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Fish oils as potent rumen methane inhibitors and associated effects on rumen fermentation in vitro and in vivo☆

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Abstract

The effect of two fish oil (FO) types, FOa (*n*-3-eicosapentanoic acid (EPA), 18.1%; *n*-3-docosahexanoic acid (DHA), 11.9%) and FOb (EPA, 5.4%; DHA, 7.5%) and quantity (0, 12.5, 25, 50, 75, 100 and 125 mg FO) on rumen fermentation patterns was evaluated with 24 and 48 h batch in vitro incubations and compared with fermentation shifts induced by soybean oil (SO). The 48 h incubation was essentially a batch culture as it was re-inoculated with rumen fluid, buffer and hay after 24 h. Shifts in rumen fermentation pattern were only observed during the second step of the 48 h incubation for the three oils. The inhibition of rumen methane (CH_4) production was influenced by both oil (FOa > SO > FOb) and concentration, although amounts higher than 75 or 25 mg for FOa and FOb, respectively, did not decrease further CH_4 production. A maximal CH_4 inhibition of 80% was observed. CH_4 inhibition seems proportional to the relative amount of polyunsaturated fatty acids (PUFA) and their rate of lipolysis. Lower CH_4 production was accompanied by increased propionate and reduced acetate production. SO supplementation was associated with lower net VFA productions (−17%), whereas none of the amounts of FO reduced VFA production. In vivo effects of rumen FOa injection (18 ml, twice daily at 1 h after feeding) on rumen fermentation and NDF digestibility were studied in a cross-over trial with four rumen canulated wethers, offered a hay/concentrate (65/35, w/w) diet, at their maintenance energy requirements. In vivo FOa injection did not alter faecal NDF digestibility (48.5±4.5% versus 46.5±8.2% for control and FOa,

Abbreviations: 2Hr, hydrogen recovery; ARDOM, apparently rumen degraded organic matter; DHA, docosahexaenoic acid (C22:6*n*-3); DM, dry matter; DMI, dry matter intake; EPA, eicosapentaenoic acid (C20:5*n*-3); FA, fatty acids; FAME, fatty acid methylesters; FFA, free fatty acids; FO, fish oil; ISO, International Organisation for Standardisation; LW, liveweight; NDF, neutral detergent fibre; PUFA, polyunsaturated fatty acids; SO, soybean oil; VFA, volatile fatty acids

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respectively, $P > 0.05$), despite a lower 48 h rumen in sacco degradability (57.5 ± 3.3% versus 51.6±5.8%, $P < 0.05$). FOa-induced higher rumen propionate concentrations (211.8±18.0 versus 262.7 ± 20.8 mmol/mol total VFA, $P < 0.05$), suggesting a depression of rumen methanogenesis, confirmed during simultaneous in vitro incubations (344.2 ± 43.4 versus 287.7 ± 36.6 mmol/mol total VFA, $P < 0.05$). Reduced biohydrogenation of FO PUFA, resulting in rumen accumulation of unesterified EPA and DHA might be nutritionally and ecologically relevant in terms of an increased post-ruminal supply of these PUFA for incorporation into animal products and through reduced rumen methanogenesis, the latter representing both an energy loss for the animal and an important greenhouse gas.

Keywords: Rumen; Methane; Fermentation pattern; Fish oil; Lipolysis

1. Introduction

No rumen methane (CH_4) suppressing feed additives besides ionophores are generally available (Annison and Bryden, 1998). However restrictions on the use of chemicals in animal nutrition, due to European legislation and growing consumer scepticism, have increased interest in feeding and biological strategies to suppress rumen methanogenesis (Demeyer and Fievez, 2000). In this context, lipid supplementation seems to be a promising dietary strategy (Jouany, 1994; Spears, 1996) due to toxic effects of free fatty acids (FFA) on both methanogens (Prins et al., 1972) and protozoa (Czerkawski et al., 1975). The severity of the inhibitory effect of fat on rumen methanogenesis is, at least partly, determined by the source and quantity of fat used (Dong et al., 1997; Machmüller et al., 1998). Fat-induced reduction of rumen methanogenesis is accompanied with a shift to increased propionate production (Demeyer and Van Nevel, 1995), but often with negative side effects such as reduced level of intake or fibre digestibility (Zinn, 1989; Doreau et al., 1997). Some researchers suggest that dramatic CH_4 reduction by dietary fat feeding will only occur when fibre digestion is inhibited (Johnson and Johnson, 1995). As a consequence, Mathison et al. (1998) concluded that ration-induced reduction in methanogenesis could not be justified when supplementation of these higher cost ingredients does not increase the metabolisable energy content of the diet. Nevertheless, fish oil (FO) supplementation has been reported to increase rumen propionate concentrations (Shaw and Ensor, 1959; Sutton et al., 1975; Doreau and Chilliard, 1997; Keady and Mayne, 1999; Wachira et al., 2000), suggesting reduced rumen methanogenesis (Van Nevel et al., 1974), without effect on ruminal (Keady and Mayne, 1999) or whole tract (Doreau and Chilliard, 1997) fibre digestibility. Moreover, the effectiveness of the *n*-3-polyunsaturated fatty acids (PUFA), particularly eicosapentaenoic acid (EPA; C20:5*n*-3) and docosahexaenoic acid (DHA; C22:6*n*-3), in reducing the risk of coronary heart disease (Heyden, 1994; Wiseman, 1997) is attracting increasing attention, and attempts to incorporate these fatty acids (FA) in ruminant dairy and meat products (Hagemeister et al., 1988; Demeyer and Doreau, 1999; Raes et al., 2001a).

