Methane output and rumen microbiota in dairy cows in response to long-term supplementation with linseed or rapeseed of grass silage- or pasture-based diets

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ABSTRACT

The long-term (>12 months) effect of fat supplementation on methane (CH_4) output and rumen microbiota was measured in dairy cows. Twenty lactating dairy cows were fed indoor with grass silage/hay, then outdoor with pasture-based diets, and allocated to three dietary treatments for two consecutive entire lactations. Diets were not supplemented (C) or supplemented with 2-3% dry matter of extruded linseed (EL) or extruded rapeseed (ER). Individual intake, milk and CH_4 production (sulphur hexafluoride tracer technique) were measured on five consecutive days in both periods. Rumen pH, volatile fatty acid (VFA) concentrations and microbial numbers were measured on rumen fluid samples collected before feeding. For both periods, CH_4 yield (g/kg dry matter intake) was lower for EL (-15%; $P < 0.001$), and numerically higher for ER (+14%) compared to C. The unexpected effect of ER on CH_4 emissions remains difficult to explain. Measured as g/kg fat corrected milk, CH_4 output remained lower for EL (-26%, $P < 0.05$) than for C and ER, for both periods. Diet EL did not modify rumen VFA profiles and microbial numbers compared to C. Our work highlights the long term efficacy of EL supplementation of grass-based diets as a CH_4 mitigation option.

Keywords: dietary fat; linseed; rapeseed; methane; long-term supplementation; dairy cow.

INTRODUCTION

Enteric methane (CH_4) production represents a major contribution to greenhouse gas emissions by ruminants. Supplementation with fat is the most effective dietary strategy to mitigate ruminal methanogenesis (Beauchemin *et al.,* 2009a; Martin *et al.,* 2010; Grainger & Beauchemin, 2011). There have been few direct comparisons between different fat sources when added to different basal diets. Linseed, which is rich in linolenic acid (C18:3 n-3), is often used as a supplement due to its nutritional benefits and antimethanogenic effect. Linolenic acid decreased the number or activity of protozoa, cellulolytic bacteria and methanogens, directly or indirectly affecting methanogenesis (Morgavi et al., 2010). Rapeseed, which is rich in C18:1 n-9, also reduces CH4 production in vivo (Machmüller et al., 2000; Beauchemin et al., 2009b), but the ruminal mechanisms are unknown. Studies reporting a persistent effect of lipids on CH4 yield have been carried out, with coconut oil, which is rich in C12:0 and C14:0 (Jordan et al., 2006) or cottonseeds which are rich in C18:2n-6 (Holter et al., 1992; Grainger et al., 2010), supplemented for one to four months. Woodward et al. (2006) reported no effect on CH4 emissions of a mixture of linseed/fish oil added to a pasture diet for three months. The effect of fat sources rich in linolenic or oleic acids on CH4 emissions has not been tested for periods longer than 12 months. The aim of this experiment was to measure the effect on CH4 emissions of long-term (>12 months) supplementation of fat as linseed and rapeseed, added to lactating cow diets based on grass silage or pasture. A secondary aim was to measure the effect on selected microbial communities.

MATERIAL AND METHODS

Animals, experimental design and diets

Twenty Holstein dairy cows were allocated to three dietary treatments during two successive lactations. Diets were not supplemented (C, n = 8) or supplemented with extruded linseed (EL, n = 8) or with extruded rapeseed (ER, n = 4). The measurement periods took place indoor, 15 months after the beginning of the experiment on animals fed grass silage/hay (P1), and outdoor two months later on cows fed pasture-based diets (P2).

During P1, all cows received 48% cocksfoot (*Dactylis glomerata*) first-cut silage, 12% second-cut cocksfoot hay, and 40% concentrates on a dry matter (DM) basis. The proportion of concentrates was maintained at a constant level by adjustment of the offered amounts to forage intake on the previous week. The composition of concentrates was calculated to provide approximately 3% added fat to diets EL and ER. Animals received forage *ad libitum* at 10:00 h and concentrates twice daily in equal amounts at 10:00 h and 17:30 h.

