Effects of chemically or technologically treated linseed products and docosahexaenoic acid addition to linseed oil on biohydrogenation of C18:3n-3 in vitro

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ABSTRACT

Rumen biohydrogenation kinetics of C18:3n-3 from several chemically or technologically treated linseed products and docosahexaenoic acid (DHA; C22:6n-3) addition to linseed oil were evaluated in vitro. Linseed products evaluated were linseed oil, crushed linseed, formaldehyde treated crushed linseed, sodium hydroxide/formaldehyde treated crushed linseed, extruded whole linseed (2 processing variants), extruded crushed linseed (2 processing variants), micronized crushed linseed, commercially available extruded linseed, lipid encapsulated linseed oil, and DHA addition to linseed oil. Each product was incubated with rumen liquid using equal amounts of supplemented C18:3n-3 and fermentable substrate (freeze-dried total mixed ration) for 0, 0.5, 1, 2, 4, 6, 12, and 24 h using a batch culture technique. Disappearance of C18:3n-3 was measured to estimate the fractional biohydrogenation rate and lag time according to an exponential model and to calculate effective biohydrogenation of C18:3n-3, assuming a fractional passage rate of 0.060/h. Treatments showed no differences in rumen fermentation parameters, including gas production rate and volatile fatty acid concentration. Technological pretreatment (crushing) followed by chemical treatment applied as formaldehyde of linseed resulted in effective protection of C18:3n-3 against biohydrogenation. Additional chemical pretreatment (sodium hydroxide) before applying formaldehyde treatment did not further improve the effectiveness of protection. Extrusion of whole linseed compared with extrusion of crushed linseed was effective in reducing C18:3n-3 biohydrogenation, whereas the processing variants were not different in C18:3n-3 biohydrogenation. Crushed linseed, micronized crushed linseed, lipid encapsulated linseed oil, and DHA addition to linseed oil did not reduce C18:3n-3

biohydrogenation. Compared with the other treatments, docosahexaenoic acid addition to linseed oil resulted in a comparable *trans*11,*cis*15-C18:2 biohydrogenation but a lesser *trans*10+11-C18:1 biohydrogenation. This suggests that addition of DHA in combination with linseed oil was effective only in inhibiting the last step of biohydrogenation from *trans*10+11-C18:1 to C18:0.

Key words: biohydrogenation, in vitro, linseed, fatty acid

INTRODUCTION

Changing the fatty acid (FA) profile of bovine milk fat toward a more nutritionally beneficial profile has received increasing attention. Increasing the proportion of unsaturated FA (UFA) in milk fat through the diet of dairy cows is considered an improvement of the dietary value of milk (Jenkins and Bridges, 2007). Dietary UFA, however, are subject to extensive biohydrogenation by ruminal microorganisms, yielding *trans*-FA intermediates and saturated FA as end products (Harfoot and Hazlewood, 1997). The extent of lipolysis and biohydrogenation is determined by several factors, including the nature of dietary FA, the retention time in the rumen, and the composition of the microbial population (Jenkins et al., 2008). Fats in the ruminant diet are mainly derived from forages, grains, and oil supplements, especially vegetable oils. The latter, however, have only a limited use in dairy diets because they tend to reduce fibrolytic activity and fiber digestion in the rumen (Harfoot and Hazlewood, 1997). The rumen environment, therefore, has to be protected against adverse effects of oil supplements and oil supplements have to be protected against ruminal biohydrogenation to increase postruminal UFA flow. Three main protection technologies can be distinguished: 1) chemical protection (e.g., formaldehyde treatment of oilseeds); 2) alterations of FA structure through formation of calcium salts and amides of FA; and 3) technological treatments of oilseeds (e.g., ex-

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trusion, cracking; Fievez et al., 2007). Several in vitro and in vivo studies evaluated the potential of these protection technologies to increase postruminal UFA flow. Sinclair et al. (2005) showed that formaldehyde treatment reduced biohydrogenation of C18:3n-3 from linseed only when it was preceded by chemical pretreatment (sodium hydroxide or formic acid) to induce permeability of the seed coat. Technological pretreatment, such as crushing, might be able to induce the same permeability of the seed coat and result in effective UFA protection after formaldehyde treatment. Technological treatments, such as extrusion, increased the proportions of biohydrogenation intermediates in vitro (Enjalbert et al., 2003) and in milk fat (Bayourthe et al., 2000; Chouinard et al., 2001; Akraim et al., 2007). The effect of extrusion conditions, such as temperature, was evaluated in these studies, but oilseeds were always ground before extrusion. Therefore, extrusion of whole linseed versus crushed linseed in combination with different processing conditions, such as steam and water percentage, might result in differences in postruminal UFA flow. Protection of UFA in a sphere of FA with a high melting point was hypothesized as a possible protection technology (Jenkins and Bridges, 2007). However, to our knowledge no studies have been reported in which this technology was applied to protect seed oil. Besides protecting oilseeds against biohydrogenation, complete biohydrogenation toward stearic acid can be inhibited by the addition of docosahexaenoic acid (DHA; C22:6n-3). Recent research suggests that DHA provokes accumulation of various *trans*-FA, including *trans*-11-C18:1, in vitro (Vlaeminck et al., 2008) and in vivo (Boeckaert et al., 2008) through changes in the rumen microbial population. The effect of DHA addition on the biohydrogenation of C18:3n-3 from linseed oil, however, has not been studied.

Metabolism of UFA in the rumen may be studied by time series of in vitro incubations to allow estimation of kinetic parameters such as fractional rate of biohydrogenation and lag time (Fievez et al., 2007). Such kinetic parameters and assumptions on fractional passage rate enable calculation of effective biohydrogenation of UFA. Furthermore, fermentation parameters such as gas production, VFA concentration, and OM degradability can be measured to examine the effects of UFA supplementation on ruminal fermentation.

