Incremental effect of a calcium salt of *cis*-monounsaturated fatty acids supplement on milk fatty acid composition in cows fed maize silage-based diets

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ABSTRACT

In most Western countries, saturated fatty acid (SFA) intake exceeds recommended levels, which is considered a risk factor for cardiovascular disease (CVD). As milk and dairy products are major contributors to SFA intake in many countries, recent research has focused on sustainable methods of producing milk with a lower saturated fat concentration by altering dairy cow diets. Human intervention studies have shown that CVD risk can be reduced by consuming dairy products with reduced SFA and increased *cis*-monounsaturated fatty acid (MUFA) concentrations. This milk fatty acid profile can be achieved by supplementing dairy cow diets with *cis*-MUFA-rich unsaturated oils. However, rumen exposure of unsaturated oils also leads to enhanced milk *trans* fatty acid (TFA) concentrations. Because of concerns about the effects of TFA consumption on CVD, feeding strategies that increase MUFA concentrations in milk without concomitant increases in TFA concentration are preferred by milk processors. In an attempt to limit TFA production and increase the replacement of SFA by *cis*-MUFA, a preparation of rumen-protected unsaturated oils was developed using saponification with calcium salts. Four multiparous Holstein-Friesian cows in mid-late lactation were used in a 4 × 4 Latin square design with 21-d periods to investigate the effect of incremental dietary inclusion of a calcium salt of *cis*-MUFA product (Ca-MUFA; 20, 40, and 60 g/kg of dry matter of a maize silage-based diet), on milk production, composition, and fatty acid concentration. Increasing Ca-MUFA inclusion reduced dry matter intake linearly, but no change was observed in estimated ME intake. No change in milk yield was noted, but milk fat and protein concentrations were linearly reduced. Supplementation with Ca-MUFA re-

sulted in a linear reduction in total SFA (from 71 to 52 g/100 g of fatty acids for control and 60 g/kg of dry matter diets, respectively). In addition, concentrations of both *cis*- and *trans*-MUFA were increased with Ca-MUFA inclusion, and increases in other biohydrogenation intermediates in milk fat were also observed. The Ca-MUFA supplement was very effective at reducing milk SFA concentration and increasing *cis*-MUFA concentrations without incurring any negative effects on milk and milk component yields. However, reduced milk fat and protein concentrations, together with increases in milk TFA concentrations, suggest partial dissociation of the calcium salts in the rumen.

Key words: milk fatty acid, calcium salt, monounsaturated fatty acid, *trans* fatty acid

INTRODUCTION

Dietary fat content and composition is considered one of the most important modifiable determinants of cardiovascular disease (CVD) risk, with a 1% reduction in intake of energy from SFA predicted to result in a 3% decrease in CVD risk, based on its effect on low density lipoprotein-cholesterol (Givens, 2009). The United Kingdom exceeds its dietary target for SFA intake, by around 10% on average (Bates et al., 2010). In the United Kingdom, milk and dairy products contribute about 27% of total SFA consumed (Bates et al., 2010), with higher contributions in other EU countries (Hulshof et al., 1999). Intervention studies have shown that replacing SFA with *cis*-MUFA in dairy products has favorable effects on serum cholesterol levels, which may lead to a reduction in CVD risk over a longer period (Wood et al., 1993; Tholstrup et al., 1998; Seidel et al., 2005).

Numerous studies have shown the potential of dairy cow nutrition to produce milk with reduced SFA and increased MUFA (e.g., Kirchgessner et al., 1967; Ashes et al., 1992; Jenkins, 1998). A series of studies at the University of Reading demonstrated that the inclusion

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of crushed rapeseeds in the diet of the dairy cow can reduce SFA from 70 to 55 or 60% of total FA while increasing *cis*-MUFA from 20 to 33% of total FA (Givens et al., 2009). However, the increase in *cis*-MUFA concentration in response to feeding full-fat rapeseed was accompanied by an increase in *trans*-18:1 concentration, which is a consequence of rumen biohydrogenation of the rapeseed oil fed. An alternative approach is to feed rumen-protected sources of *cis*-MUFA, which should minimize the development of *trans*-monoenes and maximize the increase in *cis*-MUFA concentration in milk. In previous studies, a variety of approaches have been used to reduce the rumen biohydrogenation of rapeseed or canola oil FA with varying success (e.g., Chouinard et al., 1997; Enjalbert et al., 1997). Calcium salts of canola oil FA have been effective in elevating *cis*-MUFA concentration of milk (Ferlay et al., 1992), but not without an increase in *trans*-isomer concentrations (Enjalbert et al., 1997; Chouinard et al., 1997, 1998), suggesting that Ca-salts of FA from rapeseed or canola oil are at least partially susceptible to rumen biohydrogenation. This may be due in part to the differences in the dissociation constant of the Ca-salts formed, which vary with the degree of their unsaturation (Chouinard et al., 1997 and 1998).

The objectives of the present study were to measure the effect of feeding increasing amounts of a Ca-salt of high *cis*-MUFA on feed intake, milk yield and composition, and milk FA composition in lactating Holstein cows.