During P2, all cows were managed on the same paddock of cocksfoot grass and grazed 20 hours a day. They received individually a total of 4.5 kg

TABLE 1: Ingredients and chemical composition of experimental diets. C = Control diet; EL = Diet supplemented with extruded linseed; ER = Diet supplemented with extruded rapeseed.

Component	Grass silage-based diets Indoor (Period 1)			Pasture-based diets Outdoor (Period 2)		
	C	EL	ER	C	EL	ER
Ingredients (g/kg DM)						
Grass silage + cocksfoot hay (45/15)	570	560	560	vi-	-	-
Cocksfoot grass	-	-	-	790	780	780
Concentrate mixture[1]	430	440	440	210	220	220
Chemical composition (g/kg DM)						
Organic matter	936	937	932	916	913	913
Neutral detergent fibre	372	380	402	359	377	377
Starch	232	214	164	97	39	41
Crude protein (Nitrogen x 6.25)	143	140	157	207	222	221
Ether extract	34.4	64.6	63.1	28.9	48.7	47.9
C16:0	5.4	6.9	6.4	4.2	5.4	5.2
C18:0	0.8	1.8	1.1	0.4	1.2	0.7
C18:1 n-9	4.6	10.1	22.7	2.6	6.5	14.2
C18:2 n-6	13.3	16.8	16.8	5.7	8.6	9.4
C18:3 n-3	6.6	23.9	10.3	11.2	23.4	13.6
Gross energy (MJ/kg DM)	18.9	19.6	19.6	18.5	19.0	19.0

[1]Composition (% DM)
Indoor Period 1
Diet C: 90% ground pelleted wheat, 10% rapeseed meal.
Diet EL: 71.3% ground pelleted wheat, 28.7% extruded linseed/wheat (70/30).
Diet ER: 48.2% ground pelleted wheat, 20% rapeseed meal, 31.8% extruded rapeseed/wheat bran (59/41).
Outdoor Period 2
Diet C: 73.3% ground pelleted wheat, 26.7% rapeseed meal.
Diet EL: 8.9% ground pelleted wheat, 51.1% rapeseed meal, 40% extruded linseed/wheat (70/30).
Diet ER: 8.9% ground pelleted wheat, 51.1% rapeseed meal, 40% extruded rapeseed/wheat bran (59/41).

DM per day of concentrates in equal amounts after morning milking and before evening milking. The quantity of concentrates was calculated to provide approximately 2.5% added fat to the two diets. Ingredients and chemical composition of diets are detailed in Table 1.

Measurements

During P1, feed intake was measured by weighing individually offered and refused feeds for four consecutive days. During P2, concentrate intake was measured as in P1 whereas grass intake was estimated from height and density of pasture, live weight, body condition score, milk production and concentrate intake of cows (Faverdin et al., 2007). Dry matter content in feeds was measured (80°C for 48 hours) daily for grass silage and grass, and twice a week for hay and concentrates. A sub-sample of each feed was dried (60°C for 72 hours), sieved (0.8-mm screen) and analysed for organic matter (OM), nitrogen (N), neutral detergent fibre (NDF) and starch, ether extract (EE), and gross energy (GE) as described in Martin et al. (2008). Fatty acids (FA) were extracted from ground lyophilized feed samples using chloroform:methanol (2:1) mixture and quantified by gas chromatography (Loor et al., 2004). Milk production was measured every day in each period. One 30 mL aliquot of milk containing Bronopol (2-bromo-2-nitropropane-1,3-diol) was taken twice a week and stored at 4°C until analysed for fat by infrared analysis with a spectrophotometer (Association of Official Analytical Chemists, 1997). Daily CH4 production was determined on five consecutive days in both periods at two monthly intervals, using the sulphur hexafluoride (SF6) tracer technique as described by Martin et al. (2008). A calibrated SF6 permeation tube was dosed per os into the rumen of each cow one week before sampling gas in P1.The permeation rate of SF6 from the tubes was 1.621 ± 0.051 (standard deviation) mg/day. Rumen fluid samples were collected once before the morning feeding on the last day of each period of gas collection. A 50 mL sample of rumen liquid was taken by rumenocentesis (Kleen et al., 2004). Rumen liquid was immediately measured for pH using a digital pH-meter, then filtered (250 µm nylon filter). A 0.8 mL filtrate sample was kept at -20°C until volatile fatty acid (VFA) analysis (CP 9002 Gas Chromatograph; Chrompack, Middelburg, Germany) using crotonic acid as the internal standard. A second 3 mL filtrate sample was added to 3 mL of methylgreen-formalin solution (MFS) and stored in the dark until protozoal counting under a microscope. The remainder of the liquid phase was stored at -80°C until total DNA extraction (Yu & Morrison, 2004) and quantification by real time quantitative polymerase chain reaction (qPCR) of the bacterial and archaeal community (Mosoni et al., 2011). Total bacteria were quantified in duplicate using specific primers targeting the rrs gene (Edwards *et al.,* 2008) and methanogenic archaea by

