 31, 299-319.

Bhatt, R.S., Soren, N.M., Tripathi, M.K., Karim, S.A. 2011. Effects of different levels of coconut oil supplementation on performance, digestibility, rumen fermentation and carcass traits of Malpura lambs. Anim. Feed Sci. Technol. 164, 29-37.

Butsch, B.M., Bachofen, R. 1984. The membrane potential in whole cells of *Methanobacterium thermoautotrophicum*. Arch. Microbiol .138, 293-298.

Cieslak, A., Szumacher-Strabel, M., Szymankiewicz, E., Piekniewski, M., Oleszak, P., Siwinski, L., Potkanski, A. 2006. Coconut oil reduces protozoa count and methane release during fermentation in a Rusitec system. J. Anim. Feed Sci. Technol. 15(1), 19-22.

Dewanckele, L., Vlaeminck, B., Hernandez-Sanabria, E., Ruiz-González, A., Debruyne, S., Jeyanathan, J., Fievez, V. 2018. Rumen biohydrogenation and microbial community changes upon early life supplementation of 22:6n-3 enriched microalgae to goats. Front. Microbiol. 9, 573.

Ding, X., Long, R., Zhang, Q., Huang, X., Guo, X., Mi, J. 2012. Reducing methane emissions and the methanogen population in the rumen of Tibetan sheep by dietary supplementation with coconut oil. Trop. Anim. Health Prod. 44, 1541-1545.

Dohme, F., Machmüller, A., Wasserfallen, A., Kreuzer, M. 2000. Comparative efficiency of various fats rich in medium-chain fatty acids to suppress ruminal methanogenesis as measured with Rusitec. Can. J. Anim. Sci.80, 473-484.

Dohme, F., Machmüller, A., Sutter, F., Kreuzer, M. 2004. Digestive and metabolic utilization of lauric, myristic and stearic acid in cows, and associated effects on milk fat quality. Arch. Anim. Nutr. 58, 99-116.

Dohme, F., Machmüller, A., Wasserfallen, A., Kreuzer, M. 2001 Ruminal methanogenesis as influenced by individual fatty acids supplemented to complete ruminant diets. Lett. Appl. Microbiol. 32(1), 47-51.

Dos Santos, N.J.A., Bezerra, L.R., Castro, D.P.V., Marcelino, P.D.R., Andrade, E.A., Virginio, G.F., Da Silva Junior, J.M., Pereira, E.S., Barbosa, A.M., Oliveira, L. 2022. Performance, digestibility, nitrogen balance and ingestive behavior of young feedlot bulls supplemented with palm kernel oil. Animals 12(4), 429.

Duval, S., Kindermann, M. 2016. Use of nitrooxy organic molecules in feed for reducing methane emission in ruminants, and/or to improve ruminant performance. U.S. Patent 20140147529 A1, 23 February 2016.

FAO. 2017. *The Future of Food and Agriculture-Trends and Challenges*; Food and Agriculture Organization of the United Nations, Rome, Italy. 180p. Rome, Available online: http://www.fao.org/3/y4252e/y4252e07.htm (accessed on 9 March 2021.)

Faciola, A.P.; Broderick, G.A.; Hristov, A.; Leão, M.I. 2013. Effects of lauric acid on ruminal protozoal numbers and fermentation pattern and milk production in lactating dairy cows. J. Anim. Sci. 91, 363-373.

Faciola, A.P., Broderick, G.A. 2014. Effects of feeding lauric acid or coconut oil on ruminal protozoa numbers, fermentation pattern, digestion, omasal nutrient flow, and milk production in dairy cows. J. Dairy Sci. 97, 5088-5100.

Freese, E., Sheu, C.W. Galliers, E. 1973. Function of lipophilic acids as antimicrobial food additives. Nature 241, 321.

Griinari, J.M., Corl, B.A., Lacy, S.H., Chouinard, P.Y., Nurmela, K.V.V., Bauman, D.E. 2000. Conjugated linoleic acid is synthesized endogenously in lactating dairy cows by Δ^9-desaturase. J. Nutr. 130, 2285-2291.

Hook, S.E., Wright, A.D.G., Mcbride, B.W. 2010. Methanogens: methane producers of the rumen and mitigation strategies. Archaea, 945785.

Hunt, M.C., Siponen, M. I., Alexson, S.H.E. 2012. The emerging role of acyl-CoA thioesterases and acyltransferases in regulating peroxisomal lipid metabolism. Biochim. Biophys. Acta. 1822, 1397-1410.