To our knowledge, the effectiveness of protection of UFA by several protection technologies for linseed and changes in biohydrogenation of linseed oil following DHA addition have not been compared under identical experimental conditions. The objective of the current study was to investigate, by means of in vitro incubations, whether several chemically or technologically treated linseed products and addition of DHA to lin-

seed oil can effectively change rumen biohydrogenation kinetics of C18:3n-3.

MATERIALS AND METHODS

Animals and Diet

The experiment was approved by the Institutional Animal Care and Use Committee of Wageningen University (Wageningen, the Netherlands). Ruminal fluid was collected just before the morning feeding from 4 lactating Holstein-Friesian dairy cows (lactation stage: 176 ± 105 DIM; fat- and protein-corrected milk: 31.2 ± 11.0 kg/d; BW: 657 ± 60 kg), each fitted with a ruminal cannula and fed ad libitum a TMR diet. The TMR contained (fresh weight basis) 35.9% ryegrass silage, 54.4% maize silage, 1.1% straw, 0.4% minerals, and 8.2% concentrate (containing 32.7% soybean meal, 32.7% wheat, 32.7% rapeseed meal, and 2.0% cane molasses). A freeze-dried and ground (1 mm) sample of this TMR was used as the basal incubation substrate. After collection, rumen fluid was immediately transferred into prewarmed and CO₂-flushed thermos flasks.

Linseed Products

The linseed products tested in vitro were as follows: 1) pure linseed oil (**LO**); 2) linseed crushed in a roller mill (**CL**; 0.25 mm; Ipswich Turner, Christy Turner Ltd., Ipswich, UK); 3) formaldehyde treated crushed linseed (**FCL**; 4.5 g/kg of formaldehyde applied as formalin, according to Sinclair et al., 2005); 4) sodium hydroxide/formaldehyde treated crushed linseed (**SFCL**; 3.0 g/kg of sodium hydroxide applied as a 50:50 vol/vol solution, followed by 4.5 g/kg of formaldehyde applied as formalin, according to Sinclair et al., 2005); 5 and 6) extruded mixture of whole linseed and wheat bran [70:30 vol/vol linseed:wheat bran, prepared in a small-scale single-screw extruder line of Almex AL150 (Almex, Zutphen, the Netherlands) equipped with a pellet press of Robinson/Heesen V2/30 (Heesen, Boxtel, the Netherlands) and cooler unit; **EL1**: 6% steam and 2% water, 127°C for 20–30 s; **EL2**: 2% steam and 6% water, 130°C for 20–30 s]; 7 and 8) extruded mixture of crushed linseed and wheat bran (70:30 vol/vol crushed linseed:wheat bran, extruder line and pellet press as described previously; **ECL1**: 6% steam and 2% water, 115°C for 20–30 s; **ECL2**: 2% steam and 6% water, 118°C for 20–30 s); 9) micronized crushed linseed (**MCL**; heated with infrared gas generators to 115–120°C for 90 s; gas-heated infrared irradiation belt, HOAF/WU-design, Oldenzaal, the Netherlands); 10) commercially available extruded linseed product

Table 1. Fatty acid composition (g/100 g of fatty acids) and total fatty acid content (mg/g of DM) of TMR and linseed products¹

Fatty acid	TMR	LO	CL	MCL	ECL1	ECL2	CEL	EL1	EL2	LELO	DHA Gold ²
C14:0	0.66	0.05	0.05	0.05	0.08	0.07	0.08	0.10	0.08	0.25	13.78
C16:0	16.74	5.54	5.46	5.55	6.14	6.05	6.43	6.58	6.18	7.95	32.22
<i>cis</i> 9-C16:1	0.44	0.06	0.07	0.07	0.07	0.07	0.06	0.09	0.07	0.54	0.22
C18:0	2.81	3.70	3.58	3.58	3.46	3.51	3.34	3.39	3.53	3.77	0.64
<i>cis</i> 9-C18:1	13.60	19.32	18.29	18.59	18.47	18.54	17.77	19.15	18.17	27.08	0.09
<i>cis</i> 11-C18:1	1.80	0.73	0.72	0.72	0.74	0.75	0.72	0.79	0.76	2.28	0.37
C18:2n6	26.54	16.16	15.94	15.82	17.06	16.98	19.09	17.57	17.32	18.01	0.08
C18:3n-3	25.08	53.41	54.70	54.59	52.87	52.96	51.36	51.13	52.75	38.48	0.26
C20:0	0.73	0.13	0.13	0.13	0.12	0.12	0.13	0.17	0.15	0.19	0.11
C22:0	1.10	0.13	0.13	0.13	0.12	0.12	0.13	0.16	0.14	0.12	0.03
C20:5n-3	0.19	ND ³	ND	ND	ND	ND	0.03	ND	ND	0.03	1.67
C22:5n-3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.47
C22:6n-3	ND	ND	ND	ND	ND	ND	0.05	ND	ND	ND	42.59
Other	10.31	0.76	0.94	0.76	0.87	0.83	0.79	0.88	0.83	1.31	7.46
Total fatty acids	17.6	950.0 ⁴	292.3	298.9	191.7	192.6	179.3	NT ⁵	NT	118.3	NT

¹LO: linseed oil; CL: crushed linseed; MCL: micronized crushed linseed; ECL1: extruded crushed linseed 1; ECL2: extruded crushed linseed 2; CEL: commercial extruded linseed product; EL1: extruded linseed 1; EL2: extruded linseed 2; LELO: lipid encapsulated linseed oil.

²Martek Biosciences Corp., Columbia, MD.

³ND: not detected.

⁴CVB, 2007.

⁵NT: no total fatty acid content determined because of incomplete fatty acid extraction.