TABLE 2: Dry matter (DM) intake, milk production and methane (CH_4) output in dairy cows in response to long-term supplementation with linseed or rapeseed of grass silage- or pasture-based diets. Bolding of P values indicates significance (P< 0.05). C = Control diet; EL = Diet supplemented with extruded linseed; ER = Diet supplemented with extruded rapeseed.

Parameter	Grass silage-based diets Indoor (Period 1)			Pasture-based diets Outdoor (Period 2)			Standard error	P value		
	C	EL	ER	C	EL	ER		Diet	Period	Diet* Period
Total DM intake (kg/d)	18.3[a]	17.0[b]	19.1[a]	20.4	20.6	20.3	0.40	0.12	**<0.001**	**<0.05**
Forage[1] (kg/d)	10.4	9.5	10.6	16.0	16.1	15.9	0.35	0.34	**<0.001**	0.16
Concentrate (kg/d)	7.9[a]	7.5[a]	8.5[b]	4.4	4.5	4.4	0.14	0.06	**<0.001**	**<0.05**
Gross energy intake (MJ/d)	346[a]	333[a]	375[b]	377	392	386	7.52	0.09	**<0.001**	**<0.05**
Milk yield (kg/d)	26.8	28.0	29.5	24.5	23.8	22.7	1.68	0.98	**<0.001**	0.09
4% fat corrected milk (kg/d)	23.0	24.0	26.4	21.6	23.0	22.0	1.50	0.68	**<0.001**	0.07
CH_4 (g/d)	480[a]	383[b]	547[c]	467[a]	395[b]	557[c]	23.2	**<0.001**	0.83	0.77
CH_4 (g/kg DM intake)	26.2[a]	22.5[b]	28.8[c]	22.9[a]	19.2[b]	27.5[c]	1.18	**<0.001**	**0.01**	0.66
CH_4 (% Gross energy intake)	7.7[a]	6.3[b]	8.1[a]	6.8[a]	5.6[b]	8.0[a]	0.34	**<0.001**	**0.05**	0.54
CH_4 (g/kg milk)	18.7	13.9	18.7	20.2[ab]	16.8[b]	25.0[a]	1.80	0.06	**<0.001**	**<0.05**
CH_4 (g/kg 4% fat corrected milk)	21.9[a]	16.3[b]	21.2[a]	22.7[a]	17.4[b]	25.8[a]	1.98	**0.05**	**0.01**	0.14

[1]Forage intake for outdoor period was estimated according to the model of Faverdin *et al.* (2007).
Different superscripts within rows, within the same period, indicate significant differences between diets (P <0.05).

TABLE 3: Rumen fluid fermentation and microbiota parameters in dairy cows in response to long-term supplementation with linseed or rapeseed of grass silage- or pasture-based diets. Bolding of P values indicates significance (P< 0.05). C = Control diet; EL = Diet supplemented with extruded linseed; ER = Diet supplemented with extruded rapeseed.

