Effects of different oils and plant extracts on *in vitro* ruminal methane production

T.M. STORLIEN[1], O.M, HARSTAD[1], N. NARVAEZ[2], Y. WANG[2] and T.A. MCALLISTER[2*]

[1]*Department of Animal and Aquacultural Sciences, Norwegian University of Life Sciences, P.O. Box 5003, N-1432 Ås, Norway*

[2]*Agriculture and Agri-Food Canada, Lethbridge Research Centre, Lethbridge, Alberta, Canada T1J 4B1*

Correspondence: T. A. McAllister, Agriculture and Agri-Food Canada, Lethbridge Research Center, 5403-1st Ave., South, Lethbridge, Alberta, Canada T1J 4B1 Tel.: +403-317-2240; fax: +403-317-2182. E-mail: tim.mcallister@agr.gc.ca

ABSTRACT: Effects of oils and plant extracts on CH_4 production from a barley grain (47%) - barley silage diet (53%; DM basis) was investigated. Exp. 1 used ruminal fluid alone (control), canola (CO), sunflower (SO), cod liver (CLO) oil or a (70:30) mixture of CO and CLO (MIX) at 2.5 or 5% of DM. CH_4/g DMD increased ($p<0.05$) for CO_5, $SO_{2.5,}$ CLO_5 and MIX_5. In Exp 2, MIX_5, hop extract at 7.5 ($H_{7.5}$) and 15.0 ($H_{15.0}$) and steroidal saponin extract at 2.4 ($S_{2.4}$) and 4.8 ($S_{4.8}$) % DM with and without MIX_5 were examined. $H_{7.5}$–MIX and $S_{2.4}$-MIX increased CH_4/g DMD ($p<0.05$). Addition of oil resulted in a decline in DMD in both experiments. None of the additives reduced CH_4/g DMD. Further studies to determine if more than one mitigation additive has detrimental or synergistic impacts on CH_4 produced per unit DMD are required.

Keywords: oil, plant extracts, methane, rumen

Introduction

Earlier studies indicated that lipids, saponins and tannins can inhibit methane (CH_4) production (Hess et al., 2003; Beauchemin et al., 2008; Wang et al., 2008). Both short-chain and unsaturated long-chain fatty acids can also inhibit CH_4 production in the rumen, mainly due to their toxicity towards protozoa and methanogens (Odongo et al., 2007). Canola oil and sunflower oil are sources of fatty acids that are commonly included in ruminant diets. Canola oil is rich in oleic (C18:1), linoleic (C18:2) and linolenic (C18:3) acids, whereas sunflower oil contains high level of C18:2 (Padley et al., 1994). Cod liver oil, which contains a high proportion of C18:3 and C20- fatty acids (Guil-Guerrero & El-Hassan, 2001), are of interest not only for their potential to reduce CH_4 emission (Fievez et al., 2003), but also for their ability to favorably alter the fatty acid composition of meat and milk (Bauman et al., 2006).

Saponins and tannins inhibit enteric CH_4 production mainly by reducing protozoal populations and altering the volatile fatty acid profile (Hess et al., 2003; Wang et al., 2008).

In a previous study (Holtshausen et al. 2009) saponins decreased acetate:propionate ratio, and accordingly CH_4 production. Research by Wang et al. (2008) showed that tannins can also suppress CH_4 production. However, few studies have been conducted to determine if combinations of these mitigation strategies result in additive reductions in CH_4. Thus, the main objective of this study was to investigate the CH_4 mitigation properties of canola oil, sunflower oil, cod liver oil, hop extract and saponin, and to determine if combinations of these additives cause synergistic reductions in CH_4 production during *in vitro* ruminal fermentation.

Materials and Methods

Substrate and additives

Two experiments using ground barley grain (47%) and barley silage (53%) on DM basis as substrate were conducted. Additives studied in Exp 1 were: canola oil (CO), sunflower oil (SO), cod liver oil (CLO) and CO + CLO (70:30, MIX) at two concentrations; 2.5 and 5.0% of substrate DM. In Exp 2, MIX (see Exp 1) (5% of substrate DM); hop extract (H) at 7.5 ($H_{7.5}$) and 15.0 ($H_{15.0}$) and steroidal saponin extract (S) at 2.4 and 4.8% of substrate DM were examined. Hop extract and S were examined at each level without and with MIX (5% of substrate DM; H-MIX and S-MIX, respectively) using the same substrate as in Exp 1. The hop extract was obtained by extracting ground hop pellet (Teamaker, USA) with 70% acetone aqueous solution and the saponin extract was obtained using procedure as described by Wang et al. (2000). In both experiments, three replicate incubations per additive and concentration were conducted.