Hristov, A.N., Oh, J., Giallongo, F., Frederick, T.W., Harper, M.T., Weeks, H.L., Branco, A.F., Moate, P.J., Deighton, M.H., Williams, S.R.O. 2015. An inhibitor persistently decreased enteric methane emission from dairy cows with no negative effect on milk production. Proc. Natl. Acad. Sci. USA 112, 10663-10668.

Hristov, A.N., Lee, C., Cassidy, T., Long, M., Heyler, K., Heyler, K., Corl, B., Forster, R., 2011. Effects of lauric and myristic acids on ruminal fermentation, production, and milk fatty acid composition in lactating dairy cows. J. Dairy Sci. 94, 382-395.

Hristov, A.N., Vander Pol, M., Agle, M., Zaman, S., Schneider, C., Ndegwa, P., Vaddella, V.K. Johnson, K., Shingfield, K.J., Karnati, S.K.R. 2009. Effect of lauric acid and coconut oil on ruminal fermentation, digestion, ammonia losses from manure, and milk fatty acid composition in lactating cows. J. Dairy Sci. 92, 5561-5582.

Hristov, A.N., Varga, G., Cassidy, T., Long, M., Heyler, K., Kar- nati, K.R., Corl, B., Hovde, C.J., Yoon. I. 2010. Effect of yeast culture on ruminal fermentation and nutrient utilization in dairy cows. J. Dairy Sci. 93, 682-692.

Hollmann, M., Beede, D.K., 2012. Comparison of effects of dietary coconut oil and animal fat blend on lactational performance of Holstein cows fed a high-starch diet. J. Dairy Sci. 95, 1484-1499.

Hollmann, M., Allen, M.S., Beede, D.K. 2011. Diet fermentability influences lactational performance responses to corn distillers grains: A meta-analysis. J. Dairy Sci. 94, 2007-2021.

Hollmann, M., Powers, A.C., Fogiel, A.C., Liesman, J.S., Bello, N.M., Beede, D.K. 2011. Enteric methane emissions and lactational performance of Holstein cows fed different concentrations of coconut oil. J. Dairy Sci. 95, 2602-2615

Jenkins, T.C., Harvatine, K.J. 2014. Lipid feeding and milk fat depression. Vet. Clin. North Am. Food Anim. Pract. 30, 623-642.

Jordan, E., Kenny, D., Hawkins, M., Malone, R., Lovett, D.K., Mara, F.P.O. 2006. Effect of refined soy oil or whole soybeans on intake, methane output, and performance of young bulls. J. Anim. Sci. 84(9), 2418-2425.

Kongmun, P., Wanapat. M., Pakdee, P., Navanukraw, C., Yi, Z. 2011. Manipulation of rumen fermentation and ecology of swamp buffalo by coconut oil and garlic powder supplementation. Livest. Sci. 135, 84-92.

Liu, Y. 2010. Taxonomy of methanogens in: Handbook of Hydrocarbon and Lipid Microbiology, Vol. 1, Springer-Verlag, Berlin Heidelberg, New York, pp. 550-558.

Lingen, H.J., Van Plugge, C.M., Fadel, J.G., Kebreab, E., Bannink, A., Dijkstra, J. 2016. Thermodynamic driving force of hydrogen on rumen microbial metabolism: a theoretical investigation. PLoS ONE 10, e0161362.

Lila, Z.A., Mohammed, N., Ksanda, S., Kamada, T., Itabashi, H. 2003. Effect of sarsaponin on ruminal fermentation with particular reference to methane production *in vitro*. J. Dairy Sci. 86, 3330-3336.

Lauri A., Mariani P. O. 2009. Potentials and limitations of molecular diagnostic methods in food safety. Genes Nutr. 4, 1-12.

Machmüller, A., Ossowski, D.A., Wanner, M., Kreuzer, M. 1998. Potential of various fatty feeds to reduce methane release from rumen fermentation *in vitro* (Rusitec). Anim. Feed Sci. Technol. 71(1-2), 117-30.

Machmüller, A. Kreuzer, M. 1999. Methane suppression by coconut oil and associated effects on nutrient and energy balance in sheep. Can. J. Anim. Sci. 79, 65-72.

Machmüller, A., Ossowski, D.A., Kreuzer, M. 2000. Comparative evaluation of the effects of coconut oil, oilseeds and crystalline fat on methane release, digestion and energy balance in lambs. Anim. Feed Sci. Technol. 85, 41-60.

Machmüller, A., Dohme, F., Soliva, C.R., Wanner, M., Kreuzer, M. 2001. Diet composition affects level of ruminal methane suppression by medium-chain fatty acids. Aust. J. Agric. Res. 52, 713-722.