MATERIALS AND METHODS

Design, Animals, and Management

The effect of incremental doses of a high-C18:1 Ca-salt product was measured using a balanced 4 × 4 Latin square design experiment with 21-d experimental periods. Four multiparous, mid- to late-lactation Holstein-Friesian cows were used with (mean ± SE) liveweight of 727 ± 23.0 kg, parity of 5.8 ± 0.75, milk yield of 29 ± 2.9 kg/d, and 244 ± 25.1 DIM at the start of the study. Cows were housed in individual tiestalls equipped with a rubber mattress and bedded with wood shavings. Clean water and trace mineralized blocks (Rockies, Tithebarn Ltd., Cheshire, UK) were available *ad libitum*. Cows were milked *in situ* at 0530 h and 1530 h.

Experimental Diets

Diets were offered as a TMR using computerized feeders (Insentec, Marknesse, the Netherlands; forage:concentrate ratio 50:50 on a DM basis) with the forage component consisting of maize silage and grass

silage (750 and 250 g/kg of forage DM, respectively). Treatments consisted of a control diet containing no supplemental lipid (control) or the same basal diet with Ca-salts containing a high proportion of *cis*-MUFA (**Ca-MUFA**; Volac International Ltd., Royston, UK) fed at incremental inclusion rates of 20, 40, and 60 g/kg of ration DM (CS2, CS4, and CS6, respectively), to provide supplement intakes of 440, 880, and 1320 g/day at a predicted mean daily DM intake of 22 kg. The supplement was produced by industrial saponification of an 18:1-rich oil, resulting in a vigorous reaction leading to a product rich in fine particles. The supplement was incrementally included in rations over the first 4 d of each experimental period. Ingredients and chemical composition of the experimental diets are shown in Table 1. The supplements were added to the basal TMR, rather than replacing specific components, and thus diluted the other ingredients proportionally. Feed refusals were removed and weighed before the morning milking.

Experimental Measurements, Sample Collection, and Chemical Analysis

Feed intake was recorded daily. Representative samples of the 4 TMR, individual forages (maize silage, grass silage) and concentrates [concentrates, Sopralin (Trouw Nutrition, Belfast, UK), Ca-MUFA] were taken on d 16 to 20 of each experimental period, bulked and stored in sealed bags at −20°C. A representative sample of refused feed was taken during the last 5 d of each experimental period and analyzed for DM content (100°C) to determine individual DM intakes. Samples of forages and concentrates were stored frozen until analyzed for NDF, ADF, OM, CP, water-soluble carbohydrates, starch, and estimated ME concentrations according to reference procedures as outlined elsewhere (Kliem et al., 2008).

Milk yields were recorded daily throughout the study. Samples of milk preserved with potassium dichromate (1 mg/mL, Broad Spectrum Microtabs, D&F Control Systems Inc., Dublin, CA) for the determination of fat, CP, and lactose by infrared spectroscopy (Milkoscan FT6000 infrared analyzer, Foss Ltd., Warrington, UK) were collected during the last 3 d of each experimental period. Additional samples of unpreserved milk were also collected on the last day of each period and stored at −20°C until composited according to yield, and submitted for FA analysis.

Lipids in 1 mL of milk and appropriate sample weights of forage, concentrate, and lipid supplement samples were extracted and transesterified to fatty acid methyl esters (**FAME**), and then FAME were separated using standard methods (Kliem et al., 2008).

Table 1. Ingredients and chemical composition of experimental diets (g/kg of DM or as stated)

Item	Treatment ¹			
	Control	CS2	CS4	CS6
Ingredients				
Maize silage	375	367.6	360.6	353.8
Grass silage	125	122.5	120.2	117.9
Maize meal	162	158.8	155.8	152.8
Soybean meal	100	98.0	96.2	94.3
Sugar beet pulp, molassed	112	109.8	107.7	105.7
Wheat feed	45	44.1	43.2	42.4
Molasses	15	14.7	14.4	14.2
Salt ²	5	4.9	4.8	4.7
Minerals ³	10	9.8	9.6	9.4
Protected soybean meal ⁴	46	45.1	44.2	43.4
Dicalcium phosphate	5	4.9	4.8	4.7
Ca-salts of <i>cis</i> -MUFA ⁵	0	20	40	60
Chemical composition				
DM (g/kg fresh)	640	646	653	661
OM	925	923	922	922
CP	175	171	168	165
NDF	315	309	303	297
ADF	142	139	136	134
Starch	235	230	226	221
Water soluble carbohydrates	31	30	30	29
ME (MJ/kg of DM) ⁶	11.8	12.2	12.7	13.1
FA				
16:0	3.2	4.6	6.0	7.4
18:0	0.45	0.77	1.10	1.42
18:1 <i>cis</i> -9	4.0	15.0	26.0	37.0
18:2n-6	11.1	12.6	14.0	15.4
18:3n-3	1.7	1.7	1.6	1.6
Total FA	22.9	37.7	52.5	67.3

¹Where CS2, CS4, and CS6 are diets containing 20, 40, and 60 g/kg of DM calcium salt of *cis*-monounsaturated FA, respectively.

²Pioneer Rocksalt, Broste Ltd., Norfolk, UK.

³Dairy Direct, Bury St. Edmunds, Suffolk, UK.

⁴Sopralin, Trouw Nutrition, Belfast, UK.

⁵Oil content of 763 g/kg DM, comprising 72.6% *cis*-9 18:1, 10.9% 18:2n-6, 9.7% 16:0, 2.0% 12:0, 2.2% 18:0, as well as minor (<1.0%) amounts of 10:0, 14:0, 20:0, *cis*-11 18:1, and *cis*-11 20:1.