There are few experimental data on effects of the specific FO FA, EPA and DHA, and level, on rumen digestion and fermentation patterns, including rumen methanogenesis. Hence, during a batch in vitro experiment, effects of two types and seven levels of FO inclusion on rumen fermentation characteristics was studied and compared with rumen fermentation

shifts induced by soybean oil (SO). Simultaneously, lipolysis and hydrogenation of both FO types and SO was evaluated and has been reported elsewhere (Dohme et al., 2001). Finally, the in vivo occurrence of the in vitro effects of FOa was determined.

2. Material and methods

2.1. Ethical approval of animal experiments

The research protocol of the experiments and animal treatments were approved by the Institutional Animal Care and Use Committee of Ghent University, Belgium (acceptance no. 98/34). All animal experiments were supervised by a qualified veterinarian.

2.2. In vitro incubation

2.2.1. Animals and sampling

Mixed rumen fluid, approximately 0.5 l, from four wethers with permanent rumen canula, was used as in vitro inoculum. Animals were fed ad libitum a grass hay diet every 6 h using automatic feeders. Rumen contents were sampled just before the morning feeding at 10:00 h. Immediately after collection, samples were filtered through a metallic sieve (mesh width 1 mm) and, under CO_2 flushing, were diluted five-fold with a phosphate buffer (1.4 g NH_4Cl + 28.8 g $Na_2HPO_4 \cdot 12H_2O$ + 6.2 g NaH_2PO_4 in 1 l distilled water; pH = 7.0) at 39 °C.

2.2.2. In vitro incubations

Effect of type, as well as concentration, of FO on the rumen fermentation pattern was determined after incubation of rumen contents in batch culture flasks for 0, 24 and 48 h (Van Nevel and Demeyer, 1977). The 48 h incubations were essentially batch incubations which were re-inoculated with rumen contents, phosphate buffer and hay after 24 h and incubated for another 24 h (Fig. 1). Two FO types (FOa, Pronova Biocare, Sandefjord, Norway, and FOb, Technological Laboratory of the Danish Ministry of Fisheries, Lyngby, Denmark) with different FA composition (Table 1) were applied at seven levels (0, 12.5, 25, 50, 75, 100 and 125 mg) in triplicate. Twelve incubation runs were completed on different days. These 12 incubation runs included three repetitions of four treatments. Every incubation run contained a control without oil supplementation (zero oil control) and three levels of either FOa or FOb. In half of the incubation runs, incubation flasks with SO were added (Fig. 1). Oil was added at increasing volumes (0–2.5 ml) of an oil–hexane solution with hexane evaporated by N_2 flushing. Afterwards, 1 mm ground (Brabender, Duisburg, Germany) hay (0.400 g) with 25 ml of the mixture of rumen inoculum and phosphate buffer was added. One-third of the flasks were not incubated (blanks) and immediately acidified with 0.5 ml phosphoric/formic (10/1, v/v) acid to a final concentration of 2% to prevent fermentation (Non-incubated subsamples, Fig. 1). The headspace of the other flasks was filled with CO_2 gas (99.99% purity, Air Liquide, Aalter, Belgium) and flasks were incubated for 24 h in a shaking waterbath at 39 °C. Afterwards, half of the incubated flasks were acidified and used for gas and volatile fatty acid (VFA) analysis (24 h incubated subsamples, Fig. 1) whereas

Table 1
Fatty acid composition (g/100 g FAME) of the oils used (mean (S.D.)[a], $n = 3$)

	14:0	16:0	16:1	18:0	18:1 (c + t)[b]	18:2*n*-6	18:3*n*-3	18:4*n*-3	20:1	20:5*n*-3	22:1	22:4*n*-6	22:5*n*-3	22:6*n*-3
SO	0.1 (0.0)	11.6 (0.1)	0.1 (0.0)	3.7 (0.1)	24.0 (0.1)	53.2 (0.3)	6.2 (0.2)	[c]	[c]	[c]	0.8 (0.2)	[c]	[c]	[c]
FOa	7.7 (0.0)	17.0 (0.2)	9.2 (0.0)	3.4 (0.1)	15.0 (0.0)	3.6 (0.0)	1.0 (0.3)	2.7 (0.0)	1.6 (0.0)	18.7 (0.1)	2.2 (0.0)	1.2 (0.0)	2.5 (0.0)	11.7 (0.0)
FOb	8.4 (0.3)	13.5 (0.1)	4.6 (0.1)	1.2 (0.0)	12.5 (0.0)	1.8 (0.1)	1.2 (0.1)	3.1 (0.0)	14.0 (0.1)	5.8 (0.0)	23.0 (0.3)	1.2 (0.0)	0.7 (0.0)	7.6 (0.1)

[a] Standard deviations of 0.0 indicate values were lower than 0.05.
[b] Sum of cis and trans isomers of C18:1.
[c] Not detectable using current method (Raes et al., 2001b).