Machmüller, A., Soliva, C.R., Kreuzer, M. 2003a. Effect of coconut oil and defaunation treatment on methanogenesis in sheep. Reprod. Nutr. Develop. 43, 41-55.

Machmüller, A. 2006. Medium-chain fatty acids and their potential to reduce methanogenesis in domestic ruminants. Agric. Ecosys. Environ. 112, 107-114.

Martin, C., Rouel J., Jouany, J.P., Doreau, M., Chilliard, Y. 2008. Methane output and diet digestibility in response to feeding dairy cows crude linseed, extruded linseed, or linseed oil. J. Anim. Sci. 86(10), 2642-2650.

Nocker, A., Sossa, K.E., Camper, A.K. 2007. Molecular monitoring of disinfection efficacy using propidium monoazide in combination with quantitative PCR. J. Microbiol. Methods 70, 252-260.

Odongo, N.E., Or-Rashid, M.M., Kebreab, E., France, J., McBride, B.W. 2007. Effect of supplementing myristic acid in dairy cow rations on ruminal methanogenesis and fatty acid profile in milk. J. Dairy Sci. 90, 1851-1858.

Pilajun, R., Wanapat, M. 2011. Methane production and methanogen population in rumen liquor of swamp buffalo as influenced by coconut oil and mangosteen peel powder supplementation. J. Anim. Vet. Adv. 10, 2523-2527.

Patra, A.K and Yu, T.Z. 2013. Effects of coconut and fish oils on ruminal methanogenesis, fermentation, and abundance and diversity of microbial populations *in vitro*. J. Dairy Sci. 96(3), 1782-1792.

Pathak, S., Awuh, J.A., Leversen, N.A., Flo, T.H., Asjø, B. 2012. Counting mycobacteria in infected human cells and mouse tissue: a comparison between qPCR and CFU. PLoS One 7, e34931.

Reveneau, C. Karnati, S.K.R., Oelker, E.R. Fiekins, J.L. 2012. Interaction of unsaturated fat or coconut oil with monensin in lactating dairy cows fed 12 times daily. I. Protozoal abundance, nutrient digestibility, and microbial protein flow to the omasum. J. Dairy Sci. 95(4), 2046-60.

Rico, D.E., Parales, J.E., Corl, B.A., Lengi, A., Chouinard, P.Y., Gervais, R. 2020. Abomasally infused SFA with varying chain length differently affect milk production and composition and alter hepatic and mammary gene expression in lactating cows. Br. J. Nutr. 124, 386-395.

Rother M., Krzycki, J.A. 2010. Selenocysteine, pyrrolysine, and the unique energy metabolism of methanogenic archaea. Archaea 453642.

Sauer, F.D., Erfle, J.D., van Mahade, S. 1979. Methane synthesis without the addition of adenosine triphosphate by cell membranes isolated from *Methanobacterium ruminantium*. Biochem. J. 178, 165-172.

Schönheit, P., Beimborn D.B., Perski, H.J. 1984. Potassium accumulation in growing *Methanobacterium thermoautotrophicum* and its relation to the electrochemical proton gradient. Arch. Microbiol. 140: 247-251.

Patra, A., Park, T., Yu, Z. 2017. Rumen methanogens and mitigation of methane emission by anti-methanogenic compounds and substances. J. Anim. Sci. Biotechnol. 8, 13.

Soliva, C.R., Hindrichsen, I.K., Meile, L., Kreuzer, M., Machmüller, A. 2003. Effects of mixtures of lauric and myristic acid on rumen methanogens and methanogenesis *in vitro*. Lett. Appl. Microbiol. 37, 35-39.

Sontakke, S., Cadenas, M, Maggi, R, Diniz, P., Breitschwerdt, E.B. 2009. Use of broad range 16S rDNA PCR in clinical microbiology. J. Microbiol. Methods, 76(3), 217-225.

Schonfeld, P., Wojtczak, L. 2016. Short- and medium-chain fatty acids in energy metabolism: the cellular perspective. J. Lipid. Res. 57(6), 943-954.

Xuan, Z., Meile, L., Kreuzer, M., Zeitz, J.O. 2013. The effect of saturated fatty acids on methanogenesis and cell viability of *Methanobrevibacter ruminantium*. Archaea 106916.

Vvay, D., Teter, B.B., Erdman, R.A. 2012. Milk fat responses to dietary supplementation of short- and medium-chain fatty acids in lactating dairy cows. J. Dairy Sci. 95, 5194-5202.