⁶Calculated using ME reference value for the Ca-salt *cis*-MUFA supplement of 33.25 MJ/kg of DM.

The distribution of conjugated linoleic acid (**CLA**) isomers in milk FAME was determined by HPLC using 4 silver impregnated silica columns (Chromspher 5 Lipids, 250 × 4.6 mm; 5 µm particle size; Varian, Oxford, UK) coupled in series using 0.1% (vol/vol) of acetonitrile in heptanes as the mobile phase (Shingfield et al., 2003). Isomers were identified using an authentic CLA methyl ester standard (O-5632, Sigma-Aldrich, YA-Kemia Limited, Helsinki, Finland) and chemically synthesized *trans*-9,*cis*-11 CLA (Shingfield et al., 2005). Identification was verified using *cis*-9,*trans*-11 CLA as a landmark isomer.

Milk FA composition was expressed as a weight percentage of total FA using response factors derived from the analysis of a butter oil reference standard (CRM 164; Community Bureau of Reference, Brussels, Belgium). Concentrations of specific conjugated isomers in milk fat were calculated based on propor-

tionate peak area responses determined by HPLC and the sum of *trans*-7,*cis*-9 CLA, *trans*-8,*cis*-10 CLA, and *cis*-9,*trans*-11 CLA weight percentage determined as a single peak by gas chromatography.

Statistical Analysis

Intake, milk production, and milk FA composition data were averaged for each cow and the means (n = 16) analyzed using the mixed procedure of SAS (version 8.2, SAS Institute Inc., Cary, NC) and a model testing fixed effects of treatment and period and random effects of cow, with period as a repeated effect within cow. Compound symmetry, heterogeneous compound symmetry, first-order autoregressive, heterogeneous first-order autoregressive, or an unstructured covariance structure was used for repeated measures analysis, based on goodness of fit criteria for each variable ana-

Table 2. Effect of calcium salts of *cis*-monounsaturated fatty acids (Ca-MUFA) supplementation on DM and FA intake and milk and constituent yield (LSM results)

Item	Treatment ¹					P-value ²		
	Control	CS2	CS4	CS6	SEM ³	Diet	LIN	QUAD
DM intake (kg/d)	21.2	20.3	20.0	19.8	1.76	0.180	0.050	0.451
FA intake (g/d)								
16:0	67.7	93.4	120.6	146.8	10.44	<0.001	<0.001	0.950
18:0	9.6	15.7	22.0	28.0	1.92	<0.001	<0.001	0.985
18:1 <i>cis</i> -9	85.4	304.5	521.3	731.6	46.82	<0.001	<0.001	0.887
18:2n-6	236	255	281	305	24.3	0.003	<0.001	0.724
18:3n-3	36.0	33.8	32.7	31.6	2.88	0.014	0.003	0.396
Total FA	486	765	1,052	1,330	91.6	<0.001	<0.001	0.996
Calcium salt intake (g/d)	0	405	802	1,186	73.9	<0.001	<0.001	0.848
Yield								
Milk (kg/d)	25.5	25.6	24.8	26.3	3.86	0.762	0.709	0.492
Fat (g/d)	967	923	845	936	142.7	0.148	0.284	0.081
Protein (g/d)	883	854	796	864	125.6	0.301	0.430	0.160
Lactose (g/d)	1,053	1,059	1,006	1,076	171.4	0.745	0.941	0.514
Concentration (g/kg)								
Fat	38.0	36.3	33.8	35.6	1.30	0.021	0.016	0.039
Protein	34.7	33.6	32.5	32.8	1.12	<0.001	<0.001	0.009
Lactose	41.3	41.5	39.5	40.8	1.21	0.514	0.445	0.588

¹Where CS2, CS4, and CS6 are diets containing 20, 40, and 60 g/kg of DM Ca-MUFA, respectively.

²Refers to the significance of overall effect of diet or linear (LIN) and quadratic (QUAD) effects of increasing supplement inclusion.

³SEM for n = 16 measurements.

lyzed. Orthogonal contrasts were used to test for linear, quadratic and cubic effects of Ca-MUFA inclusion level. In addition, treatment means were compared with the control mean using Dunnett's adjusted probability of difference comparisons in the LSMeans option of the Mixed procedure. Differences were deemed significant when $P < 0.05$.

RESULTS

Diets were formulated so that the Ca-MUFA supplement diluted the other ingredients. This meant that CP, NDF, ADF, starch, and water-soluble carbohydrate concentrations of the TMR rations decreased with increasing inclusion level of supplement (Table 1). However, this also led to successive increases in overall calculated ME content of the diets with increasing inclusion of Ca-MUFA.

For all variables, no cubic effects ($P > 0.05$) of incremental inclusion of Ca-MUFA in the diet were noted, so significance levels have not been reported. Increasing the inclusion level of Ca-MUFA supplement resulted in a linear ($P = 0.05$) reduction in DMI (Table 2). Increasing Ca-MUFA inclusion also linearly ($P < 0.01$) increased the intake of 16:0, 18:0, *cis*-9 18:1, 18:2 n-6, and total FA, but decreased ($P = 0.003$) 18:3 n-3 intake in a linear manner. Daily ME intake was also calculated, but no significant effect of diet on ME intake was observed (LSM of 250, 248, 254, and 259 MJ/d for control, CS2, CS4, and CS6, respectively; $P = 0.546$).