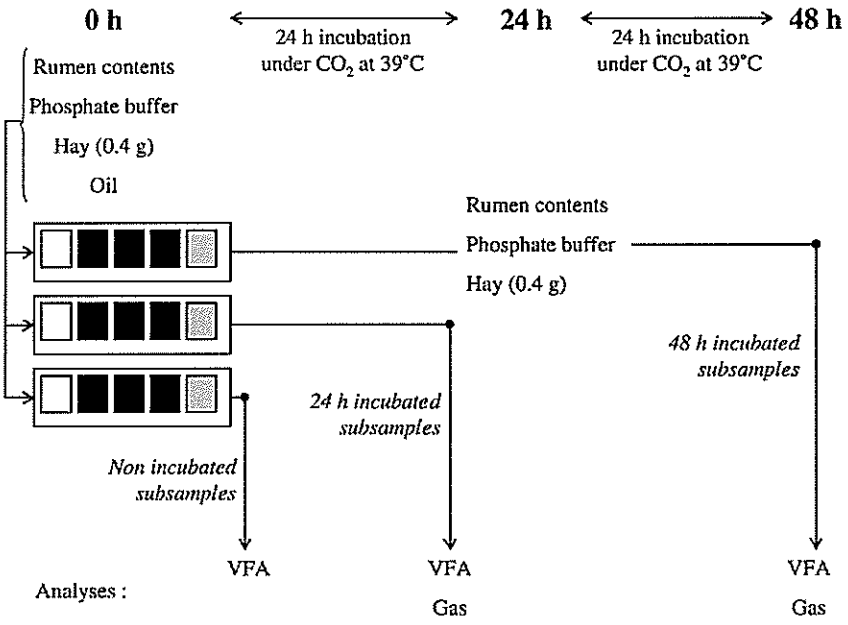

Fig. 1. Outline of in vitro incubation run: white, black and grey squares symbolise, respectively, number of subsamples without oil, with either one of the two FO types (FOa or FOb) and with SO (only in 6 out of 12 incubation runs); (●) indicates addition of phosphoric/formic acid (10/1) to stop fermentation.

an additional 25 ml of a mixture of rumen inoculum and phosphate buffer and 0.400 g hay was added to the remaining flasks, without further FO addition, which were incubated for an additional 24 h period after refilling with CO_2 (48 h incubated subsamples, Fig. 1). Consequently, the FO concentrations per millilitre of incubation liquid differed in the 24 and 48 h incubations: for the 24 h incubation concentrations corresponded to 0, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mg FO/ml incubation liquid and for the 48 h incubation to 0, 0.25, 0.5, 1.0, 1.5, 2.0 and 2.5 mg FO/ml incubation liquid. For SO supplemented flasks, the concentration corresponded to 3 and 1.5 mg SO/ml incubation liquid after 24 and 48 h of incubation, respectively. This re-inoculation of the batch incubation was utilised as previous results of our group revealed no effect on rumen fermentation parameters in 24 h batch incubations when FO triacylglycerides were supplemented to the incubation flasks, whereas changes in the fermentation pattern occurred when supplementing FFA.

2.2.3. *Analyses*

Immediately after incubation, 1 ml of the gas phase was sampled with a gastight syringe and analysed for CH_4 and hydrogen (H_2) by gas chromatography (FM, Dual Column GC 700, Avondale, Pennsylvania, USA) according to Van Nevel et al. (1970). After gas sampling, pH of the incubation liquid was measured and samples of the acidified liquid contents (as before) were centrifuged (10 min at 22,000 × *g*, MSE, Amsterdam, Netherlands),

filtered, and the filtrate used for VFA analysis by gas liquid chromatography (Van Nevel and Demeyer, 1977). To calculate the net amount of VFA produced, the amount of VFA in the corresponding non-incubated sample (Fig. 1) was subtracted.

2.2.4. Calculations

Hydrogen recovery (2Hr, %) was calculated as $(2P + 2B + 4CH_4)/(2A + P + 4B) \times 100$ with acetate (A), propionate (P), butyrate (B) and CH_4 expressed as net molar production rates. This stoichiometric recovery reflects the validity of the model of metabolic H_2 transfer in the rumen fermentation model (Demeyer, 1991). OM apparently degraded in the rumen (ARDOM) was calculated from net production of individual VFA as ARDOM (mol) = $A/2 + P/2 + B$, with A, P and B as defined before and assuming a mean molecular weight of ARDOM of 162 g/mol (Demeyer, 1991).

2.3. In vivo experiment

2.3.1. Experimental design, animals and management

Four adult cross-bred wethers (liveweight (LW) at the beginning of the trial: 71.9 ± 14.9 kg), each with a permanent rumen canula, were used in a cross-over experiment of 70 days, with two adaptation and two experimental periods each of 14 and 21 days, respectively. The LW's of the sheep were considered when allocating sheep to treatments. During experimental periods, animals were housed in metabolic cages (1.5 m × 1.0 m) to enable separate and total collection of faeces and urine. Measurements of dry matter intake (DMI), apparent faecal neutral detergent fibre (NDF) digestibility, rumen VFA and protozoal concentrations, in sacco rumen NDF degradability, rumen passage rate and total urinary excretion of purine derivatives were completed (described below). Sheep were moved to bigger cages (2.5 m × 1.5 m) adapted for individual feeding during the adaptation periods. Sheep were fed a grass hay/grain based concentrate (65/35, w/w on a DM basis) diet twice daily at 07:00 and 19:00 h according to their maintenance requirements for energy (CVB, 1995) (mean chemical characteristics of the complete diet in g/kg DM were crude ash (EEG, 1971), 80; crude protein (EC, 1993), 141; crude fat (ISO 1444–1973), 21; NDF (Goering and Van Soest, 1970; Van Soest et al., 1991; EC, 1992), 510). NDF was assayed without sodium sulfite and without alpha amylase and expressed with residual ash. FO (FOa, Pronova Biocare, Sandefjord, Norway) was added 1 h after feeding by injecting 18.0 ± 2.3 ml of FOa through the rumen canula. The exact FOa quantity injected was adapted to the individual intake of each animal in order to correspond to 4.2% (w/w) FOa supplementation on a dietary basis.