(**CEL**; containing 56.0% crushed linseed, 21.0% wheat, 15.0% sunflower cake, 4.5% field beans, 2.0% butylated hydroxytoluene (BHT), 1.0% linseed oil, and 0.5% salt; Nutex Compact, Dumoulin, Seilles, Belgium; Van et al., 2008); 11) lipid encapsulated linseed oil (**LELO**; canola meal with 12% linseed oil and 2% pork fat prepared in a twin-shaft paddle mixer-vacuum coater; Dinissen, Pegasus PG-10lab, Sevenum, the Netherlands); and 12) linseed oil + DHA addition (**LO+DHA**; 10 mg of DHA/g of incubation substrate; DHA Gold, Martek Biosciences Corp., Columbia, MD; Vlaeminck et al., 2008). All linseed and linseed oil treatments were made from a single batch of linseed and linseed oil, respectively. The FA composition of the TMR, linseed products, and DHA Gold is given in Table 1.

In Vitro Incubations

The rumen fluid of the 4 cows was mixed and strained through a double layer of cheese cloth continuously flushed with CO₂, diluted with a phosphate buffer (per liter of distilled water: 28.8 g of Na₂HPO₄·12H₂O, 6.1 g of NaH₂PO₄·H₂O, and 1.4 g of NH₄Cl, adjusted to pH 6.8 by adding NaOH solution), and placed on a stirrer to ensure complete mixing of the rumen fluid-phosphate buffer mixture (1:4). Accurately weighed treatment products (~1 g) and 50 mL of the rumen fluid-phosphate buffer mixture were added to gastight incubation flasks (150 mL) under anaerobic conditions. Each sample was incubated in duplicate in 2 separate runs on separate days. To provide equal amounts of supplemented C18:3n-3 and fermentable substrate

(Table 2), treatment products comprised 1.00 g of TMR with 0.06 g of LO; 0.89 g of TMR with 0.17 g of CL, MCL, FCL, or SFCL; 0.82 g of TMR with 0.24 g of EL1, EL2, ECL1, ECL2, or CEL; 0.50 g of TMR with 0.57 g of LELO; and 1.00 g of TMR with 0.06 g of LO + 50.6 mg of DHA Gold. Flasks were flushed with CO₂ before incubation started in a shaking water bath at 39°C for 0, 0.5, 1, 2, 4, 6, 12, and 24 h. At the end of the incubation periods, flasks were removed sequentially and immediately placed on ice. Then, flasks were opened and pH was measured. The incubation residue was collected, stored at -20°C, and freeze dried before FA analysis was carried out. In a second duplicated set of 24-h flasks, cumulative gas production (Cone et al., 1996), fluid VFA content, and OM disappearance were measured. In these 24-h flasks, first a subsample of 0.75 mL of the incubation fluid was taken for VFA analysis. These samples were stored with 85% H₃PO₄ (1:1 vol/vol) and kept in a freezer at -20°C pending VFA analysis. The incubation residue of the 24-h flasks was filtered and analyzed for ash content to determine OM degradability.

Analysis

The FA in the TMR (375 mg of freeze-dried material), treatment products (375 mg of material), and incubation residues (375 mg of freeze-dried material) were extracted with 15 mL of chloroform-methanol (2:1 vol/vol) and 375 µL of distilled water (Folch et al., 1957). The homogenized extracts were filtered and centrifuged at 800 × *g* after adding 2.2 mL of distilled water for a

Table 2. Calculated amounts of C18:3n-3 (mg/flask containing 50 mL of incubation fluid) and fermentable OM (mg/flask containing 50 mL of incubation fluid) for the control treatment (CON) and linseed treatments¹ before incubation

Parameter	CON	LO	CL	FCL	SFCL	MCL	ECL1	ECL2	CEL	EL1	EL2	LELO	LO+DHA
C18:3n-3	4.41	36.48	31.59	30.82	30.99	31.65	28.07	28.16	25.86	28.21	28.02	28.21	36.46
Fermentable OM	510	512	502	501	501	501	499	499	500	499	498	471	511

¹LO: linseed oil; CL: crushed linseed; FCL: formaldehyde treated crushed linseed; SFCL: sodium hydroxide pretreated formaldehyde treated crushed linseed; MCL: micronized crushed linseed; ECL1: extruded crushed linseed 1; ECL2: extruded crushed linseed 2; CEL: commercial extruded linseed product; EL1: extruded linseed 1; EL2: extruded linseed 2; LELO: lipid encapsulated linseed oil; LO+DHA: linseed oil and DHA Gold (Martek Biosciences Corp., Columbia, MD).

clear separation. The upper phase was removed thoroughly using repeated washing with wash solution [30 mL of chloroform, 480 mL of methanol, and 470 mL of NaCl solution (7.3 g/L of water)]. Approximately 3 mL of the lower phase, containing lipids, was collected and solvents were evaporated by vacuum centrifugation. The residual lipids were collected and FA were methylated with 0.5 mL of 0.5 *N* NaOH methanolate (10 min at 80°C) followed by 0.5 mL of 14% boron trifluoride (2 min at 80°C). Fatty acid methyl esters were collected in 1 mL of hexane. For a clear separation of the hexane layer a saturated salt solution (400 g of NaCl/L of water) was added and tubes were centrifuged at $800 \times g$ for 5 min. Residues were dissolved in 1 mL of hexane and transferred to GC vials. Fatty acid methyl esters were quantified using gas chromatography (Trace GC Ultra, Thermo Fisher Scientific, Waltham, MA) with a fused silica capillary column (100 m $\times$ 0.250 mm, 0.2 μ m film thickness; SP2560, Supelco, St. Louis, MO) using helium as a carrier gas at a constant flow of 1.5 mL/min. The flame ionization detector was set at 280°C. The time-temperature program used started with an initial temperature of 140°C for 4 min, increased 4°C/min to a final temperature of 240°C, and held at this temperature for 20 min. Fatty acid methyl esters were identified using external standards (S37, Supelco). Separation of the isomers *trans*10-C18:1 and *trans*11-C18:1 was not possible in all samples and therefore *trans*10+11-C18:1 were reported together.