Milk yield and milk constituent yields were not affected by Ca-MUFA supplementation. Milk fat and protein concentrations were linearly reduced ($P = 0.016$ and $P < 0.001$, respectively) when the Ca-MUFA supplement was fed, but the incremental effect on both milk fat and protein concentration was less for the highest dose (quadratic effects, $P = 0.039$ and $P = 0.009$, respectively).

Increasing the inclusion level of Ca-MUFA in the diet linearly reduced ($P < 0.01$) all short- and medium-chain SFA concentration in milk fat, apart from 4:0 (Table 3). Supplementation with Ca-MUFA also linearly decreased ($P < 0.01$) some branched-chain SFA (13:0 anteiso, 14:0 iso, 15:0 anteiso) concentrations. Milk fat concentration of 16:0 was linearly and quadratically reduced ($P < 0.01$) with increasing Ca-MUFA supplementation. Effects on some 16:1 FA were noted, with the Ca-MUFA diets resulting in linear enhancements in *trans*-6/7 16:1, *trans*-8 16:1, *trans*-12 16:1, and *trans*-13 16:1 concentrations, and linear reductions in *cis*-13 16:1 concentrations. Because *cis*-9 16:1 co-eluted with 17:0 anteiso and *trans*-9 16:1 with 17:0 iso, no clear conclusions can be drawn as to which of these individual FA changed in concentration when their respective sums were linearly reduced ($P < 0.001$) by Ca-MUFA supplementation.

Supplementation of Ca-MUFA also resulted in linear increases ($P < 0.02$) in milk fat 18:0, total CLA, and total nonconjugated 18:2 isomer concentrations, and a linear decrease ($P < 0.01$) in 18:3 n-6 and 18:3 n-3

Table 3. Effect of calcium salts of *cis*-monounsaturated fatty acids (Ca-MUFA) supplementation on milk FA composition (LSM results as g/100 g of FA)

FA	Treatment ¹				SEM ²	P-value ³		
	Control	CS2	CS4	CS6		Diet	LIN	QUAD
4:0	3.8	3.8	3.7	3.4	0.22	0.247	0.098	0.278
6:0	2.7	2.5	2.3	2.0	0.13	0.005	<0.001	0.448
8:0	1.4	1.2	1.1	1.0	0.08	0.002	<0.001	0.935
10:0	3.2	2.6	2.20	1.8	0.21	<0.001	<0.001	0.404
10:1 <i>cis</i> -9	0.36	0.28	0.22	0.17	0.029	<0.001	<0.001	0.368
12:0	3.9	3.2	2.7	2.4	0.27	<0.001	<0.001	0.214
12:1 <i>cis</i> -9	0.12	0.10	0.08	0.05	0.011	<0.001	<0.001	0.0374
13:0	0.13	0.10	0.06	0.06	0.017	<0.001	<0.001	0.031
13:0 anteiso	0.10	0.08	0.07	0.05	0.010	0.001	<0.001	0.458
14:0	11.5	10.3	9.4	8.2	0.43	<0.001	<0.001	1.000
14:0 iso	0.13	0.11	0.09	0.08	0.012	0.005	<0.001	0.944
14:1 <i>trans</i> -9	0.25	0.21	0.16	0.13	0.007	<0.001	<0.001	0.671
14:1 <i>cis</i> -9	1.32	1.18	1.02	0.83	0.085	<0.001	<0.001	0.407
15:0	1.02	0.84	0.77	0.64	0.077	<0.001	<0.001	0.158
15:0 anteiso	0.46	0.42	0.37	0.31	0.016	<0.001	<0.001	0.159
15:1 <i>trans</i> -5	0.034	0.038	0.041	0.034	0.0054	0.481	0.807	0.163
16:0	32.3	26.7	23.4	20.9	1.34	<0.001	<0.001	0.002
16:0 iso	0.30	0.29	0.26	0.26	0.022	0.069	0.016	0.470
16:1 <i>trans</i> -6+7	0.049	0.061	0.099	0.081	0.0028	<0.001	<0.001	0.001
16:1 <i>trans</i> -8	0.04	0.07	0.15	0.14	0.011	<0.001	<0.001	0.006
16:1 <i>trans</i> -9 ⁴	0.31	0.30	0.27	0.26	0.031	0.001	<0.001	0.645
16:1 <i>trans</i> -10	0.027	0.022	0.019	0.024	0.0061	0.543	0.480	0.257
16:1 <i>trans</i> -11	0.010	0.016	0.020	0.011	0.0049	0.020	0.156	0.005
16:1 <i>trans</i> -12	0.134	0.147	0.166	0.185	0.0168	0.022	0.004	0.768
16:1 <i>trans</i> -13	0.002	0.012	0.005	0.019	0.0033	<0.001	<0.001	0.068
16:1 <i>cis</i> -9 ⁵	2.1	1.8	1.7	1.4	0.15	<0.001	<0.001	0.765
16:1 <i>cis</i> -13	0.16	0.11	0.08	0.05	0.024	<0.001	<0.001	0.056
3,7,11,15 tetramethyl 16:0	0.052	0.063	0.056	0.041	0.002	<0.001	<0.001	<0.001
17:0	0.46	0.40	0.33	0.28	0.016	<0.001	<0.001	0.640
17:1 <i>cis</i> -7	0.17	0.14	0.11	0.09	0.010	<0.001	<0.001	0.582
18:0	7.8	9.0	9.3	9.4	0.65	0.052	0.017	0.135
18:1 <i>trans</i> total	3.4	5.4	7.9	9.6	0.14	<0.001	<0.001	0.119
18:1 <i>cis</i> total	16.5	21.5	25.2	28.8	1.01	<0.001	<0.001	0.095
Non-CLA 18:2 total ⁶	2.9	3.0	3.1	3.3	0.20	0.028	0.006	0.405
CLA total	0.77	1.29	1.60	1.84	0.095	<0.001	<0.001	0.006
18:3n-6	0.033	0.024	0.020	0.018	0.0020	0.004	<0.001	0.067
18:3n-3	0.225	0.215	0.165	0.189	0.0190	0.010	0.006	0.101
19:0 ⁷	0.042	0.051	0.057	0.058	0.0020	0.001	<0.001	0.050
20:0	0.103	0.105	0.106	0.098	0.0093	0.532	0.401	0.284
20:1 <i>cis</i> -9	0.075	0.076	0.072	0.065	0.0044	0.036	0.011	0.140
20:1 <i>cis</i> -11	0.027	0.035	0.053	0.085	0.0102	0.021	0.004	0.247
20:2n-6	0.040	0.038	0.033	0.022	0.0046	0.126	0.033	0.367
20:3n-6	0.139	0.115	0.106	0.088	0.0116	<0.001	<0.001	0.089
20:4n-6	0.136	0.110	0.100	0.078	0.0049	<0.001	<0.001	0.590
22:0	0.035	0.033	0.027	0.026	0.0043	0.009	0.002	0.414
22:4n-6	0.020	0.020	0.021	0.014	0.0018	0.042	0.157	0.143
22:5n-3	0.040	0.032	0.028	0.023	0.0041	0.023	0.004	0.662
24:0	0.020	0.017	0.005	0.002	0.0023	0.001	<0.001	1.000
Σ SFA	71.0	63.1	57.1	51.6	1.66	<0.001	<0.001	0.008
Σ SFA ≤ 14:0	27.2	24.1	21.5	18.8	1.05	<0.001	<0.001	0.738
Σ <i>trans</i> total	6.1	9.6	12.9	15.6	0.74	<0.001	<0.001	<0.001
Σ <i>trans</i> MUFA	4.3	6.2	8.7	10.5	0.22	<0.001	<0.001	0.691
Σ <i>cis</i> MUFA	19.9	24.4	26.7	30.8	0.91	<0.001	<0.001	0.073
Σ n-6 PUFA	3.1	3.0	3.1	3.2	0.20	0.048	0.033	0.050
Σ n-3 PUFA	0.40	0.41	0.42	0.38	0.013	0.001	0.054	<0.001
n-6:n-3	7.3	7.1	8.1	8.2	0.43	0.028	0.010	0.500