Yabuuchi, Y., Matsushita, Y., Otsuka, K., Fukamachi, K., Kobayashi, Y. 2006. Effects of supplemental lauric acid-rich oils in high-grain diet on *in vitro* rumen fermentation. Anim. Sci. J. 77, 300-307

Yang, S.L., Bu, D.P., Wang, J.Q., Hu, Z.Y., Li, D., Wei, H.Y. 2009. Soybean oil and linseed oil supplementation affect profiles of ruminal microorganisms in dairy cows. Anim. 3(11), 1562-1569.

Summary

In view of the efforts to reduce the emission of greenhouse gasses into the earth's atmosphere, methane produced by ruminants is considered a relevant target to mitigate methane emission originating from anthropogenic sources. In the current thesis, the potential of krabok oil was investigated to reduce methane production by ruminants. Typically, krabok oil has similar amounts of lauric acid (C12:0) compared to coconut oil but krabok oil also contains ~44% myristic acid (C14:0) and previous research indicated that C14:0 and C12:0 synergistically affected rumen methanogenesis when they were applied as pure fatty acids. In the current thesis it was attempted to assess this synergism *in vitro* and *in vivo*, with the use of two natural fatty acid sources, *i.e.,* krabok- and coconut oil. In the first *in vitro* experiment, rumen methanogenesis and the biohydrogenation of unsaturated long chain fatty acids were assessed because unsaturated fatty acids can act as a hydrogen sink and thus might explain a reduced methane production. The study consisted of five treatments, *i.e.*, a basal substrate, without supplementation of coconut or krabok oil, a basal substrate supplemented with either coconut oil or krabok oil. Both, coconut and krabok oil were supplemented in two doses, providing either 80 or 120 mg of C12:0+C14:0 per 100 ml of incubation fluid. The results of this study indicated that both krabok and coconut oil reduced methane production which was coincided with a shift from acetate and butyrate production to propionate. The observed shift in volatile fatty acids was similar between the two supplemental oils but the effects were more pronounced in combination with unsaturated fatty acids originating from linseed and sunflower oil. The overall effect of either krabok oil or coconut oil on rumen biohydrogenation, however, was limited.

In view of the confirmed inhibitory effect of krabok oil on rumen methanogenesis, a subsequent study was conducted to test the defaunating effect of krabok oil *in vivo*. The rationale of this notion is related with the fact that methanogenic *Archaea* live to a great extent in symbiosis with rumen protozoa. Three rumen-cannulated beef cows were used to assess the potential of krabok and coconut oil to affect rumen protozoa numbers with experimental treatments arranged in a 3×3 Latin square design. The experimental diets consisted of a concentrate supplemented with either 25.5 g/kg of tallow (control) or the same quantity of coconut oil or krabok oil. Supplemental krabok oil as well as coconut were found to decrease the protozoa numbers, but the effect of coconut oil was more pronounced. The ciliate counts were, however, not significantly different between treatment although the ciliate counts were strongly related to total protozoa counts. Except for propionic acid, the rumen concentrations of the remaining volatile fatty acids were similar between treatments. The two oils did not influence the amylolytic, cellulolytic or proteolytic bacteria counts. Cluster analysis of the denaturing gel electrophoresis profiles of ciliate communities showed a clustering of the coconut oil containing diet and the tallow containing diet.

In the aforementioned study, the defaunating effect of krabok oil was lower compared to coconut oil which might have been caused by the low dose of supplemental fat, *i.e.,* 19.2 g/kg dry matter. Therefore, a second *in vivo* experiment was conducted using a higher dosage of supplemental fat, *i.e.,* 35 g/kg dry matter. In addition, an in-depth assessment of the methanogenic community in the rumen was performed so as to potentially gain more insight