2.3.2. Sampling and analyses

Rumen samples were collected once weekly 6 h after the 07:00 h feeding. Acidified (phosphoric/formic acid, 10/1, v/v, final concentration 2%) rumen samples were used for VFA analysis as described above. Total urine was collected once a week for 24 h and analysed for purine derivatives as described by Fievez et al. (2001a). During the same 24 h, total faeces were collected, weighed and a representative subsample (5%) dried (65 °C for 72 h) and stored for determination of NDF. Rumen degradability of hay NDF was estimated from in sacco incubations of hay for 6 and 48 h (Ørskov and McDonald, 1979). Once a week, four

polyamide bags (Solana, Edegem, Belgium; pore size 50 μm) with approximately 2 g of 2 mm ground (Brabender, Duisburg, Germany) hay were introduced through the rumen canula. After removal from the rumen, bags were washed as described by Mbanzamihigo et al. (2002) and residues analysed for NDF. Rumen passage rate (k_p, %/h) was determined using the Cr-mordanted hay procedure (Udén et al., 1980). Once a week, 40 g of Cr-mordanted hay particles between 0.212 and 1 mm were introduced into the rumen through the rumen canula. One litre rumen samples were removed 2, 5, 8, 11, 23, 35, 47 and 71 h after administration. Subsamples of 0.1 l were stored at −18 °C until analysis and the remainder re-introduced into the rumen. Cr-concentrations were determined using atomic absorption spectrometry (357.9 nm) (Varian AA-1475, Sint-Katelijne-Waver, Belgium). Rumen samples for determination of rumen protozoa concentrations were collected weekly, 3 h after the 07:00 h feeding. Sample preparation, storage and analyses were as described by Fievez et al. (2001b).

2.3.3. In vitro incubations

As no in vivo CH_4 measurements were made during this experiment, simultaneous in vitro incubations were completed to estimate the effect of FOa addition on rumen CH_4 production (Mbanzamihigo et al., 1995). Once a week, rumen contents were collected from each sheep before the 07:00 h feeding. In vitro incubations were performed in duplicate with rumen contents of each individual sheep as described above for the 24 h in vitro incubations, but no additional FOa or substrate was added. CH_4, H_2 and VFA were analysed after 24 h incubation as described for the in vitro experiment.

2.4. Statistics (SPSS software for windows, release 9.0, SPSS Inc., USA)

Run effects within the in vitro experiment were first evaluated, as not all treatments appeared in each incubation run. For each group of three incubation runs, means of the fermentation parameters for the zero oil controls were compared using a one-way Anova according to: $Y_i = \mu + A_i + \varepsilon_i$, where Y_i is the value of fermentation parameter for zero oil control; A_i the effect of group of incubation runs; ε_i the residual error. As results for the zero oil controls did not differ among runs, incubation runs were combined for further statistical analyses.

To determine the effect of FO type and amount in the in vitro experiment, or animal and oil supplementation in the in vivo experiment, data were statistically evaluated using the general linear model (GLM) procedures (univariate) according to: $Y_{ij} = \mu + A_i + B_j + AB_{ij} + \varepsilon_{ij}$, where Y_{ij} is the studied parameters; A_i the effect of FO type for in vitro experiment or animal effect in vivo; B_j the effect of amount of FO added in vitro or effect of FO supplementation in vivo; AB_{ij} the interactions between different factors; ε_{ij} the residual error.

Within each FO type, level of oil addition was evaluated using a one-way Anova and a post hoc Duncan test according to: $Y_i = \mu + A_i + \varepsilon_i$, where Y_i is the fermentation parameter studied; A_i the effect of amount of FO added; ε_i the residual error.

To compare the three oil types (FOa, FOb and SO) in vitro, one-way Anova and a post hoc Duncan test was used according to: $Y_i = \mu + A_i + \varepsilon_i$, where Y_i the studied parameters; A_i the effect of oil type; ε_i the residual error.

Table 2
Evaluation of incubation run differences on total VFA production (VFA_{tot}) (μmol/incubation), relative CH_4 and individual VFA production (mmol/mol total VFA), relative hydrogen accumulation (mmol/mol total VFA), hydrogen recovery (2Hr, %) and pH of zero oil controls incubated during second 24 h incubation period in 48 h in vitro incubations (mean (S.D.), $n = 12$)

No. of group run	VFA_{tot}	Acetate	Propionate	Butyrate	CH_4	H_2	2Hr	pH
I	2474 (378)	612 (28)	216 (15)	97 (15)	248 (62)	0.16 (0.20)	88.3 (10.7)	5.70 (0.12)
II	2560 (121)	547 (57)	237 (14)	121 (27)	191 (81)	6.1 (10.3)	82.0 (13.3)	5.69 (0.13)
III	2484 (352)	612 (43)	216 (21)	98 (9)	232 (26)	0.01 (0.00)	85.6 (10.4)	5.74 (0.03)
IV	2461 (190)	595 (43)	218 (18)	106 (12)	239 (11)	0.12 (0.11)	87.3 (5.0)	5.81 (0.10)
P-value	0.98	0.30	0.36	0.36	0.60	0.43	0.88	0.51

3. Results

3.1. Effect of incubation run

In order to evaluate whether it was justified to combine different incubation runs for further statistical analysis, run effects were evaluated using results of the zero oil controls (Table 2). As the results for the zero oil controls did not differ ($P > 0.1$ for any fermentation parameter) incubation runs were combined for evaluation of effects of type and level of FO on in vitro fermentation pattern.

3.2. Effect of type and level of FO on rumen fermentation pattern in 24 and 48 h laboratory batch culture

Compared to the incubated blank without oil, oil addition did not change total (1790 ± 296 μmol/incubation, $n = 54$) or individual VFA production (588 ± 47, 237 ± 33, 98 ± 12 mmol/mol total VFA, for acetate, propionate and butyrate, respectively, $n = 54$), CH_4 production (256 ± 59 mmol/mol total VFA, $n = 54$) or H_2 accumulation (0.81 ± 0.46 mmol/mol total VFA, $n = 54$) during the first 24 h incubation period. However, significant lipolysis and hydrogenation of all three oils (FOa, FOb and SO) occurred (Dohme et al., 2001).