¹Where CS2, CS4, and CS6 are diets containing 20, 40, and 60 g/kg of DM Ca-MUFA, respectively.²SEM for n = 16 measurements.³Refers to the significance of overall effect of diet or linear (LIN) and quadratic (QUAD) effects of increasing supplement inclusion.⁴Co-elutes with 17:0 iso.⁵Co-elutes with 17:0 anteiso.⁶CLA = conjugated linoleic acid. All 18:2 isomers excluding CLA.⁷Co-elutes with *cis*-15 18:1.

concentrations (Table 3). Effects of Ca-MUFA supplementation on some of the longer chain FA present in milk at low concentrations were also noted. Overall, increasing inclusion of Ca-MUFA caused a linear and quadratic reduction ($P < 0.001$) in total SFA, and a linear reduction ($P < 0.001$) in SFA <14:0 (Table 3). Total *trans*- and *cis*-MUFA linearly increased ($P < 0.001$) with increasing Ca-MUFA supplementation. Overall, a slight increase ($P = 0.033$) in total n-6 PUFA and a decrease ($P = 0.001$) in total n-3 PUFA was observed, so that the n-6:n-3 ratio increased linearly ($P < 0.05$) with increasing Ca-MUFA inclusion level.

Increasing the inclusion level of Ca-MUFA in the dairy cow diet resulted in linear increases ($P < 0.01$) in all 18:1 *trans* isomers measured, and also 18:1 *cis*-9, 18:1 *cis*-11, and 18:1 *cis*-13 ($P < 0.001$; Table 4). Quadratic responses ($P < 0.05$) were also noted for *trans*-5 18:1, *trans*-9 18:1, *trans*-10 18:1, *trans*-12 18:1, *trans*-13/14 18:1, and *trans*-16/*cis*-14 18:1 (the latter isomers co-eluted). This resulted in an overall linear enhancements ($P < 0.001$) in total *trans*-18:1 and total *cis*-18:1 (Table 3). Supplementation with Ca-MUFA caused variable responses in 18:2 isomers (Table 5). Linear increases ($P < 0.01$) were observed in *trans*-9, *cis*-12 18:2, and *trans*-11,*cis*-15 18:2, and a linear and quadratic enhancement in *cis*-9,*trans*-12 18:2 and *cis*-9,*cis*-12 18:2 concentrations with increasing supplement inclusion level. However, increasing Ca-MUFA inclusion caused a linear and quadratic reduction ($P < 0.01$) in *trans*-11,*trans*-15 and *cis*-10,*trans*-14 18:2.