in the mode of action of krabok oil. All experimental treatments contained 35 g supplemental fat per kg dry matter: a control diet with tallow; a diet with supplemental coconut oil; and a diet with supplemental krabok oil. A 4th treatment consisted of a diet with similar amounts of medium chain fatty acids (*i.e.,* C10:0+C12:0+C14:0) from coconut- and krabok oil whereby extra tallow was supplied so as to maintain a constant fat content between the experimental treatments. Eight rumen-cannulated bulls, two bulls per treatment were used. The bulls were fed a total mixed ration which predominantly consisted of cassava chips, rice straw, tomato pomace, rice bran and soybean meal. Both krabok oil and coconut oil caused greater concentrations of rumen volatile fatty acids and shifted the proportions of individual volatile fatty acids from acetate to propionate. Protozoal numbers were reduced by either source of medium chain fatty acids and the strongest reduction was observed when supplemental krabok oil was fed. The abundance of methanogenic *Archaea* was likewise affected by the experimental diets. The denaturing gradient gel electrophoresis profiles of the rumen archaeal population did not result in a grouping of treatments but in case the diets were supplemented with krabok oil, the richness and evenness were increased within the archaeal community.
For practical reasons, it was considered opportune to evaluate the inhibitory effect of whole krabok seed (contains ~ 56% krabok oil) on methane production. Furthermore, the appropriate inclusion level of dietary krabok oil to mitigate methane production was not settled yet. Therefore, a dose-response relationship between krabok oil and methane production was also investigated using a fully automated gas production test. The experimental substrates contained either krabok oil (KO), whole krabok seed (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 fiber, and acid detergent fibre constant to prevent biased results through potential differences in fermentability between WKS, its derivatives, and the basal substrate. Both, the absolute (ml/g fat-free OM) and the relative (% of total gas production methane production were reduced at the highest inclusion level of WKS or its derivatives. At the same time, the highest inclusion levels FER, FER+KO, and WKS, but not KO, reduced total gas production and total volatile fatty acids. The data indicated that at least 94 g KO/kg dry matter is needed to inhibit methane production. Furthermore, supplementation of KO in the form of WKS is not warranted because the fat extracted residue of WKS is poorly degraded during fermentation.
In current thesis, it was shown that krabok oil is instrumental in mitigating methane emission. However, in view of the potential issues related to fiber digestibility and feed intake, future studies should be conducted to evaluate the practical use of krabok oil in formulating ruminant diets.

Samenvatting

Methaan geproduceerd door herkauwers levert een belangrijke bijdrage aan de uitstoot van broeikasgassen. Het is daarom van belang om middelen te onderzoeken die deze methaanproductie kunnen remmen. In dit proefschrift is daartoe het potentieel van krabokolie onderzocht. In vergelijking met kokosolie, bevat krabokolie een vergelijkbare hoeveelheid laurinezuur (C12:0), maar krabokolie bevat daarnaast ~44% myristinezuur (C14:0). Eerder onderzoek toonde aan dat de vetzuren C14:0 en C12:0 elkaar versterken om de methaan vorming vanuit de pens te remmen. In het huidige proefschrift zijn twee natuurlijke vetzuurbronnen, *i.e.*, krabok- en kokosolie gebruikt om de wisselwerking tussen C14:0 en C12:0 verder te onderzoeken.

Het eerste onderzoek betrof een laboratoriumstudie waarin naast de methaanvorming in de pens, ook de hydrogenering van onverzadigde langketen vetzuren werd onderzocht. Een toename in de verzadigsgraad van langketen vetzuren kan namelijk een mogelijke verklaring zijn voor een verlaagde methaanproductie. In dit onderzoek werden vijf substraten onderzocht; een basis substraat, zonder toevoeging van kokos- of krabokolie, en een basis substraat gesuppleerd met kokosolie of krabokolie waarbij de oliën in twee doses werden gesuppleerd, namelijk 80 of 120 mg C12:0+C14:0 per 100 ml incubatievloeistof. De resultaten van dit onderzoek gaven aan dat zowel krabok- als kokosolie de methaanproductie verminderden, hetgeen samenviel met een verschuiving van de acetaat- en butyraatproductie naar de productie van propionzuur. De waargenomen verschuiving in vluchtige vetzuren was vergelijkbaar tussen de twee gesuppleerde oliën, maar de effecten waren meer uitgesproken in combinatie met onverzadigde vetzuren afkomstig van lijnzaad- en zonnebloemolie. Het effect van krabokolie of kokosolie op de biohydrogenering van langketen onverzadigde vetzuren in de pens bleek echter beperkt.

Omdat het remmende effect van krabokolie op de methaanvorming in het eerste onderzoek werd bevestigd, werd een vervolgstudie uitgevoerd om het effect van krabokolie op de in de pens levende protozoën te testen in een voedingsonderzoek bij runderen. De protozoën in de pens leven namelijk voor een groot deel in symbiose met de organismen die methaan produceren, *i.e.*, de *methanogene Archaea*. Het idee is dat een verlaging van de protozoënpopulatie in de pens daarom zal samengaan met een verlaging van de Archaea populatie in de pens. Drie vleeskoeien, voorzien van een penscanule, werden gebruikt om het potentieel te onderzoeken van krabok- en kokosolie om de populatie protozoën in de pens te verlagen. De experimentele rantsoenen bestonden uit een mengvoer gesuppleerd met ofwel 25,5 g/kg rundvet (controle) of met dezelfde hoeveelheid kokosolie of krabokolie. De gesuppleerde hoeveelheden krabokolie en kokosnoot bleken inderdaad het aantal protozoën te verminderen, maar het effect van kokosolie op de protozoën populatie was meer uitgesproken. De ciliaten zijn een belangrijke groep binnen de protozoënpopulatie in de pens maar de ciliatentellingen waren echter niet statistisch significant verschillend tussen de behandelingen, hoewel de ciliatentellingen sterk gerelateerd waren aan het totale aantal protozoën. Met uitzondering van propionzuur waren de concentraties van de resterende vluchtige vetzuren in de pensvloeistof vergelijkbaar tussen de behandelingen. De twee oliën