The pH was measured using an electronic pH meter (pH electrode HI 1230, Hanna Instruments BV, IJsselstein, the Netherlands). The concentration of VFA was determined using gas chromatography (GC type Fisons HRGC MEGA2, Fisons Instruments, Milan, Italy) as described by Taweel et al. (2005). Ash was determined by combustion at 550°C (ISO 5984; ISO, 2002).

Calculations and Statistics

All statistical analyses were carried out in SAS version 9.1 (SAS Institute, Cary, NC). Total C18 FA remained constant over the 24-h period and therefore individual C18 FA were calculated as proportions of total C18 FA.

Disappearance of C18:3n-3 from the incubation flasks at each sampling time was calculated relative to the 0-h time point. This disappearance of C18:3n-3 was then used to estimate the fractional biohydrogenation rate and lag time according to an exponential model with the NLIN procedure of SAS. Lag time was constrained to be positive. Effective C18:3n-3 biohydrogenation was calculated according to Dhanoa et al. (1999) assuming a fractional passage rate of 0.060/h.

The individual FA and pH measured at the different sampling times were analyzed using the MIXED procedure of SAS. The statistical model included the fixed effects of incubation run, treatment, time, and the interaction between treatment and time. Posthoc analyses were carried out using the Tukey test to test pairwise comparisons. Least squares means are reported, and significance was declared at $P < 0.05$.

Gas production profiles obtained with the automated system were fitted by iteration for individual incubation flasks to a generalized Michaelis-Menten model without lag time (France et al., 2000) with the NLIN procedure. The gas production profiles were characterized by the cumulative gas production (**OMCV**; mL/g of incubated OM), the estimated asymptotic gas production (parameter A; mL/g of incubated OM), a constant that determines the sharpness of the switching characteristic of the profile (parameter B), the time after incubation at which half of the asymptotic gas production has been reached (parameter C; h), the maximum rate of gas production (mL/h), and the time to reach the maximum rate of gas production (**Tmax**; h). Parameters B and C were constrained to be positive. Biohydrogenation kinetic parameters, gas production kinetic parameters, VFA concentration, and OM degradability were analyzed with the MIXED procedure, with incubation run and treatment as the fixed variables.

RESULTS

Rumen Fermentation Pattern

Fluid pH decreased significantly ($P < 0.001$) with time for all treatments (results not shown), from an

average of 6.61 at 0 h to 6.18 at 24 h of incubation. An effect of treatment on fluid pH ($P < 0.001$) was found; however, after 24 h of incubation the vessel fluid pH was >6.10 for all treatments (results not shown). The concentration of total VFA; the molar proportions of acetate, butyrate, and propionate; and the ratio of nonglucogenic to glucogenic VFA were not influenced by treatment ($P > 0.05$; Table 3). However, the molar proportions of isobutyrate, valerate, and isovalerate were increased ($P < 0.001$) in LELO compared with the other treatments, although valerate proportion was similar for LELO and CEL. Organic matter degradability was influenced ($P = 0.016$) by linseed treatment; CEL showed a greater OM degradability than FCL and the other treatments showed intermediate results (Table 3).

The effects of the various linseed products on gas production parameters are shown in Table 4. No effects ($P > 0.05$) of the linseed treatments were found on OMCV. A tendency ($P < 0.10$) was found toward a greater OMCV for the LELO treatment compared with the FCL treatment. The LO and FCL treatments showed a significantly greater value for parameter A compared with the ECL2 and EL2 treatments. The value for parameter B was significantly greater in the CL, ECL2, and CEL treatments compared with the LO, FCL, and MCL treatments, and the value for parameter C was greatest in the FCL treatment compared with the other treatments. The maximum rate of gas production was greater in the ECL1, ECL2, CEL, and LELO treatments compared with the FCL, SFCL, and MCL treatments, and the Tmax was greatest in the SFCL treatment, although Tmax was similar for SFCL and CL, FCL, and LO+DHA.

FA Composition

Figures 1, 2, 3 and 4 show the changes in vessel proportions of C18:3n-3, *trans*11,*cis*15-C18:2, *trans*10+11-C18:1, and C18:0, respectively, during the 24 h of incubation. Proportion of C18:3n-3 decreased with time ($P < 0.001$), and this decrease was influenced by treatment (interaction treatment $\times$ time; $P < 0.001$). After 24 h of incubation the proportion of C18:3n-3 relative to 0 h was greater in the EL1, EL2, FCL, and SFCL treatments compared with the CEL and LELO treatments (Table 5), with intermediate values for the other treatments. Biohydrogenation intermediate *trans*11,*cis*15-C18:2 reached a peak value after 12 h of incubation for the LO, CEL, and LELO treatments. Proportion of *trans*11,*cis*15-C18:2 continued to increase until 24 h of incubation for the other linseed treatments. The CL, ECL1, ECL2, MCL, and CEL treatments resulted in greater proportions of

*trans*11,*cis*15-C18:2 after 24 h of incubation compared with the LO, EL1, and EL2 treatments ($P < 0.05$). Proportion of *trans*10+11-C18:1 increased during the 24 h of incubation for all treatments except for LO, where a maximum proportion of 11.1 g of *trans*10+11-C18:1/100 g of total C18 FA was reached after 12 h of incubation. Proportion of *trans*10+11-C18:1 at 24 h was greater for the CEL, ECL1, ECL2, LELO, and LO+DHA treatments compared with the FCL, SFCL, EL1, and EL2 treatments ($P < 0.05$). Compared with the LO treatment, all linseed treatments except CEL and LELO showed a lesser C18:0 proportion after 24 h of incubation ($P < 0.05$). This decrease was more important for the LO+DHA treatment, which showed only a slight increase in C18:0 proportion during the 24 h of incubation.

The biohydrogenation kinetic parameters for C18:3n-3 are presented in Table 5. The fractional biohydrogenation rate was greater ($P < 0.001$) for the LELO treatment compared with the other treatments; only CEL showed intermediate results. The lag time was not different between the various linseed treatments ($P > 0.05$). The calculated effective biohydrogenation was significantly influenced by chemical and technological treatment of linseed. The FCL, EL1, and EL2 treatments resulted in a lesser effective biohydrogenation of C18:3n-3 compared with the LO, ECL1, ECL2, CEL, and LELO treatments.