With regard to CLA isomer composition, linear and in some cases quadratic increases ($P < 0.01$) were noted in

the concentrations of several isomers (*trans*-11,*trans*-13 CLA; *trans*-10,*trans*-12 CLA; *trans*-9,*trans*-11 CLA; *trans*-8,*trans*-10 CLA; *trans*-7,*trans*-9 CLA; *trans*-10,*cis*-12 CLA; *trans*-8,*cis*-10 CLA; *trans*-7,*cis*-9 CLA; and *cis*-9,*trans*-11 CLA; Table 5).

DISCUSSION

Feeding *cis*-MUFA-rich lipid sources to dairy cows is an effective strategy to reduce milk fat SFA and increase *cis*-MUFA concentrations. However, this is also commonly associated with increases in *trans* fatty acid (TFA) concentrations via rumen biohydrogenation of *cis*-MUFA and PUFA present in the oil (Chilliard et al., 2007). One of the issues associated with creating calcium salts of unsaturated FA (particularly highly unsaturated FA) to protect against this rumen metabolism is apparent dissociation. In vitro studies have reported that the greater the unsaturation of a calcium salt, the greater the risk of dissociation. Sukhija and Palmquist (1990) found that across a range of pH values, calcium salts of soybean oil (containing proportionately less *cis*-9 18:1 and more 18:2 n-6) dissociates more readily than calcium salts of oils containing more *cis*-9 18:1 and less PUFA. A later study, by Van Nevel and Demeyer (1996), reported that between pH of 6.9 and 6.3, calcium salts of PUFA are partially protected against biohydrogenation; but protection varies with degree of unsaturation, with 18:2 n-6 calcium salts protected to a greater extent than 18:3 n-3 calcium salts. These studies suggest that calcium salts of predominantly *cis*-9 18:1 are probably better protected than calcium salts of

Table 4. Effect of calcium salts of *cis*-monounsaturated fatty acids (Ca-MUFA) supplementation on milk fat 18:1 isomer composition (LSM results as g/100 g of FA)

FA	Treatment ¹					P-value ³		
	Control	CS2	CS4	CS6	SEM ²	Diet	LIN	QUAD
<i>trans</i> -4 18:1	0.013	0.037	0.073	0.116	0.0145	<0.001	<0.001	0.073
<i>trans</i> -5 18:1	0.000	0.010	0.085	0.110	0.0085	<0.001	<0.001	<0.001
<i>trans</i> -6-8 18:1	0.23	0.58	1.01	1.33	0.078	<0.001	<0.001	0.197
<i>trans</i> -9 18:1	0.22	0.52	0.78	0.96	0.060	<0.001	<0.001	0.006
<i>trans</i> -10 18:1	0.37	0.76	0.88	1.09	0.075	<0.001	<0.001	0.033
<i>trans</i> -11 18:1	1.1	2.1	3.1	4.0	0.33	<0.001	<0.001	0.277
<i>trans</i> -12 18:1	0.44	0.74	1.03	1.26	0.082	<0.001	<0.001	0.010
<i>trans</i> -13-14 18:1	1.2	1.2	1.5	1.6	0.073	0.019	0.004	0.515
<i>trans</i> -16 18:1/ <i>cis</i> -14 18:1	0.24	0.29	0.31	0.30	0.028	0.009	0.004	0.022
<i>cis</i> -9 18:1	15.4	20.2	23.8	27.2	0.94	<0.001	<0.001	0.109
<i>cis</i> -11 18:1	0.49	0.58	0.68	0.76	0.041	<0.001	<0.001	0.481
<i>cis</i> -12 18:1	0.36	0.36	0.34	0.33	0.035	0.134	0.036	0.900
<i>cis</i> -13 18:1	0.076	0.123	0.157	0.189	0.0078	<0.001	<0.001	0.090
<i>cis</i> -15 18:1	0.042	0.051	0.057	0.058	0.0020	0.014	<0.001	0.050
<i>cis</i> -16 18:1	0.047	0.060	0.067	0.052	0.0028	0.008	0.096	0.002

¹Where CS2, CS4, and CS6 are diets containing 20, 40, and 60 g/kg of DM Ca-MUFA, respectively.

²SEM for n = 16 measurements.

³Refers to the significance of overall effect of diet or linear (LIN) and quadratic (QUAD) effects of increasing supplement inclusion.