Ruminal fluid collection and incubation

Ruminal contents were collected via the rumen fistula of two non-lactating dairy Holstein cows, 2 h after the morning feeding. Cows were fed a diet consisting of (on DM basis) 74%

barley silage, 5% hay, 17% rolled barley and 4% mineral-vitamin supplement. Donor cows were cared for in accordance with the guidelines of the Canadian Council on Animal Care (1993). Ruminal contents were strained through four layers of cheesecloth and mixed 1:2 vol/vol with Menke's buffer (Menke et al., 1979). Inoculum (40ml) was dispensed under O_2-free CO_2 into 125 ml vials containing 500 mg of substrate ground through a 1 mm screen. Vials were immediately sealed and placed in an incubator (Forma Scientific Division of Mallinckroot. Inc., Ohio, SA) at 39˚C for 24 h in Exp 1, and for 12 h and 48 h in Exp 2. At 6, 12, 24 and 48 h (Exp 2 only) of incubation, total gas production in each vial was measured using a water displacement device as described by Wang et al. (2000). A gas sample from each vial prior to gas measurement was withdrawn using a gas-tight syringe for measuring CH_4 concentration. At the end of each experiment, the cultures were transferred into tubes and centrifuged at 500 x g at 4˚C for 10 min in preparation for determination of residual DM, and supernatant were sampled and stored at –40˚C prior to analysis for VFA.

Analytical procedures

Methane was analyzed by gas chromatography (GC) as described by Chaves et al. (2006), and VFA as described by Wang et al. (2000).

Calculations

Dry matter digestibility (DMD) was calculated as the difference between the amount of DM weighed into the vials minus the DM residue remaining after incubation and correction for DMD in the blank. Methane production was calculated from the CH_4 concentration in the sample and total volume of gas produced. Methane production measured at different incubation time points were added and expressed as ml/g substrate DM at the beginning and ml/g substrate DMD at the end of each experiment.

Statistical analysis

The results were analyzed using PROC MIXED and pair wise comparison (Tukey) of the means of the treatments in SAS version 9.1 (SAS Institute Inc., 2007). Significance was considered at $p<0.05$.

Results

Effects of oils on CH_4 production, DM digestibility and fermentation characteristics (Exp1)

Treatments had no effect on CH_4 production per g substrate DM ($p<0.05$), but all decreased ($p<0.05$) DMD (Table I). Compared to the control, addition of 5% CO resulted in the most pronounced depression of DMD, while 2.5% CLO caused the least reduction. Increasing the level of oils from 2.5 to 5% of substrate DM caused an almost two-fold reduction in DMD. Sunflower oil was an exception, where supplementation with 5% inhibited DMD to a lesser extent than 2.5%. Thus, CH_4 production per g DMD increased ($p<0.05$) for 5% addition of CO, CLO and MIX, and for 2.5% addition of SO. There were no consistent interactions with mixing CLO and CO in proportion 30:70. Only the $SO_{2.5}$ altered ($p<0.05$) total VFA concentration. Neither the pure oils nor the MIX altered acetate:propionate ratio ($p>0.05$) (Table I).

Effects of hop extract and saponin with and without oils on CH_4 production, DM digestibility and fermentation characteristics (Exp2)

All treatments nominally reduced CH_4 production per g DM, a reduction that reached significance ($p<0.05$) for $H_{15.0}$, $H_{15.0}$-MIX, S and S-MIX after 48 h of incubation (Table II). However, all treatments reduced ($p<0.05$) DMD, resulting in increased ($p<0.05$) production of CH_4 per unit DMD for H-MIX and $S_{2.4}$ -MIX (after 48 h incubation). The additional effects of

supplementing H and S with MIX, were not consistent, but overall tended to be additive. Hop extract$_{7.5}$, H$_{7.5}$-MIX, S$_{4.8}$ and S$_{4.8}$-MIX affected acetate: propionate ratio (p<0.05), and there was an increase (p<0.05) in total VFA concentration with MIX, H-MIX and S-MIX treatments (Table II).