Parameter	Grass silage based diets Indoor (Period 1)			Pasture based diets Outdoor (Period 2)			Standard error	P value		
	C	EL	ER	C	EL	ER		Diet	Period	Diet* Period
pH	6.88	6.83	6.61	6.79	6.84	6.61	0.10	0.12	0.77	0.86
Total VFA (mmol)	109[a]	99.7[a]	139[b]	108[a]	107[a]	148[b]	8.97	**<0.05**	0.49	0.83
Acetate (% of total)	63.5	61.8	65.2	60.8	60.6	59.7	0.93	0.46	**<0.01**	0.22
Propionate (% of total)	21.4[a]	22.9[a]	17.0[b]	18.6	18.8	17.7	0.87	0.06	**<0.001**	**<0.01**
Butyrate (% of total)	10.5	11.1	13.2	14.0	14.0	16.1	0.90	0.19	**<0.001**	0.91
Acetate/propionate	3.0[a]	2.7[a]	3.8[b]	3.4	3.3	3.4	0.17	0.06	0.24	**<0.01**
(Acetate+butyrate)/propionate	3.5[a]	3.2[a]	4.7[b]	4.1	4.0	4.3	0.22	0.07	**<0.05**	**<0.05**
Total protozoa (Log_{10} cells/mL)	4.25	4.39	4.81	4.81	4.99	5.14	0.18	0.26	**<0.001**	0.56
Holotrichs (Log_{10} cells/mL)	3.43	3.24	3.91	3.39	3.50	3.91	0.18	0.10	0.68	0.15
Entodiniomorphs (Log_{10} cells/mL)	4.16	4.35	4.74	4.84	4.98	5.10	0.19	0.27	**<0.001**	0.64
Total bacteria (rrs Log_{10} copies/mL)	11.8	11.8	11.6	12.2	12.4	12.4	0.11	0.51	**<0.001**	0.20
Methanogens (mcrA Log_{10} copies/mL)	6.67	6.68	6.70	7.78	7.83	7.65	0.15	0.89	**<0.001**	0.82

Different superscripts within rows, within the same period, indicate significant differences between diets (P <0.05).

targeting the methyl coenzyme-M reductase (mcrA) gene (Denman *et al.*, 2007). Standard curves (10^8 to 10^3 rrs copies) targeting total bacteria were prepared with equal amounts of the rrs DNA fragment amplified from genomic DNA of *Prevotella bryantii* B14 (DSM 11371). The methanogenic archaea were quantified relative to PCR products corresponding to almost the entire sequence of mcrA gene of *Methanobrevibacter ruminantium* 1093 (DSM).

Statistical analyses

Data were analysed using the MIXED procedure of SAS (2000). The statistical model included period, diet and their interaction as fixed effects, and animal as a random effect. Differences between diets were determined by a Tukey t-test. Results were considered significant for P value ≤0.05.

RESULTS

The measured forage intake and total dry matter intake (DMI) during P1 were significantly lower than the estimated grass and total intake during P2 (Table 2). In contrast, the concentrate intake was higher during P1 than P2, leading to a lower forage/concentrate ratio in P1 compared to P2 (Table 1). In P1, total DMI was similar for C and ER and significantly lower for EL. In P2, estimated intakes of concentrate and forage and estimated total DMI were the same for all diets. Milk and 4% fat corrected milk (FCM) yields were similar between diets, but decreased in P2 ($P < 0.001$; Table 2).

The amount of CH_4 emitted daily by dairy cows differed between diets ($P < 0.001$, but there was no significant Diet x Period interaction; Table 2). Methane emissions (g/d and g/kg DMI) for EL were 15-18% lower than C, while those for ER were 15-17% higher ($P < 0.001$). Loss of CH_4 as a percentage of GE intake was similar for C and ER, but was less for EL ($P < 0.001$). Methane emission per kg of milk or kg of 4% FCM was similar for C and ER, but was 26% less for EL ($P<0.05$).

Rumen pH averaged 6.76 for all diets (Table 3). Total VFA concentration was similar for C and EL and higher for ER ($P < 0.05$). No effect of fat was observed on the molar proportion of acetate and butyrate, but proportion of propionate decreased with ER compared to C in P1 ($P < 0.01$). As a consequence, the (acetate + butyrate)/propionate ratio was higher ($P < 0.01$) with diet ER compared to the two other diets. The VFA profiles of the ruminal fluid differed also between the two periods ($P < 0.001$). There was no effect of fat supplementation on the concentration of total protozoa, total bacteria and methanogens.