Table 5. Effect of calcium salts of *cis*-monounsaturated fatty acids (Ca-MUFA) supplementation on milk fat 18:2 isomer composition (LSM results as mg/100 g of FA)

FA	Treatment ¹					P-value ³		
	Control	CS2	CS4	CS6	SEM ²	Diet	LIN	QUAD
<i>trans</i> -11, <i>trans</i> -15 18:2	88.9	94.3	91.9	64.7	10.16	0.005	0.005	0.006
<i>cis</i> -9, <i>trans</i> -13 18:2	145	182	175	164	11.0	0.053	0.184	0.018
<i>trans</i> -9, <i>cis</i> -12 18:2	8.6	22.7	37.5	39.4	6.09	0.010	0.002	0.237
<i>trans</i> -11, <i>cis</i> -15 18:2	29.6	35.7	42.8	46.8	4.64	<0.001	<0.001	0.481
<i>cis</i> -9, <i>trans</i> -12 18:2	30.9	49.5	60.3	67.3	1.57	<0.001	<0.001	0.006
<i>cis</i> -10, <i>trans</i> -14 18:2	99.9	110.1	90.1	80.1	0.80	<0.001	<0.001	<0.001
<i>cis</i> -9, <i>cis</i> -12 18:2	2,485	2,426	2,535	2,705	200.7	0.007	0.003	0.018
<i>trans</i> -12, <i>trans</i> -14 CLA ⁴	2.2	3.8	3.2	4.9	0.75	0.285	0.120	0.842
<i>trans</i> -11, <i>trans</i> -13 CLA	5.5	10.8	14.0	13.5	0.96	0.001	<0.001	0.013
<i>trans</i> -10, <i>trans</i> -12 CLA	4.7	10.7	15.7	15.2	2.00	0.020	0.005	0.138
<i>trans</i> -9, <i>trans</i> -11 CLA	14.9	20.7	24.6	27.8	1.24	<0.001	<0.001	<0.001
<i>trans</i> -8, <i>trans</i> -10 CLA	7.5	12.2	19.2	22.0	1.78	0.003	<0.001	0.593
<i>trans</i> -7, <i>trans</i> -9 CLA	3.5	6.3	8.9	10.4	0.85	0.006	<0.001	0.431
<i>trans</i> -12, <i>cis</i> -14 CLA	1.5	2.4	1.4	1.8	0.77	0.708	0.970	0.710
<i>trans</i> -11, <i>cis</i> -13 CLA	4.0	5.7	8.7	9.3	1.21	0.095	0.021	0.668
<i>trans</i> -10, <i>cis</i> -12 CLA	2.5	7.3	8.8	9.9	0.76	<0.001	<0.001	0.027
<i>trans</i> -9, <i>cis</i> -11 CLA	12.9	18.8	20.2	26.6	4.44	0.109	0.310	0.025
<i>trans</i> -8, <i>cis</i> -10 CLA	6.6	15.0	16.7	21.0	3.17	0.025	0.005	0.410
<i>trans</i> -7, <i>cis</i> -9 CLA	56.7	139.0	209.2	235.2	9.18	<0.001	<0.001	0.007
<i>cis</i> -12, <i>trans</i> -14 CLA	1.2	1.6	2.0	3.7	1.08	0.408	0.140	0.532
<i>cis</i> -11, <i>trans</i> -13 CLA	1.4	5.8	6.8	1.7	2.82	0.528	0.882	0.172
<i>cis</i> -9, <i>trans</i> -11 CLA	662	1,179	1,585	1,866	135.7	<0.001	<0.001	0.163
<i>cis</i> -11, <i>cis</i> -13 CLA	2.6	1.6	2.0	1.2	0.79	0.484	0.218	0.871
<i>cis</i> -10, <i>cis</i> -12 CLA	1.3	3.1	2.5	3.2	1.74	0.610	0.330	0.635
<i>cis</i> -9, <i>cis</i> -11 CLA	1.9	3.7	2.2	2.3	0.92	0.243	0.947	0.193

¹Where CS2, CS4, and CS6 are diets containing 20, 40, and 60 g/kg DM calcium salt, respectively.²SEM for n = 16 measurements.³Refers to the significance of overall effect of diet or linear (LIN) and quadratic (QUAD) effects of increasing supplement inclusion.⁴CLA = conjugated linoleic acid.

PUFA. Apart from degree of unsaturation, particle size is thought to affect dissociation of calcium salt supplements. Block et al. (2008) reported a higher rate of increase in rumen concentration of 18:0 when smaller particles of a calcium salt supplement were incubated in vitro compared with larger particles of the same supplement.

The FA profile of the Ca-MUFA supplement in the present study was different to that of rapeseed oil (Glasser et al., 2008), with a higher *cis*-9 18:1 content, around half the 18:2 n-6 content, and much less 18:3 n-3. In fact, the Ca-MUFA profile was similar to that of high oleic acid (low PUFA) rapeseed types, which have been used in dairy cow diets to try to reduce milk fat PUFA concentrations and thereby address product shelf-life issues (Jenkins, 1998; Looor et al., 2002). It was anticipated that the high oleic acid content of the Ca-MUFA, when rumen-protected, would increase transfer of *cis*-9 18:1 from the diet to milk fat by minimizing dissociation and exposure to rumen microorganisms.

Increasing inclusion of Ca-MUFA resulted in a linear reduction in DMI. Lin et al. (1996) reported a lack of DMI response when feeding incremental amounts (25 and 49 g/kg of DM) of calcium salts of a high oleic acid

sunflower oil, and Chouinard et al. (1998) reported no significant differences in DMI between a control diet and diets containing calcium salts of different lipid sources (40 g/kg of DM). Incremental infusion of high oleic acid sunflower oil into the abomasum of dairy cows, thus bypassing the effects of the rumen, was reported to decrease DMI (Drackley et al., 2007), with the authors concluding that monounsaturated free FA decrease DMI in a dose-dependent manner. This, together with the fact that in the present study increasing Ca-MUFA supplementation had no effect on ME intake, suggests that the DMI was suppressed due to both increasing energy density of the diet and also a greater proportion of unsaturated FA leaving the rumen.