Discussion

Effect of oils on CH_4 production, DM digestion and fermentation

In vitro (Machmüller et al., 1998; Jalc et al., 2007) and *in vivo* (Beauchemin et al., 2008; Beachemin et al., 2009) studies have shown that addition of lipids to ruminant diets results in a decrease in CH_4 production and frequently in digestibility. Our results indicated that oils had no effect on CH_4 emission per unit of available substrate DM, but they did reduce DMD. The fact that VFA concentrations remained similar even between treatments that differed in DMD suggests that the oils themselves underwent significant fermentation (Table I). It is known that oils may increase the efficiency of microbial growth (Dewhurst et al. 2000), and the present results suggest that this activity was directed towards the soluble DM fraction. Few studies have examined the impact of potential CH_4 inhibitors in combination, but our results suggest the effects of a mixture of CO and CLO on CH_4 production, DMD and VFA concentration are not additive (Table I).

Effect of hop extract and saponin on CH_4 production, DM digestion and fermentation

To our knowledge, this is the first study examining the effect of a combining H and S with lipids on CH_4 production. This is an interesting perspective, since H, S and lipids are all natural components of feed (Van Soest, 1994). The most striking results obtained are the relatively small effects of H and S alone as compared to MIX, H-MIX and S-MIX (Table II). However, no or modest effects of S on CH_4 production are in agreement with the results

obtained in other related *in vitro* studies (Hess et al., 2003; Pen et al., 2006; Goel et al., 2008; Holthausen et al., 2009) as well as in an experiment with dairy cows (Holtshausen et al., 2009)

The hop extract used in this study contained approximately 125 mg condensed tannin/g extract. Inclusion of H at 15% of substrate DM depressed CH_4 production per unit DM substrate, but not per unit DMD. This response may be arising from the tannins present in hop extract. Wang et al. (2008) studied the effects of phlorotannins from the seaweed, *Ascophyllum nodosum* on *in vitro* CH_4 production and digestibility of acid detergent fiber in a barley silage - alfalfa hay diet and also found that these compounds reduced CH_4 production per unit DM in accordance with our results. However, unlike with phlorotannins, the isolated tannin fraction from hops did not alter the ratio of acetate: propionate in the present study.

The pronounced negative effect of lipids on DMD is noteworthy, but is in line with other results as discussed in Exp 1. As in Exp 1, there was no evidence of an additive effect of combining either H or S with a mixture of CO and CLO on CH_4 production, DMD or VFA concentrations.

Conclusions

Results from the present *in vitro* studies showed that neither hop nor saponin extracts reduced CH_4 production without causing a significant reduction in the microbial digestion of DM. In contrast to most other studies, canola oil, sunflower oil, cod liver oil and a mixture of the two latter had no significant mitigating effects on CH_4 production in spite of significant negative effect on DM digestibility. There were no consistent synergetic effects of combining hop extract and saponin with a mixture of canola oil and cod liver oil. The response of both the individual oils and their mixtures on CH_4 production per unit of DM digested requires further investigation.

Acknowledgements

This work was supported by the Agriculture and Agri-Food Canada, Norwegian Research Council,-Norwegian Dairy Co-operative, -Agricultural Purchasing and Marketing Corporation, - Meat and Egg Co-operative and Norwegian Meat and Poultry Research Centre. The authors also acknowledge the technical assistance of Darryl Vedres and Zhong Xu.

References

Bauman, D.E., Lock, A. L., Corl, B. A., Ip, C., Salter A. M., & Parodi, P.W. (2006). Milk fatty acids and human health: potential role of conjugated linolenic acid and trans fatty acids. In: Ruminant Physiology. Digestion, metabolism and impact of nutrition on gene expression, immunology and stress (Ed. K. Sejrsen, T. Hvelplund, M.O. Nielsen). Wageningen Academic Publishers, pp. 536-537.

Beauchemin, K.A., Kreuzer, M., O'Mara F., & McAllister, T. A. (2008). Nutritional management for enteric methane abatement: a review. *Australian Journal of Experimental Agriculture*, *48*, 21-27.