DISCUSSION

The lower forage and DMI intake observed during P1 with EL in comparison to C and ER is consistent with the results of Martin *et al.* (2008) for EL, and Bayourthe e*t al.* (2000) for ER added to corn-silage-based diets.

Supplementation with EL significantly decreased the amount of CH_4 per day, per unit of intake and per unit of milk without altering cow milk yield. There was a decrease in CH_4 yield (g/kg DMI) of 6% with each 1% fat added in EL. A similar decrease in CH_4 yield (-4.8% per 1% unit of fat added) was reported previously for dairy cows fed diets supplemented with EL (Martin *et al.,* 2010).

Irrespective of the diet, the effect of fat supplementation on CH_4 emissions differed between the fat sources, but the reduction in CH_4 emissions reported with EL was not observed with ER. The increased CH_4 emission with ER supplementation does not agree with a recent meta-analysis of the literature that reported a similar effect of fat source ($n = 65$; $P = 0.46$) on CH_4 yield (g/kg DMI) for diets containing up to 80 g fat/kg DM (Grainger & Beauchemin, 2011). In this study, the increment of CH_4 emissions with ER remains difficult to explain.

Supplementation with EL decreased methanogenesis after more than one year of continuous fat supplementation of grass-based diets. These data are the first to report such a long-term effect of fat supplementation on methanogenesis. Woodward *et al.* (2006) reported no effect on CH_4 emissions of a mixture of linseed/fish oil after three months of supplementation in diets of grazing dairy cows. However, their experiment did not have sufficient power in the design to detect the expected reduction in CH_4 emissions (Grainger *et al*., 2010). In their review, Grainger & Beauchemin (2011) mentioned a change in the effect of fat supplementation on CH_4 emissions over time in trials carried out over short- and medium-term of one to four months in cattle. The inhibitory effect of whole cottonseed increased from seven to 16 weeks (Holter *et al.,* 1992), and from six to 12 weeks of treatment (Grainger *et al.,* 2010) in dairy cows. Another experiment did not report the significance of the interaction between weeks of treatment and CH_4 reduction using coconut oil fed to beef cattle (Jordan *et al.,* 2006). In our study, we can make no conclusion on a possible interaction with time of EL or ER supplementation on CH_4 emissions because we only measured CH_4 for the first time after 15 months of fat supplementation.

Physico-chemical parameters of pH, VFA concentration and composition, as well as protozoa, bacteria and methanogen numbers in the ruminal fluid pre-feeding were unaffected by EL feeding. Mosoni *et al.* (2008) also reported no effect of 2% to 6% fat as EL fed to dairy cows on the number of protozoa, cellulolytic ruminococci and methanogens from the solid and liquid phase of rumen contents taken before feeding. They explained the observed reduction in methanogenesis with EL by a decrease in protozoal numbers observed after feeding. The study of the microbial mechanisms involved in methanogenesis would have probably been more relevant after feeding when production of CH_4 is maximal. Our work supports the view that the total number of rumen methanogens is not the key factor that affects CH_4 production (Morgavi *et al.,* 2010). A decrease in methanogenesis may be related to a decrease in methanogen's activity and changes in their diversity as observed in bulls fed a starch-rich diet supplemented with EL (Popova *et al.,* 2011). A lower amount of OM fermented in the rumen with EL, related to the lower intake, at least partly resulted in the lower CH_4 production.

In conclusion, this study showed that adding 2-3% fat to dairy cow diets reduced CH_4 output without altering animal performances. This effect was observed with extruded linseed after more than one year of continuous fat supplementation of grass-based diets, highlighting for the first time a long-term effect. This decrease in methanogenesis with linseed was not explained by fermentative and microbial parameters measured in this work. The mitigating effect of fat was not observed for rapeseed. The effects of the two sources of fat tested were similar with both grass silage- and pasture-based diets.

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