FOa decreased rumen methanogenesis by up to 80% during the second 24 h incubation period (Table 3). Lower rumen CH_4 production was reflected in increased propionate, and decreased acetate, production. Some accumulation of H_2 gas, which increased with increasing amounts of FOa added, was observed during one incubation run. Inhibition of rumen CH_4 production was higher in incubations containing FOa than in those containing comparable amounts of FOb. CH_4 production was not inhibited further by supplementation above 75 mg for FOa and 25 mg for FOb (Table 3). There was no influence of FO inclusion on rumen butyrate production for FOa, and little for FOb. Addition of FO did not reduce net VFA production, suggesting that degradation of the hay was not suppressed by oil supplementation. Generally, 2Hr were in the range of values expected during rumen in vitro incubations (i.e. 85–95%; Demeyer, 1991), although higher concentrations of FOa tended to reduced 2Hr in some cases. pH values were not affected by the level of oil added, but were

Table 3
Effect of type and amount (mg) of FO supplementation during the second 24 h incubation period in 48 h in vitro incubations on total VFA production (VFA_{tot}) (μmol/incubation), relative CH_4 and individual VFA production (mmol/mol total VFA), relative hydrogen accumulation (mmol/mol total VFA), hydrogen recovery (2Hr, %) and pH (mean (S.D.))

Amount FO	VFA_{tot}	Acetate	Propionate	Butyrate	CH_4	H_2	2Hr	pH
FOa								
0.0 ($n = 6$)	2509 (278)	579.6 a (53.9)	226.5 a (17.5)	108.9 (23.5)	219.6 a (71.5)	3.1 (7.3)	85.2 ab (11.3)	5.70 (0.11)
12.5 ($n = 3$)	2389 (206)	559.3 ab (16.6)	270.4 ab (24.6)	93.2 (6.8)	195.9 a (36.0)	0.4 (0.2)	85.7 ab (4.9)	5.76 (0.04)
25.0 ($n = 3$)	2151 (374)	506.5 ab (59.1)	314.1 ab (59.4)	89.7 (11.0)	198.9 a (51.5)	0.8 (0.2)	95.2 a (8.5)	5.74 (0.06)
50.0 ($n = 3$)	2228 (270)	478.9 bc (78.1)	361.2 bc (81.5)	81.1 (6.0)	140.2 ab (89.8)	1.1 (0.5)	87.7 ab (7.4)	5.75 (0.08)
75.0 ($n = 3$)	2269 (86)	410.8 c (8.5)	371.9 bc (59.8)	104.4 (51.3)	57.0 b (34.4)	64.9 (111)	77.6 b (8.0)	5.75 (0.05)
100.0 ($n = 3$)	2159 (56)	405.9 c (5.6)	377.7 c (67.0)	104.8 (42.0)	55.5 b (38.4)	71.9 (122.6)	78.6 b (10.1)	5.77 (0.05)
125.0 ($n = 3$)	2118 (335)	418.4 c (30.4)	376.0 c (91.2)	98.9 (42.9)	63.0 b (15.2)	51.3 (86.6)	78.3 b (7.1)	5.78 (0.02)
FOb								
0.0 ($n = 6$)	2473 (253)	603.1 a (39.2)	214.7 a (17.8)	102.1 ab (10.2)	235.1 a (18.5)	0.06 a (0.09)	86.1 (7.4)	5.77 (0.07)
12.5 ($n = 3$)	2484 (501)	556.8 ab (71.0)	257.2 ab (41.8)	103.0 ab (12.0)	193.4 ab (10.2)	0.001 a (0.003)	84.0 (10.6)	5.78 (0.05)
25.0 ($n = 3$)	2320 (647)	552.1 ab (48.9)	278.9 ab (39.8)	83.7 a (7.5)	169.6 b (0.5)	0.004 a (0.005)	81.9 (7.8)	5.89 (0.16)
50.0 ($n = 3$)	2319 (493)	528.5 ab (54.7)	293.2 b (35.8)	91.2 ab (4.6)	168.6 b (16.9)	0.002 a (0.004)	84.4 (8.8)	5.79 (0.05)
75.0 ($n = 3$)	2375 (190)	493.9 b (62.2)	299.7 b (56.2)	101.7 ab (6.6)	149.0 b (28.9)	0.3 ab (0.2)	82.8 (9.5)	5.87 (0.05)
100.0 ($n = 3$)	2448 (96)	477.3 b (6.1)	323.9 b (21.4)	92.3 ab (22.4)	147.1 b (49.9)	0.6 b (0.2)	86.1 (9.3)	5.85 (0.06)
125.0 ($n = 3$)	2427 (390)	497.6 b (52.5)	291.1 b (43.6)	108.3 b (7.7)	153.8 b (34.2)	0.8 c (0.5)	82.2 (7.5)	5.87 (0.06)
Statistics[a]								
FO	0.19	**	**	0.98	**	0.07	0.97	***
Amount	0.72	***	***	0.61	***	0.60	0.59	0.18
FO × amount	0.97	0.67	0.69	0.96	0.13	0.62	0.56	0.83

(a, b, c) Different letters within the same column for each fish oil indicate significant differences between different oil concentrations ($P < 0.05$).
[a] *** $P < 0.001$; ** $P < 0.01$; when not significant, P-value is shown.