Beauchemin, K.A., McGinn, S. M., Benchaar, C., & Holthausen, L. (2009). Crushed sunflower, flax or canola seeds in lactating dairy cow diets: Effects on methane production, rumen fermentation, and milk production. *Journal of Dairy Science*, *92*, 2118-2127.

Canadian Council on Animal Care (CCAC). (1993). Guide to the care and use of experimental animals. Vol. 1. 2nd ed. E. D. Olfert, B. M. Cross, and A. A. McWilliam, ed. CCAC, Ottawa, ON, Canada.

Chaves, A.V., Thompson, L. C., Iwaasa, A. D., Scott, S., Olson, M. E., Benchaar, C., Veira, D. M., & McAllister, T. A. (2006). Effect of pasture type (alfalfa vss. grass) on

methane and carbon dioxide production by yearling beef heifers. *Canadian Journal of Animal Science, 86*, 409-418.

Dewhurst, R.J., Davies, D. R., & Merry, R. J. (2000). Microbial protein supply from the rumen. *Animal Feed and Science Technology*, *85*, 1-21.

Fievez, V., Dohme, F., Danneels, M., Raes K., & Demeyer, D. (2003). Fish oils as potent rumen methane inhibitors and associated effects on rumen fermentation *in vitro* and *in vivo*. *Animal Feed and Science Technology*, *104*, 41–58.

Goel, G., Makkar, H.P.S., & Becker, K. (2008). Changes in microbial community structure, methanogenesis and rumen fermentation in response to saponin-rich fractions from different plant materials. *Journal of Applied Microbiology*, *105*, 770-777.

Guil- Guerrero, J.L., & El-Hassan, B. (2001). Purification Process for Cod Liver Oil Polyunsaturated Fatty Acids. *Journal of the American Oil Chemists' Society*, *78*, 477–484.

Hess, H.D., Kreuzer, M., Diaz, T. E., Lascano, C., Carulla, J., Soliva, C. R., & Machmuller, A. (2003). Saponin rich tropial fruits affect fermentation and methanogenesis in faunated and defaunated rumen fluid. *Animal Feed and Science Technology*, *109*, 79-94.

Holtshausen, L., Chaves, A.V., Beauchemin, K. A., McGinn, S. M., McAllister, T. A., Cheeke P. R., & Benchaar, C. (2009). Feeding saponin-containing *Yucca schidigera* and *Quillaja saponaria* to decrease enteric methane production in dairy cows. *Journal of Dairy Science, 92*, 2809-2821.

Jalc, D., Certik, M., Kundrikova, K., & Namestkova, P. (2007). Effect of unsaturated C_{18} fatty acids (oleic, linoleic and α- linolenic acid) on ruminal fermentation and production of fatty acid isomers in an artificial rumen. *Veterinarni Medicina*, *52*, 87-94.

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). *Animal Feed and Science Technology*, *71*, 117-130.

Menke, K.H., Raab, L., Salewski, A., Steingass, H., Fritz, D., & Schneider, W. (1979). The estimation of the digestibility and metabolizable energy content of ruminant feedingstuffs from the gas production when they are incubated with rumen liquor *in vitro*. *Journal of Agricultural Science*, *93*, 217-222.

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. *Journal of Dairy Science*, *90*, 1851-1858.

Padley, F. B., Gunstone, F. D., & Harwood, J. L. (1994). Occurrence and characteristics of oils and fats. In: The Lipid Handbook, 2nd ed. (Ed. F. D. Gunstone, J. L. Harwood and F. B. Padley). Chapman and Hall, pp. 119-123.

Pen, B., Sar, C., Mwenya, B., Kuwaki, K., Morikawa, R., & Takahashi, J. (2006). Effects of *Yucca schidigera* and *Quillaja saponaria* extracts on *in vitro* ruminal fermentation and methane emission. *Animal Feed and Science Technology*, *129*, 175-186.

SAS Institute Inc. (2007).SAS/STAT® User's Guide. Cary, NC: SAS Institute Inc.

Van Soest, P.J. (1994). Nutritional ecology of the ruminant, 2nd ed. Ithaca, New York.