hadden ten opzichte van de controle geen invloed op het aantal zetmeel-, vezel- of eiwitsplitsende bacteriën in de pens.
In bovengenoemde studie was het remmende effect van krabokolie lager dan dat van kokosolie wat mogelijk veroorzaakt werd door een te lage dosis gesuppleerd vet, namelijk 19,2 g/kg droge stof. Daarom werd een tweede voedingsproef uitgevoerd met een hogere dosering gesuppleerd vet, namelijk 35 g/kg droge stof. In een poging om meer inzicht te krijgen in het werkingsmechanisme van krabokolie werd in dit experiment de methaan producerende populatie in de pens nader bestudeerd. De experimentele voeders bestonden uit een controle rantsoen met rundvet; een rantsoen met kokosolie; en een rantsoen met krabokolie. Een vierde behandeling bestond uit een dieet met vergelijkbare hoeveelheden middellange keten vetzuren uit kokos- en krabokolie, waarbij extra rundvet werd toegevoegd om zo een constant vetgehalte tussen de experimentele voeders te verkrijgen. Er werden acht pensgecannuleerde stieren gebruikt met twee stieren per behandeling. De stieren kregen een gemengd rantsoen dat voornamelijk bestond uit cassavechips, rijststro, tomatenpuree, rijstzemelen en sojameel. Zowel krabokolie als kokosolie veroorzaakten hogere concentraties aan vluchtige vetzuren in de pensvloeistof en tevens verschoof de verhouding tussen individuele vluchtige vetzuren van acetaat naar propionaat. Het aantal protozoën werd verminderd door zowel krabokolie als kokosolie maar de sterkste vermindering van het aantal protozoën werd waargenomen wanneer krabokolie aan de dieren werd verstrekt. De Archaeapopulatie werd op een vergelijkbare manier beïnvloed door de experimentele rantsoenen. Op basis van gradiënt-gelelektroforeseprofielen van de Archaeapopulatie in de pens kon er geen groepering worden vastgesteld tussen de experimentele voeders, maar in het geval dat de rantsoenen werden gesuppleerd met krabokolie, nam zowel de rijkdom en uniformiteit van de Archaeapopulatie toe.
Om praktische redenen werd het opportuun geacht om ook het remmende effect van het krabokzaad (bevat ~ 56% krabokolie) op de methaanproductie te evalueren. Aangezien er tevens nog geen gegevens beschikbaar waren over de dosis-responsrelatie tussen het gehalte aan krabokolie in de voeding en de methaanproductie, is ook dit aspect onderzocht onder laboratoriumcondities. De experimentele substraten bevatten ofwel krabokolie, krabokzaad, het residu van krabokzaad na vetextractie of het residu+krabokolie. Gepaste hoeveelheden krabokzaad, of de hiervan afgeleide producten, werden aan een basis substraat toegevoegd om zo een laag, gemiddeld of hoog gehalte aan krabokolie te bereiken (*i.e.*, 37-46, 90-94 en 146-153 g krabokolie/kg droge stof). De experimentele substraten werden zodanig geformuleerd dat de hoeveelheden geïncubeerde vetvrij organische stof, ruw eiwit, en plantaardige vezels constant waren. Zowel de absolute (ml/g vetvrije organische stof) als de relatieve (% van de totale gasproductie) methaanproductie werden verminderd bij het hoogste gehalte aan krabokzaad en de hiervan afgeleide producten. Echter, zowel de totale gasproductie en de productie van vluchtige vetzuren was verlaagd wanneer het substraat hoge gehalten aan krabokzaad of het vetvrije residu van krabokzaad, bevatten. De gegevens gaven verder aan dat er minimaal 94 g krabokolie per kg droge stof nodig is om de methaanproductie te remmen. Op basis van de uitkomsten van dit experiment is het niet gewenst om krabokzaad te gebruiken om de methaanproductie te remmen omdat het krabokzaad slecht wordt gefermenteerd.

In het huidige proefschrift werd aangetoond dat krabokolie een belangrijke rol kan spelen bij de vermindering van de methaanproductie bij herkauwers. Er zijn echter nog kanttekeningen te plaatsen m.b.t. de praktische toepassing van krabokolie omdat hoge gehalten aan krabokolie mogelijk de verteerbaarheid van plantaardige vezels en de voeropname negatief zou kunnen beïnvloeden. Toekomstige studies moeten uitwijzen in hoeverre krabokolie kan worden toegepast in de praktijk om "laag methaanemissie" rantsoenen te formuleren voor herkauwers.