Table 4
Effect of SO supplementation (75 mg) during the second 24 h incubation period in 48 h in vitro incubations on total VFA production (VFA_{tot}) (μmol/incubation), relative CH_4 and individual VFA production (mmol/mol total VFA), relative hydrogen accumulation (mmol/mol total VFA), hydrogen recovery (2Hr, %) and pH (mean (S.D.), $n = 6$)

Supplementation	VFA_{tot}	Acetate	Propionate	Butyrate	CH_4	H_2	2Hr	pH
None	2500 (157)	570.8 (52.2)	225.2 (19.9)	115.4 (20.4)	215.0 (57.9)	0.0[a] (0.0)	88.2 (3.8)	5.75 (0.12)
SO	2053 (272)	475.5 (38.4)	400.7 (71.3)	78.5 (17.1)	95.3 (36.5)	0.1 (0.2)	85.2 (6.2)	5.89 (0.14)
Statistics[b]	**	**	***	**	**	0.34	0.38	0.10

[a] Means and standard deviations of 0.0 indicate values were lower than 0.05.
[b] Significantly different through SO supplementation: *** $P < 0.001$; ** $P < 0.01$; when not significant, P-value is shown.

higher for the incubation runs in which FOb was administered. The biological importance of this relatively small difference is assumed to be negligible.

3.3. Effect of SO on rumen fermentation pattern in 24 and 48 h laboratory batch culture

Supplementation of 75 mg of SO (Table 4) significantly decreased total VFA ($P < 0.01$), acetate ($P < 0.01$), butyrate ($P < 0.01$) and CH_4 production ($P < 0.01$), while propionate production was significantly increased ($P < 0.001$). The decrease of rumen methanogenesis (about 57%) was lower than the reduction induced by FOa (up to 80%, Table 3). No H_2 accumulation nor change in 2Hr was observed and nor did SO supplementation induce a significant change in pH.

3.4. Effect of FOa on in vivo rumen function and total digestibility

Increased ($P < 0.001$) rumen propionate and reduced ($P < 0.001$) acetate concentrations (Table 5) suggest that FOa injection through the rumen canula reduced rumen methanogenesis. This was confirmed during simultaneous 24 h in vitro incubations of rumen contents from these sheep (Table 6), where similar effects in molar proportions of acetate and propionate were observed. The FOa-induced depression of rumen CH_4 production was not associated with defaunation as reduced protozoal numbers were observed in only one of the four sheep. However, the partial defaunation (i.e. reduction of protozoal numbers by 63%) in the rumen of this sheep was associated with lower rumen butyrate concentrations and increased urinary excretion of purine derivatives. FOa did not alter in vivo NDF digestibility (Table 7), although rumen degradability of hay NDF after 48 h of in sacco incubation (Table 7) was lower and no differences were observed after 6 h of in sacco incubation. Longer rumen retention, as suggested from the tendency ($P = 0.11$) of reduced rumen outflow rates, is consistent with the lower DMI ($P < 0.001$) when FOa was supplemented. The decrease in DMI reflects reduced hay intake as the concentrate was consumed on all occasions.

Table 5
Effect of in vivo FOa injection (18 ml) twice daily on rumen concentrations of total VFA (VFA_{tot}), acetate, propionate, butyrate and protozoa and rumen pH for rumen contents sampled 6 h after feeding (mean (S.D.), $n = 12$)

FO	VFA_{tot} (mM)	Acetate (mmol/mol VFA_{tot})	Propionate (mmol/mol VFA_{tot})	Butyrate (mmol/mol VFA_{tot})	Protozoa (10^6/ml RC[a])	pH
−	112.7 (14.2)	670.2 (29.0)	211.8 (18.0)	118.1 (12.8)	1.63 (0.35)	6.64 (0.10)
+	108.4 (19.5)	629.7 (21.7)	262.7 (20.8)	107.0 (11.2)	1.59 (0.52)	6.62 (0.21)
Statistics[b]						
FO	0.12	***	***	**	0.76	0.80
Animal	***	***	**	***	*	***
FO × animal	0.13	0.05	0.66	**	0.05	0.53

[a] Rumen contents.
[b] *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; when not significant, P-value is shown.

Table 6
Effect of in vivo FOa injection (18 ml) twice daily on fermentation characteristics of simultaneous in vitro incubations with rumen contents of four sheep studied in the in vivo experiment (mean (S.D.), $n = 24$)

FO	VFA_{tot} (μmol/incubation)	Acetate (mmol/mol VFA_{tot})	Propionate (mmol/mol VFA_{tot})	Butyrate (mmol/mol VFA_{tot})	CH_4
−	1120 (196)	602.7 (33.1)	184.3 (22.8)	157.4 (25.3)	344.2 (43.4)
+	1145 (294)	522.5 (31.2)	231.4 (42.2)	170.8 (22.8)	287.7 (36.6)
Statistics[a]					
FO	0.52	***	***	0.37	***
Animal	***	0.11	***	***	*
FO × animal	***	0.08	**	0.10	**

[a] *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; when not significant, P-value is shown.

Table 7
Effect of in vivo FOa injection (18 ml) twice daily on dry matter intake (DMI) of the basal hay/concentrate diet, rumen outflow rate, urinary excretion of purine derivatives (PD), rumen NDF degradability after 6 and 48 h in sacco incubation and total tract NDF degradability (mean (S.D.))

FO	DMI (g/d)	Outflow (%/h)	Urinary PD (mmol/kg DMI)	NDF degradability (%)		
				In sacco		Total
				6 h	48 h	
−	810 (118)	3.82 (1.41)	4.62 (1.28)	27.6 (8.0)	57.5 (3.3)	48.5 (4.5)
+	755 (94)	2.84 (0.67)	5.20 (1.28)	25.5 (3.8)	51.6 (5.8)	46.5 (8.2)
n	64	12	12	12	12	12
Statistics[a]						
FO	***	0.11	0.15	0.47	**	0.49
Animal	***	0.15	*	0.94	0.20	0.40
FO × animal	***	0.38	*	0.80	*	0.41

[a] *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; when not significant, P-value is shown.