DISCUSSION

Modeling Biohydrogenation Kinetic Parameters

The results of our experiment show a large variation in the rate and extent of biohydrogenation of C18:3n-3 across substrates. Fractional biohydrogenation rate of C18:3n-3 was significantly affected by treatment, but lag time was not affected. Enjalbert et al. (2003) reported a negative lag time in their in vitro experiment to evaluate biohydrogenation kinetics of UFA. However, such results are physiologically not acceptable and can be indicative of an inappropriate model or data that are not compatible with the requirements of the model (Dhanoa et al., 1996). Sinclair et al. (2005) evaluated the biohydrogenation of protected linseed sources in vitro and reported fractional biohydrogenation rates without accounting for a lag time. Lag time is related to the time needed for lipolysis, is dependent on fat source, and might be associated with DM digestibility and time for the microbes to adapt to the substrate and incubation conditions (Ribeiro et al., 2007). In our experiment, the model with a lag time constrained to be positive resulted in a better model fit than without a lag time, and estimated lag time differed significantly from zero in 13 of the 24 replicates (results not shown).

Table 3. Effect of control treatment (CON) and linseed treatments¹ on OM degradability (%), concentration of total VFA (mmol/L), and VFA molar proportions (mmol/mol) after 24 h of incubation

Parameter	Treatment													SED ²	Significance ³
	CON	LO	CL	FCL	SFCL	MCL	ECL1	ECL2	CEL	EL1	EL2	LELO	LO+DHA		
OM degradability	68.4	64.5 ^{ab}	65.2 ^{ab}	59.8 ^b	62.3 ^{ab}	65.6 ^{ab}	63.1 ^{ab}	65.4 ^{ab}	66.0 ^a	60.7 ^{ab}	60.7 ^{ab}	65.4 ^{ab}	64.6 ^{ab}	1.6	0.016
Total VFA	95	100	98	94	96	100	101	104	98	97	99	96	103	4.9	0.688
Acetate	605	605	591	600	598	596	590	592	582	604	603	585	594	8.7	0.202
Propionate	278	278	282	282	281	282	288	287	287	277	278	277	286	4.5	0.145
Butyrate	83	81	85	85	84	82	84	83	89	84	84	85	84	3.0	0.669
Isobutyrate	5	5 ^{cd}	7 ^{bc}	5 ^d	6 ^{bcd}	7 ^b	6 ^{bcd}	6 ^{bcd}	7 ^{bc}	6 ^{bcd}	6 ^{bcd}	10 ^a	6 ^{bcd}	0.5	<0.001
Valerate	19	19 ^b	20 ^b	19 ^b	19 ^b	20 ^b	19 ^b	19 ^b	21 ^{ab}	19 ^b	18 ^b	25 ^a	19 ^b	1.2	<0.001
Isovalerate	11	11 ^{bcd}	14 ^b	10 ^d	11 ^{bcd}	13 ^{bc}	12 ^{bcd}	12 ^{bcd}	14 ^b	11 ^{cd}	11 ^{bcd}	18 ^a	11 ^{bcd}	1.0	<0.001
NGR ⁴	2.65	2.63	2.57	2.62	2.61	2.57	2.52	2.54	2.52	2.67	2.65	2.56	2.55	0.05	0.112

^{a-d}Means within a row with different superscripts differ ($P < 0.05$).

¹LO: linseed oil; CL: crushed linseed; FCL: formaldehyde treated crushed linseed; SFCL: sodium hydroxide pretreated formaldehyde treated crushed linseed; MCL: micronized crushed linseed; ECL1: extruded crushed linseed 1; ECL2: extruded crushed linseed 2; CEL: commercial extruded linseed product; EL1: extruded linseed 1; EL2: extruded linseed 2; LELO: lipid encapsulated linseed oil; LO+DHA: linseed oil and DHA Gold (Martek Biosciences Corp., Columbia, MD).

²SED: standard error of difference.

³Significance of treatment.

⁴NGR: nonglucogenic to glucogenic VFA ratio calculated as [acetate + 2 × (butyrate + isobutyrate) + valerate + isovalerate]/(propionate + valerate + isovalerate).

Table 4. Effect of control treatment (CON) and linseed treatments¹ on gas production kinetics² during 24 h of incubation

Parameter	Treatment													SED ³	Significance ⁴
	CON	LO	CL	FCL	SFCL	MCL	ECL1	ECL2	CEL	EL1	EL2	LELO	LO+DHA		
OMCV	126	119	120	107	114	113	116	115	119	117	110	127	110	5.34	0.059
A	169	162 ^a	151 ^{abc}	162 ^a	154 ^{abc}	154 ^{abc}	141 ^{abc}	137 ^{bc}	138 ^{abc}	150 ^{abc}	136 ^c	161 ^{ab}	144 ^{abc}	6.86	0.002
B	1.49	1.44 ^{bc}	1.61 ^a	1.41 ^c	1.55 ^{abc}	1.44 ^{bc}	1.55 ^{ab}	1.61 ^a	1.62 ^a	1.46 ^{abc}	1.54 ^{abc}	1.52 ^{abc}	1.53 ^{abc}	0.04	<0.001
C	11.51	11.42 ^{bc}	10.13 ^{bcd}	14.61 ^a	11.96 ^b	11.61 ^{bc}	8.85 ^d	8.76 ^d	8.32 ^d	9.90 ^{bcd}	9.57 ^{cd}	9.82 ^{bcd}	10.80 ^{bcd}	0.61	<0.001
Tmax	3.67	3.47 ^{bcd}	4.07 ^{ab}	3.92 ^{abc}	4.40 ^a	3.53 ^{bcd}	3.28 ^{cd}	3.49 ^{bcd}	3.34 ^{bcd}	3.14 ^d	3.46 ^{bcd}	3.42 ^{bcd}	3.76 ^{abcd}	0.21	<0.001
Rmax	9.29	8.88 ^{abcd}	9.20 ^{abcd}	7.16 ^e	7.90 ^{de}	8.21 ^{cde}	9.84 ^a	9.71 ^{ab}	10.40 ^a	9.40 ^{abc}	8.78 ^{abcd}	10.06 ^a	8.37 ^{bcd}	0.40	<0.001

^{a-e}Means within a row with different superscripts differ ($P < 0.05$).