Wang, Y., McAllister, T. A., Yanke, L. J., Xu, Z. J., Cheeke, P. R., & Cheng, K.-J. (2000). *In vitro* effects of steroidal saponins from *Yucca schidigera* extract on rumen microbial protein synthesis and ruminal fermentation. *Journal of the Science of Food and Agriculture*, *80*, 2114-2122.

Wang, Y., Xu, Z., Bach, S. J., & McAllister, T. A. (2008). Effects of phlorotannins from *Ascophyllum nodosum* (brown seaweed) on *in vitro* ruminal digestion of mixed forage or barley grain. *Animal Feed and Science Technology*, *145*, 375-395.

Table I. Effect of canola oil (CO), sunflower oil (SO), cod liver oil (CLO) and CLO + CO (30:70) (MIX), added at 2.5 and 5.0% of substrate dry matter (DM) on methane production, DM digestibility (DMD) and volatile fatty acids (VFA) production, after 24h of incubation.

	Control	CO		SO		CLO		MIX		SEM
24 h		2.5	5	2.5	5	2.5	5	2.5	5	
CH_4 (ml/g substrate DM)	47[a]	47[a]	45[a]	46[a]	46[a]	49[a]	49[a]	45[a]	46[a]	6.182
CH_4 (ml/g substrate DMD)	79[a]	111[a]	175[b]	127[b]	110[a]	105[a]	142[b]	105[a]	160[b]	0.297
DMD (mg/g substrate DM)	596[a]	425[b]	261[b]	363[b]	420[b]	467[b]	343[b]	435[b]	302[b]	19.3
VFA (mmol/g substrate DMD)	8.9[a]	11.2[a]	16.1[a]	18.1[b]	7.4[a]	13.9[a]	14.5[a]	12.3[a]	15.5[a]	0.8
Acetate:propionate	1.9	1.7	1.4	2.2	1.2	2.4	1.7	2	1.5	0.1

[a,b] Different letter within the same row indicate significant different from control ($p<0.05$).

Table II. Effect of cod liver oil + canola oil (30:70) (MIX, 5% of substrate DM), hop extract (H) at 7.5 and 15 and saponin (S) at 2.4 and 4.8% of substrate DM, H + MIX (H-MIX) and S + MIX (S-MIX) on methane production, DM digestibility (DMD), Volatile fatty acids (VFA) after 12h and 48h of incubation.

	Control	MIX	H		H-MIX		S		S-MIX		SEM
		5	7.5	15	7.5	15	2.4	4.8	2.4	4.8	
12 h incubation:											
CH_4 (ml/g substrate DM)	48^a	46^a	47^a	43^b	45^a	44^a	41^b	33^b	41^b	34^b	0.95
CH_4 (ml/g substrate DMD)	87^a	166^a	92^a	92^a	228^b	263^b	86^a	70^a	240^b	150^a	13.73
DMD (mg/g substrate DM)	557^a	282^b	511^a	470^b	205^b	171^b	481^a	467^b	182^b	228^b	27.62
VFA (mmol/g substrate DMD)	7.6^a	14.6^a	12.6^a	12.1^a	28.1^b	36.5^b	7.2^a	9.4^a	22.9^a	14.0^a	1.995
Acetate:propionate	2.8^a	2.7^a	2.9^a	3.0^a	3.3^a	3.2^a	2.2^b	1.8^b	2.5^a	2.0^b	0.095
48 h incubation:											
CH_4 (ml/g substrate DM)	62^a	60^a	62^a	52^b	59^a	57^b	52^b	44^b	51^b	43^b	1.24
CH_4 (ml/g substrate DMD)	111^a	216^a	121^a	111^a	301^b	336^b	108^a	95^a	299^b	190^a	17.7
DMD (mg/g substrate DM)	739^a	405^b	673^b	582^b	437^b	329^b	609^b	591^b	334^b	338^b	26.7
VFA (mmol/g substrate DMD)	12.0^a	22.6^b	21.8^a	20.2^a	29.0^b	32.5^b	12.7^a	16.9^a	25.5^b	25.3^b	1.3
Acetate:propionate	2.5^a	2.3^a	3.0^b	2.7^a	2.9^b	2.6^a	2.1^a	1.8^b	2.1^a	1.8^b	0.0785

^{a,b} Different letter within the same row indicate significant different from control ($p<0.05$).