4. Discussion

4.1. Effect of type and level of FO and SO on rumen fermentation pattern in laboratory batch culture

Unsaturated FFA inhibit bacterial activity particularly during the lag-phase of microbial development (Maczulac et al., 1981), making bacteria most sensitive to the presence of FFA in the beginning of the incubation. However, during the first 24 h incubation period of the current study, FO supplementation did not change the fermentation pattern, despite the release of EPA and DHA from the triacylglyceride fraction (Dohme et al., 2001). Nevertheless, the lipolysis process might have been relatively slower in the beginning of the incubation as the bacteria of the rumen inoculum were not adapted to FO. Consequently, by the time considerable amounts of FFA were released in the incubation medium, the activity of cellulolytics and methanogens might have been suppressed. Indeed, the ratio of feed to

incubation medium (1.6 g hay per 100 ml) was relatively high in the current in vitro incubations, resulting in relatively low pH values, which might have influenced the microbial flora in terms of stimulation of the amylolytic bacteria and suppression of cellulolytics and methanogens as reflected in the relatively low molar proportion of acetate for the zero oil control. The latter bacteria are the more sensitive to the adsorption of the surface active FFA on the microbial cell wall.

In the second 24 h incubation period, changes in rumen fermentation pattern were induced by type and level of FO. Maximal rumen CH_4 inhibition of 80% occurred with FOa when the incubation liquid was supplemented with 1.5 mg FO/ml. Higher FO concentrations did not induce additional reduction. As expected, reduced CH_4 production was accompanied by increased propionate and reduced acetate production (Wolin, 1960; Czerkawski et al., 1966), the former providing the main alternative sink for metabolic H_2 during rumen fermentation (Van Nevel et al., 1974). A similar shift in rumen VFA concentrations measured in vivo has been reported (Shaw and Ensor, 1959; Sutton et al., 1975; Doreau and Chilliard, 1997; Keady and Mayne, 1999; Wachira et al., 2000), although other studies reported no effect of FO inclusion on the composition of VFA in the rumen liquor (Doreau, 1992; Keady and Mayne, 1999). This varying potential to modify rumen fermentation has been attributed both to different proportions of long chain FA (i.e. $C_{\geq 20}$, both saturated and unsaturated) and different degrees of unsaturation in FO long chain PUFA (Doreau, 1992; Keady and Mayne, 1999). The inclusion of the two FO types allows evaluation of both hypotheses, as FOa and FOb clearly differ in both total long chain ($C_{\geq 20}$) FA content (40.7 and 55.3 g/100 g fatty acid methyl esters (FAME), respectively) and in the proportion of long chain PUFA in total fatty acids (34.1 and 15.3 g/100 g FAME, respectively) (Table 1). From our results, rumen CH_4 inhibition can be related to the amount of FO added (Eq. (1)). This relationship could be improved by incorporation of the amounts of EPA and DHA added (Eq. (2)) but was poorer when the sum of long chain ($C_{\geq 20}$) fatty acids (FA) (both poly and monounsaturated) was incorporated (Eq. (3)):

$$\text{CH}_4\text{-inhibition (\%)} = 7.99 + 22.33 \times \text{FO}_{\text{conc}}\ \text{(mg/ml incubation liquid)}, \quad R^2 = 0.60 \tag{1}$$

$$\text{CH}_4\text{-inhibition (\%)} = 8.81 + 97.9 \times \text{FO}_{\text{conc}} \times \sum(\text{EPA} + \text{DHA})\ \text{(\% total FAME)}, \quad R^2 = 0.73 \tag{2}$$

$$\text{CH}_4\text{-inhibition (\%)} = 11.22 + 39.85 \times \text{FO}_{\text{conc}} \times \sum \text{C}_{\geq 20}\ \text{(\% total FAME)}, \quad R^2 = 0.20 \tag{3}$$

Hence, shifts in the rumen fermentation pattern by long chain FA ($C_{\geq 20}$) seem to be determined by the amount of PUFA (i.e. EPA and DHA) rather than by the amount of FA with 20 or more carbons. Indeed, besides the specific inhibitory effect on rumen methanogens of saturated medium chain FA (Dohme et al., 2000), reduction of rumen methanogenesis by higher chain FA (mainly C_{18}) was established to be closely related to the degree of unsaturation (e.g. Demeyer and Henderickx, 1967). Current results confirm this relationship for long chain FA ($C_{\geq 20}$) present in FO. Comparison of CH_4 inhibition by FOa, FOb and SO (Table 8)

Table 8
Relative effect[a] of supplementation of SO and two types of FO (FOa and FOb) on in vitro rumen CH_4 inhibition and suppression of VFA production and their dependence of degree of unsaturation (mean (S.D.), $n = 6$ for SO and $n = 3$ for FOa and FOb)

Oil type	PUFA/TFA[b] (%)	$PUFA_{db}$[c] (no. of db/mg oil)	$(PUFA + MUFA)_{db}$[d] (no. of db/mg oil)	CH_4-inhibition (%)	VFA-reduction (%)
SO	59.1	1.25	1.50	56.7 b (17.3)	17.9 a (4.9)
FOa	38.2	2.02	2.30	70.9 ab (10.2)	11.4 b (0.8)
FOb	20.1	1.03	1.57	37.9 c (9.3)	3.5 c (3.2)

[a] (Parameter $X_{0\,mg\,oil}$ − parameter $X_{75\,mg\,oil}$) × 100/parameter $X_{0\,mg\,oil}$.
[b] TFA: total fatty acids.
[c] Number of double bounds in PUFA ($(2 \times C_{18:2}) + (3 \times C_{18:3}) + (4 \times C_{18:4}) + (5 \times C_{20:5}) + (4 \times C_{22:4}) + (5 \times C_{22:5}) + (6 \times C_{22:6})$) per mg of oil.
[d] Number of double bounds in PUFA and monounsaturated fatty acids (MUFA) ($C_{16:1} + C_{18:1} + C_{20:1} + C_{22:1}$) per mg of oil.