¹LO: linseed oil; CL: crushed linseed; FCL: formaldehyde treated crushed linseed; SFCL: sodium hydroxide pretreated formaldehyde treated crushed linseed; MCL: micronized crushed linseed; ECL1: extruded crushed linseed 1; ECL2: extruded crushed linseed 2; CEL: commercial extruded linseed product; EL1: extruded linseed 1; EL2: extruded linseed 2; LELO: lipid encapsulated linseed oil; LO+DHA: linseed oil and DHA Gold (Martek Biosciences Corp., Columbia, MD).

²OMCV: cumulative gas production (mL/g of incubated OM); A: estimate asymptotic gas production (mL/g of incubated OM); B: constant that determines the sharpness of the switching characteristic of the gas production profile; C: time after incubation at which half of the asymptotic gas production has been reached (h), Tmax: time to reach the maximum rate of gas production (h); Rmax: maximum rate of gas production (mL/h).

³SED: standard error of difference.

⁴Significance of treatment.

Unesterified FA at 0 h of incubation may contribute to the differences in observed lag times (Ribeiro et al., 2007). To account for the reported biohydrogenation rates with or without lag time, effective biohydrogenation was calculated according to Dhanoa et al. (1999) and used to compare the effectiveness of protection.

Rumen Fermentation Pattern

Troegeler-Meynadier et al. (2003) examined the effect of pH on biohydrogenation of C18:2n6 and C18:3n-3 and concluded that biohydrogenation of C18:2n6 and C18:3n-3 was inhibited when pH was below 6.0 compared with above 6.5. In the present experiment, fluid pH showed a decrease with time, but incubation flasks were buffered sufficiently to maintain a minimal pH above 6.1 after 24 h of incubation for all treatments. Therefore, it is expected that biohydrogenation of C18:3n-3 was not influenced by the pH change in time. Total VFA concentration, acetate, propionate, butyrate proportions, and the ratio of nonglucogenic to glucogenic VFA were not different between the treatments, indicating no differences in fermentation pattern. Ribeiro et al. (2005) concluded that changes in fermentation pattern likely reflect shifts in the bacterial population in response to changes in fermentable substrates. The results of the present trial therefore indicate that no shift occurred in bacterial population for the different linseed products. The proportions of valerate and branched-chain VFA did differ between the treatments, with the greatest proportions found in the LELO treatment compared with the other treatments except the CEL treatment for the valerate proportion. Valerate and branched-chain VFA result mainly from fermentation of protein (Bannink et al., 2006); therefore, these changes are probably related to the canola meal, which was used as the carrier product for the linseed oil.

Overall cumulative gas production did not differ between the treatments, whereas some of the gas production curve parameters did differ between treatments. However, no clear effect was found of one of the treatments on these parameters. Sinclair et al. (2005) did not observe differences in gas production profiles when different treatments rich in C18:3n-3 were incubated in vitro for 48 h. Organic matter degradability was greater in the CEL treatment compared with the FCL treatment. The CEL product contained 56% crushed linseed and 44% other products including wheat and sunflower cake, which might be responsible for the greater OM degradability. In view of the moderate decrease in pH and the absence of differences in total gas production and in the major VFA, it appears that no differences in rumen fermentation exist between tested linseed products.

C18:3n-3 Biohydrogenation

Gonthier et al. (2005) hypothesized that chemical treatments such as formaldehyde treatment could be more effective than heat treatment in the protection of UFA from ruminal biohydrogenation. Sinclair et al. (2005) observed in vitro that pretreatment of linseed with sodium hydroxide or formic acid followed by treatment with formaldehyde resulted in effective protection of C18:3n-3. This observation was confirmed by Fievez et al. (2007), who concluded that oilseed pretreatment, either chemically or through emulsification, is essential for the formation of the inert formaldehyde-protein matrix. In the present experiment, we hypothesized that technological pretreatment (crushing) would be as effective as chemical pretreatment (sodium hydroxide) in inducing permeability of the seed coat and thereby as effective in forming the inert formaldehyde-protein matrix resulting in protection against biohydrogenation. Indeed, formaldehyde treatment of crushed linseed showed to be effective in reducing biohydrogenation of C18:3n-3 compared with linseed oil. Additional pretreatment with sodium hydroxide before applying formaldehyde treatment did not improve the effectiveness of protection any further; numerically, additional pretreatment with sodium hydroxide resulted in lesser effective protection compared with crushing only as pretreatment. These results confirm that crushing makes the protein of the oilseed accessible to formaldehyde to form the inert formaldehyde-protein matrix. Indeed, Petit (2003) did not show an effective protection of C18:3n-3 from linseed that was protected by spraying the formalin (37% formaldehyde) on the whole seed, whereas Goodridge et al. (2001) found an increased protection of C18:3n-3 from ground linseed protected by encapsulation in a matrix of aldehyde treated protein.