Acknowledgements

I would like to acknowledge and extend my heartfelt gratitude to the following persons who have made the completion of this thesis possible:
I would like to thank the European Commission through the Asia-Link programme (Phase III) in cooperation with the Faculty of Natural Resources, Rajamangala University of Technology Isan, Sakon-Nakhon Campus, Sakon Nakhon, Thailand for granting me the scholarship to study towards a PhD degree. I would like to acknowledge the Faculty of Natural Resources, Rajamangala University of Technology Isan, Sakon Nakhon Campus Thailand for facilitating laboratory analyses, providing research facilities and for financial support. Moreover, I would like to acknowledge the Laboratory for Animal Nutrition and Animal Product Quality (Lanupro), Faculty of Bioscience Engineering, and the Laboratory of Microbial Ecology and Technology (LabMET), Faculty of Bioscience Engineering, Ghent University, Gent, Belgium for facilitating laboratory analyses and providing research facilities.
I am heartily thankful to my supervisor, Assoc. Prof. dr. Jan Thomas Schonewille, who gave me enthusiastic encouragement, guidance, support and his friendship which enabled me to develop an understanding of the research work. The thesis could not have been completed without his constructive corrections and advises. I would also like to thank Assist. Prof. dr. Chalermpon Yuangklang for acting as the co-supervisor in Thailand, for his advice and assistance in keeping my progress on schedule. I have learnt a lot from both of them. Thank you very much for fulfilling my knowledge. I am grateful to Prof. dr. Wouter H. Hendriks, my promoter, for accepting me as his PhD student, and for advice, design and support during my study and preparation of the manuscripts and thesis.
I am sincerely grateful to Dr. Robert Paling, former Director of the Bureau of International Cooperation (BIC) and his team for helping me during my stay in the Netherlands. Many thanks to the European Commission through the Asia-Link project partners: Ruminant project; Dr. Yong-Meng Goh from Faculty of Veterinary Medicine, University Putra Malaysia, Malaysia, Dr. Dam Van Tien from Faculty of Animal Science, Hue University of Agriculture and Forestry, Vietnam, Prof. dr. Stefaan de Smet and Prof. dr. Veerle Fievez of the Faculty of Bioscience Engineering, Ghent University, Belgium.
My grateful thanks are extended to Prof. dr. Veerle Fievez, Dr. Bruno Vlaeminck and Prof. dr. Stefaan de Smet, Gent University, Belgium for facilitating laboratory analyses and their kindness and hospitality during my stay in Gent, Belgium. I really appreciated it.
I would like to thank my colleagues, PhD students of the EU Asia-link project: Hasliza Abu Hassim, Phan Vu Hai, Rittichai Pilachai and Jaturong Wonsanit (Daeng), for their warm kindness and friendship. I really enjoyed working with them. Many thanks to my Thai friends in the Netherlands and Belgium for their support and friendship.
My family is the most important reason why I have been able to fulfill my studies. Thank you, my husband, Assist. Prof. dr. Pornthep Panyakaew and my daughter, Korya, Piyathida Panyakaew for your support and patience while I was away for my studies. I would like to thank my father, Mr. Noppadon Srenanul, my mother, Mrs. Loumyai Laramat, for their encouragement, support and patience throughout my studies.

Lastly, I offer my regards and blessings to all of those who supported me in any way during the completion of the thesis.

Curriculum vitae

Paiwan Panyakaew was born on the 22nd of February 1979 in Sakon Nakhon, Thailand. She obtained her Bachelor of Science degree majoring in Animal Science from Rajamangala University of Technology Isan, Sakon Nakhon campus, Thailand in 2002. During 2002-2005, she worked at the Department of Animal Science, Faculty of Natural Resources, Rajamangala University of Technology Isan, Sakon-Nakhon Campus, Sakon Nakhon, Thailand. At the same time in 2005, she obtained her Master of Science (Animal Science) degree from the Faculty of Agriculture, Khon Kaen University, Thailand. Since November 2005, she has been a lecturer at the Department of Animal Science, Faculty of Natural Resources, Rajamangala University of Technology Isan, Sakon-Nakhon Campus, Sakon Nakhon, Thailand. In 2008, she was selected to participate in the Asia-Link Programme (Phase III) in the project entitled "The conversion of local feeds into human food by the ruminant". During this three-year EU funded program, she started her research on krabok oil as tool to mitigate methane production in ruminants. In 2010, she received grants from the Lotus-Erasmus Mundus Action2 (EU-South-East Asian mobility project) for 10 months research at two European patners of the Asia-Link Programme; Ghent University, Belgium and Utrecht University, The Netherlands. Her position and academic work in dairy cows will be continued at the Department of Animal Science, Faculty of Natural Resources, Rajamangala University of Technology Isan, Sakon-Nakhon Campus, Sakon Nakhon, Thailand.