suggests that the degree of unsaturation, linked to PUFA, clearly determines the severity of the CH_4 inhibition (FOa > SO > FOb). Moreover, FFA have been identified to be more efficient CH_4 inhibitors than triacylglycerides (Van Nevel, 1991). Indeed, the power of the prediction of CH_4 inhibition (Eq. (2)) through FO supplementation, could be increased if average release of EPA and DHA was considered (i.e. amount of EPA and DHA released from triacylglycerides after 48 h of incubation expressed as % of EPA and DHA originally present as triacylglycerides, as described in more detail by Dohme et al., 2001) (Eq. (4)):

$$\text{CH}_4\text{-inhibition (\%)} = 7.02 + 132.84 \times \text{FO}_{\text{conc}} \times \sum(\text{EPA} + \text{DHA}) \times \text{release}_{\text{EPA+DHA}}, \quad R^2 = 0.803 \tag{4}$$

H_2 uptake for hydrogenation of EPA and DHA, as calculated from values reported in the concurrent experiment of Dohme et al. (2001), only correspond to $0.12 \pm 0.07\%$ of the metabolic H_2 available through CH_4 inhibition. This indicates hydrogenation of EPA or DHA and rumen methanogenesis are not competitive processes for metabolic H_2, which has been observed before for other PUFA (Czerkawski et al., 1966). Although the substantial in vitro accumulation of free EPA and DHA altered the rumen fermentation pattern, total VFA and ARDOM were not affected by FOa or FOb supplementation, which contrasts with Kowalczyck et al. (1977). On average, $42.4 \pm 4.8\%$ of hay OM was apparently degraded during in vitro incubations with or without FO, which was significantly lower (35.1%) when SO (75 mg/ml incubation liquid) was added. This shift in rumen fermentation pattern through FO supplementation without inhibition of rumen fibre degradability has been reported before (Sutton et al., 1975; Keady and Mayne, 1999) but contrasts with the general opinion that ruminal NDF digestion decreases as unsaturation of fat increases (e.g. Pantoja et al., 1994).

4.2. Effect of FOa on in vivo rumen function and total digestibility

Based upon the in vitro incubations, FOa seemed the more potent CH_4 inhibitor. Moreover, as the concurrent study (Dohme et al., 2001) revealed that relatively high amounts of

EPA and DHA might escape rumen hydrogenation, FOa might have the potential to supply relatively high quantities of long chain PUFA (i.e. EPA and DHA) to the duodenum of ruminants. The results of the in vitro incubations suggest that FOa concentrations of 1.5 mg FOa/ml incubation liquid resulted in maximal rumen CH_4 inhibition without reduction of rumen apparent OM degradability. This concentration corresponds with two daily injections of 15.7 g FOa, assuming an average rumen volume of 10.5 l, as calculated from rumen Cr-concentration decline (Udén et al., 1980). On a dietary basis, the daily amount of FOa represented 4.2% (w/w) of the DMI of hay and concentrate. This FOa supplementation did not reduce in vivo NDF digestion or NDF degradability after 6 h of in sacco incubation, which is consistent with our in vitro results and Sutton et al. (1975); Doreau (1992); Choi et al. (1998); and Keady and Mayne (1999), suggesting no effect of FO treatment on rumen OM or ADF disappearance. Nevertheless, rumen fibre degradation was reduced after 48 h of in sacco incubation. However, the tendency to a decreased rumen outflow rate might have compensated for the reduced rumen fibre digestion (Sutton et al., 1983; Van Nevel et al., 1993). Moreover, FOa injection changed the rumen fermentation pattern by increasing and decreasing in vivo propionate and acetate concentrations by 24 and 6%, respectively, corresponding with the in vitro situation between 0.25 and 0.5 mg/ml incubation liquid, which was accompanied with an 11% reduction in CH_4 production. This change is consistent with the 16% CH_4 inhibition as derived from simultaneous in vitro incubations of the rumen fluid taken from the sheep of the in vivo experiment. Similar shifts in the VFA pattern through FO supplementation have been reported by Shaw and Ensor (1959); Sutton et al. (1975); Doreau and Chilliard (1997); Keady and Mayne (1999); and Wachira et al. (2000) although rumen CH_4 productions were not quantified in these studies. Consequently, the reduced intake of hay, inducing the risk of underfeeding, might have been compensated by the supplementation of oil, delivering 154 kJ net energy/day (CVB, 1995), and by the reduction of CH_4 excretion, which represents an energy loss for the ruminant. The CH_4 depression induced by FOa injection was not associated with defaunation or changes in urinary excretion of purine derivatives. The latter results are in agreement with observations of rumen CH_4 inhibition by other fat sources without decrease of rumen protozoal numbers (Broudiscou et al., 1990; Dohme et al., 1999) and without reduction of efficiency of rumen microbial growth (Van Nevel and Demeyer, 1981).

5. Conclusions

The severity of in vitro inhibition of rumen methanogenesis by FO is determined by the level of unsaturation of the long chain fatty acids ($C_{\geq 20}$) and the amount of oil added, although supplementation above 1.5 mg FOa/ml incubation liquid did not further reduce rumen methanogenesis. CH_4 suppression was accompanied by a shift of the fermentation pattern towards propionate without any effect on total VFA production, suggesting FO supplementation did not affect the amount of ARDOM. Reduced fibre degradation with FOa supplementation was observed at 48 h rumen in sacco incubations but not at 6 h, or in vivo. Increases of rumen propionate concentrations indicate that inhibition of rumen methanogenesis occurs in vivo, although to a lesser extent than observed in vitro. Reduced biohydrogenation of FO PUFA, resulting in rumen accumulation of unesterified EPA and

DHA might be nutritionally and ecologically relevant in terms of an increased post-ruminal supply of these PUFA for incorporation into animal products and through reduced rumen methanogenesis.

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