Because of the size of whole linseed, it is expected that the seed coat will not be crushed completely during chewing and ruminating, resulting in less oil being released and available for biohydrogenation (Petit, 2003). However, the seed coat will also limit digestion postruminally, thus preventing complete release and absorption of the desired internal PUFA (Jenkins, 2006). The physical crushing of linseed may therefore contribute to an increased availability of UFA for absorption and hence, biohydrogenation, and possible transfer into milk fat (da Silva et al., 2007). Feeding ground linseed indeed increased the proportions of C18:3n-3 and *trans*-FA in milk fat compared with feeding whole linseed (da Silva et al., 2007). Compared with linseed oil, crushed linseed may result in lesser biohydrogenation because of the localization of the oil in the seed or meal (Chilliard et al., 2000). In the current experiment, crushed lin-

Figure 1. Changes in proportion of C18:3n-3 for linseed treatments during 24 h of incubation (treatment: $P < 0.001$; time: $P < 0.001$; treatment $\times$ time: $P < 0.001$; SED: 1.88). LO: linseed oil; CL: crushed linseed; FCL: formaldehyde treated crushed linseed; SFCL: sodium hydroxide pretreated formaldehyde treated crushed linseed; ECL1: extruded crushed linseed 1; ECL2: extruded crushed linseed 2; MCL: micronized crushed linseed; CEL: commercial extruded linseed product; EL1: extruded linseed 1; EL2: extruded linseed 2; LELO: lipid encapsulated linseed oil; LO+DHA: linseed oil and DHA Gold (Martek Biosciences Corp., Columbia, MD).

Figure 2. Changes in proportion of *trans*11,*cis*15-C18:2 for linseed treatments during 24 h of incubation (treatment: $P < 0.001$; time: $P < 0.001$; treatment $\times$ time: $P < 0.001$; SED: 0.48). CL: crushed linseed; FCL: formaldehyde treated crushed linseed; SFCL: sodium hydroxide pre-treated formaldehyde treated crushed linseed; ECL1: extruded crushed linseed 1; ECL2: extruded crushed linseed 2; MCL: micronized crushed linseed; CEL: commercial extruded linseed product; LO: linseed oil; EL1: extruded linseed 1; EL2: extruded linseed 2; LELO: lipid encapsulated linseed oil; LO+DHA: linseed oil and DHA Gold (Martek Biosciences Corp., Columbia, MD).

Figure 3. Changes in proportion of *trans*10+11-C18:1 for linseed treatments during 24 h of incubation (treatment: $P < 0.001$; time: $P < 0.001$; treatment $\times$ time: $P < 0.001$; SED: 1.39). CL: crushed linseed; FCL: formaldehyde treated crushed linseed; SFCL: sodium hydroxide pre-treated formaldehyde treated crushed linseed; ECL1: extruded crushed linseed 1; ECL2: extruded crushed linseed 2; MCL: micronized crushed linseed; CEL: commercial extruded linseed product; LO: linseed oil; EL1: extruded linseed 1; EL2: extruded linseed 2; LELO: lipid encapsulated linseed oil; LO+DHA: linseed oil and DHA Gold (Martek Biosciences Corp., Columbia, MD).

Figure 4. Changes in proportion of C18:0 for linseed treatments during 24 h of incubation (treatment: $P < 0.001$; time: $P < 0.001$; treatment $\times$ time: $P < 0.001$; SED: 1.88). CL: crushed linseed; FCL: formaldehyde treated crushed linseed; SFCL: sodium hydroxide pretreated formaldehyde treated crushed linseed; ECL1: extruded crushed linseed 1; ECL2: extruded crushed linseed 2; MCL: micronized crushed linseed; CEL: commercial extruded linseed product; LO: linseed oil; EL1: extruded linseed 1; EL2: extruded linseed 2; LELO: lipid encapsulated linseed oil; LO+DHA: linseed oil and DHA Gold (Martek Biosciences Corp., Columbia, MD).

Table 5. Proportion of C18:3n-3 after 24 h of incubation and estimated biohydrogenation kinetic parameters for C18:3n-3 for control treatment (CON) and linseed treatments¹

Parameter	Treatment														
	CON	LO	CL	FCL	SFCL	MCL	ECL1	ECL2	CEL	EL1	EL2	LELO	LO+DHA	SED ²	Significance ³
C18:3n-3, %	0.21	0.37 ^{abcd}	0.40 ^{abcd}	0.59 ^{abc}	0.54 ^{abc}	0.35 ^{abcd}	0.30 ^{cd}	0.31 ^{bcd}	0.22 ^d	0.66 ^a	0.63 ^{ab}	0.16 ^d	0.44 ^{abcd}	0.08	<0.001
kh, %/h	14.04	6.65 ^b	4.13 ^b	2.47 ^b	2.61 ^b	4.53 ^b	5.93 ^b	5.69 ^b	8.04 ^{ab}	1.79 ^b	2.23 ^b	13.10 ^a	4.42 ^b	1.45	<0.001
Lag time, h	0	1.49	2.11	2.99	0.06	2.01	2.00	2.12	2.00	0.25	1.49	3.43	1.37	0.99	0.149
EBH, %	70.0	43.5 ^{abc}	35.6 ^{bcd}	24.3 ^d	30.1 ^{cd}	38.1 ^{bcd}	43.7 ^{abc}	42.9 ^{abc}	50.8 ^{ab}	22.7 ^d	25.0 ^d	55.5 ^a	38.3 ^{abcd}	4.30	<0.001

^{c-d}Means within a row with different superscripts differ ($P < 0.05$).

LO: linseed oil; CL: crushed linseed; FCL: formaldehyde treated crushed linseed; MCL: micronized linseed; ECL: extruded crushed linseed; ECL1: extruded crushed linseed 1; ECL2: extruded crushed linseed 2; CEL: commercial extruded linseed product; EL1: extruded linseed 1; EL2: extruded linseed 2; LELO: lipid encapsulated linseed oil; LO+DHA: linseed oil and DHA Gold (Martek Biosciences Corp., Columbia, MD).

SED: standard error of difference.

Significance of treatment.

C18:3n-3: proportion of C18:3 of total C18 FA after 24 h of incubation relative to 0 h sample.

klh: fractional rate of biohydrogenation.

EBH: calculated effective biohydrogenation assuming a fractional passage rate of 0.06/h.