List of Publications

Peer-reviewed journals

1. **Panyakaew, P**., Goel, G., Lourengo, M., Yuangklang, C., Fievez, V. 2013 Medium-chain Fatty acids from coconut or krabok oil inhibit *in vitro* rumen methanogenesis and conversion of non-conjugated dienoic biohydrogenation intermediates. Anim. Feed Sci. Technol. 180, 18-25.
2. **Panyakaew, P**., Goel, G., Yuangklang, C., Boon, N., Schonewille, J.Th., Hendriks, W.H., Fievez, V. 2013 Effect of supplementing coconut or krabok oil, rich in medium chain fatty acids on ruminal fermentation, protozoa and archaeal population of bulls. Anim. 7(12), 1950-1958.
3. **Panyakaew, P**., Schonewille, J.Th., Cone, J.W., Pellikaan, W.F., Fievez V., Yuangklang, C., Hendriks, W.H. 2020. Isolipidic replacement of krabok oil by whole krabok seed reduces *in vitro* methanogenesis, but negatively affects fermentation. J. Anim. Physiol. Anim. Nutr. 104, 453-461.
4. **Panyakaew, P**., Schonewille, J.Th., Fievez, V., Goel G., Boon N., Yuangklang, C., Hendriks, W.H. 2020. Coconut and to a lesser extent krabok oil, depress rumen protozoa in beef cows. J. Sci. Agri. Technol. 1(1), 26-32.

Contributions to conferences and symposia

1. **Panyakaew, P**., Yuangklang C., Schonewille J.Th., Hendriks W.H. 2016. Effect of whole Krabok seed, krabok oil or krabok residue on *in vitro* methane production. 17th AAAP animal science congress. Fukuoka, Japan, 22-25 August. (Oral presentation)
2. **Panyakaew, P**., Goel G., Yuangklang C., Schonewille J.Th., Hendriks W.H., Boon N., Fievez V. 2013 Dynamics on methanogenic population, protozoa numbers and rumen fermentation in response to dietary supplementation of coconut or krabok oil. 38th ANR Forum, 6th Jun. Roeselare, Belgium. (Oral presentation)
3. **Panyakaew, P**., Goel G., Yuangklang, C., Boon, N., Fievez, V. 2012. Effect of coconut or krabok oil, rich in medium chain fatty acids on ruminal archaeal population of bulls as assessed by PCR-DGGE and Quantitative Real time PCR analysis. 15th AAAP animal science congress. Bangkok, Thailand, 26-30 November. (Oral presentation)
4. **Panyakaew, P**., Goel, G., Yuangklang, C., Boon N., Fievez, V. 2010. Effect of medium chain fatty acids from coconut oil or krabok oil on rumen protozoa in beef cows. In: Proceedings of the 7th Joint RRI-INRA Symposium. Gut microbiology: new insights into gut micrbial ecosystems, Aberdeen (UK), pp. 73.
5. **Panyakaew, P**., Goel, G., Yuangklang, C., Fievez, V. 2010. Effect of coconut or krabok oil, rich in medium chain fatty acids on rumen fermentation and ciliate protozoa in beef cows. 13th Associations of Institutions for Tropical Veterinary Medicine (AITVM) Conference, 23rd - 26th August. Bangkok, Thailand. (Oral presentation)
6. **Panyakaew, P**., Goel, G., Lourengo, M., Yuangklang, C., Fievez, V. 2009. Effect of medium-chain fatty acids from coconut oil or krabok oil on *in vitro* rumen biohydrogenation. In: Chilliard, Y., Glasser, F., Faulconnier, Y., Bocquier, F., Veissier, I., Doreau, M. (Eds.) Ruminant Physiology. Digestion, metabolism, and effects of nutrition on reproduction and welfare. Wageningen Academic Publishers, 304-305. (Poster presentation)

7. **Panyakaew, P**., Goel, G., Lourengo, M., Yuangklang, C., Fievez, V. 2008. Medium-chain fatty acids from coconut oil or krabok oil to reduce *in vitro* rumen methanogenesis. Comm. Appl. Biol. Sci, Ghent University, 73/1: 189-192. (Poster presentation)