Extrusion of oilseeds may contribute to an increased availability of UFA for absorption by rupturing the seed to liberate the oil from the seed cells. This process might influence the production of intermediates and end products of biohydrogenation (Dhiman et al., 1999; Neves et al., 2007). It was hypothesized that extrusion of whole linseed versus extrusion of crushed linseed under different processing conditions (steam and water percentage) might result in differences in postruminal UFA flow. The extruded whole linseed treatments resulted in lesser calculated effective biohydrogenation of C18:3n-3 compared with the LO, extruded crushed linseed, CEL, and LELO treatments. The extrusion process possibly ruptured the whole seeds only to a certain extent, leaving the seed coat intact in part of the linseeds. Because the intact seed coat provides an effective barrier against biohydrogenation (Jenkins, 2006), the C18:3n-3 inside the whole seeds was protected against biohydrogenation. Overprotection by the seed coat, however, might prevent the release and absorption of the C18:3n-3 postruminally, which was not measured in this study. Different conditions during extrusion (6% steam and 2% water vs. 2% steam and 6% water) did not result in differences in biohydrogenation for both the extruded whole linseed treatments and the extruded crushed linseed treatments. Chouinard et al. (2001) studied the effect of ground soybeans extruded at 120, 130, and 140°C on milk FA composition and found no differences in C18:3n-3 proportion in milk fat. In the present study, extrusion temperature was 127°C for the extruded whole linseed product with 6% steam and 2% water (EL1), 130°C for the extruded whole linseed product with 2% steam and 6% water (EL2), 115°C for the extruded crushed linseed product with 6% steam and 2% water (ECL1), and 118°C for the extruded crushed linseed product with 2% steam and 6% water (ECL2). The processing parameters resulted in a very small temperature range, which did not induce differences in biohydrogenation kinetics between the products.

Micronization is a heat treatment in which the seed is rapidly heated internally accompanied by an increase in water vapor pressure. The micronization process will cook the seed from inside out and the seed will expand to the point of eversion (Wang et al., 1997). Petit et al. (2002) showed that micronization at different temperatures (130, 140, 150, and 160°C) resulted in a similar loss of C18:3n-3 after incubation, except for the linseed

micronized at 160°C for 0.5 h, which reduced C18:3n-3 proportion because of FA oxidation. These researchers suggested that micronization temperature should not exceed 130°C to protect linseed. In the present study, a micronization temperature of 115 to 120°C was used for 90 s; however, no difference was found in effective biohydrogenation of C18:3n-3 between the MCL treatment and the LO treatment. This finding was in agreement with the results of Mustafa et al. (2003), who showed that micronized linseed (115°C for 1.5 min) was extensively biohydrogenated in the rumen.

Encapsulation of UFA in a sphere of high melting point saturated FA was hypothesized as a possible way of protecting UFA against biohydrogenation (Jenkins and Bridges, 2007). Perfield et al. (2004) showed that a lipid encapsulated conjugated linoleic acid (Balchem Encapsulates, New Hampton, NY), a stable powder at room temperature, decreased milk fat to the same extent as an amide protected conjugated linoleic acid, suggesting an equal amount of protection in the rumen and an equal postruminal availability. In the present experiment, linseed oil was incorporated into canola meal and coated with pork fat. This product, however, resulted in the greatest fractional biohydrogenation rate compared with all other linseed products and therefore clearly showed no protection against biohydrogenation.

Microbial population characteristics are associated with the extent of biohydrogenation in the rumen. Recent *in vitro* (Vlaeminck et al., 2008) and *in vivo* (Boeckaert et al., 2008) research showed that DHA addition results in a significant reduction of the conversion of *trans*11-C18:1 to C18:0 through changes in the rumen microbial population. In the current experiment, DHA (DHA Gold) was added to a linseed oil treatment. The calculated effective biohydrogenation of C18:3n-3 did not differ from that of the other treatments. The proportion of *trans*10+11-C18:1, however, was significantly greater and the proportion of C18:0 was significantly lesser after the 24-h incubation period. Vlaeminck et al. (2008) showed an increased proportion of both *trans*11-C18:1 and *trans*11,*cis*15-C18:2 when DHA was added to rumen fluid. The present study also showed an increased proportion of *trans*11,*cis*15-C18:2 after 24 h of incubation. This increased *trans*11,*cis*15-C18:2 proportion, however, was comparable to the increase in *trans*11,*cis*15-C18:2 proportion in the other treatments. This comparable increase of *trans*11,*cis*15-C18:2 in all treatments suggests that, unlike the rate of biohydrogenation of *trans*10+11-C18:1, the rate of biohydrogenation of *trans*11,*cis*15-C18:2 was not different compared with the other treatments. It was therefore suggested that the addition of DHA in combination with linseed

oil was effective only in inhibiting the last step of biohydrogenation from *trans*10+11-C18:1 to C18:0.

CONCLUSIONS

Technological pretreatment (crushing) followed by chemical treatment applied as formaldehyde of linseed was effective in protecting C18:3n-3 against biohydrogenation *in vitro*. Additional chemical pretreatment (sodium hydroxide) before formaldehyde treatment did not further improve the effectiveness of protection. Extrusion of whole linseed compared with extrusion of crushed linseed was effective in reducing C18:3n-3 biohydrogenation, whereas steam and water percentage did not influence C18:3n-3 biohydrogenation. Crushing linseed, micronizing crushed linseed, incorporating linseed oil into canola meal and coating with pork fat, and adding DHA in combination with linseed oil did not reduce C18:3n-3 biohydrogenation. Addition of DHA in combination with linseed oil resulted in a comparable *trans*11,*cis*15-C18:2 biohydrogenation and a lesser *trans*10+11-C18:1 biohydrogenation compared with the other treatments. This suggests that addition of DHA in combination with linseed oil was effective only in inhibiting the last step of biohydrogenation from *trans*10+11-C18:1 to C18:0. Regarding all evaluated linseed products, only FCL and extruded whole linseed show potential in the ruminant diet to increase postruminal C18:3n-3 flow.

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