Supplementation with Ca-MUFA had no effect on milk yield, which may reflect the consistent calculated ME intake across inclusion levels or stage of lactation. A similar result was reported by Lin et al. (1996) and Chouinard et al. (1998) following supplementation with calcium salts of oleic acid-rich oils. In the current study, energy density of the diet was increased by including Ca-MUFA at the expense of the other nutrients required for milk production, which may have masked any increases in production. Furthermore, the use of

mid- to late-lactation cows may also explain the lack of a response after Kirkland and Gordon (2001) demonstrated that cows in later lactation (411–428 DIM) partitioned less of their dietary increase in ME intake toward milk energy output than cows in early lactation.

No effect of diet on milk fat, protein, and lactose yield was observed, although milk fat and protein concentrations were reduced, and the responses were less at the higher inclusion levels. Studies have shown that including unsaturated oil supplements in dairy cow diets can suppress both fat and protein concentration in milk (Kliem, 2010), partially due to the negative effects of unsaturated oils on the rumen environment, and also due to increases in milk yield with no accompanying change in fat and protein production. In the present study, no change in milk yield was noted with increasing supplement inclusion level, which may explain the lack of effect on milk component yield. Milk fat suppression is also related to increased rumen outflow of specific unsaturated FA, which can inhibit milk fat synthesis (Chilliard et al., 2007). However duodenal infusion of oleic acid has been shown to have little effect on milk fat concentration (Enjalbert et al., 1997). In the current study, the increase in *trans*-10, *cis*-12 CLA reaching the mammary gland (reflected in increased milk concentration) may have had some effect on milk fat synthesis. In addition, the supplement diluted other dietary ingredients, leading to a reduction in fermentable energy and CP intake, which may have been the cause of lower milk protein concentration.

The effect of Ca-MUFA supplementation on short- and medium-chain SFA concentration in milk fat may be in part due to a concentration effect, reflecting the increase in concentration observed in longer chain unsaturated FA. However, a more likely explanation is the inhibition of mammary de novo synthesis by long-chain unsaturated FA which have either escaped rumen biohydrogenation or are intermediates of this process. Indeed, when taking total daily milk fat yield into account, we observed linear ($P < 0.001$) decreases in the daily output of the sum of de novo-synthesized FA 4:0 to 14:0 (300 and 190 g/d for control and CS6, respectively) and 16:0 (237 and 165 g/d for control and CS6, respectively). Increasing the amount of long chain (≥ 18 -carbon) unsaturated FA in the diet has been found to inhibit de novo synthesis (Barber et al., 1997; Chilliard et al., 2000). Studies have shown that calcium salts of unsaturated FA do reduce milk fat concentrations of SFA synthesized de novo (Chouinard et al., 1998; Harvatine and Allen, 2006). As well as short- and medium-chain SFA, calcium salt supplements also reduced the milk fat concentration of odd- and branched-chain SFA (such as 13:0 anteiso, 15:0); again, this may have been a concentration effect. However, most of these FA in

milk are known to be mainly synthesized de novo by rumen bacteria (Vlaeminck et al., 2006), suggesting that Ca-MUFA inclusion may have had some effect on the rumen environment.

The concentration of 16:0 was reduced by almost 12 g/100 g of FA. Mammary de novo synthesis accounts for about 50% of 16:0 in milk fat (Hawke and Taylor, 1995) with the remainder being supplied by the circulation. The Ca-MUFA supplement contained around 11 g/100 g of FA 16:0, and intake of this FA did increase. Therefore, the reduction in milk fat 16:0 concentration is probably related to inhibition of de novo synthesis, which was reflected in the linear reduction in daily output already mentioned. In contrast, milk fat 18:0 concentration was enhanced with increasing inclusion of Ca-MUFA supplement. Concentration of 18:0 in milk fat is usually enhanced following supplementation of dairy cow diets with 18-carbon PUFA-rich oilseed supplements (Givens and Shingfield, 2006), complete rumen biohydrogenation of which leads to increased availability of 18:0 for absorption by the small intestine. In addition, a high proportion of unprotected *cis*-9 18:1 is metabolized to 18:0 in the rumen (Harfoot and Hazlewood, 1997; Jenkins et al., 2008). In the current study, increased concentration of 18:0 in milk may have been due to increased intake, or perhaps *cis*-9 18:1 and 18:2 n-6 within the supplement were subjected to limited rumen biohydrogenation, but the increase was small when compared with earlier studies and no overall diet effect on milk output (g/d) of 18:0 ($P = 0.104$) was noted. A greater increase in milk fat 18:0 concentration was observed by Chouinard et al. (1998) after supplementing cow diets with calcium salts of rapeseed oil. A study comparing supplementary soybean oil with a calcium salt of soybean oil reported no difference in omasal flow of 18:0 (Lundy et al., 2004); however, both these previous studies suggest rumen protection was not complete for the calcium salt supplements used. Despite marginal increases in 18:0 concentration, Ca-MUFA supplementation reduced total SFA by almost 20 g/100 g of FA at the highest inclusion level (60 g/kg of DM). Supplementation with 33 g/kg of DM calcium salts of unsaturated FA reduced total (6:0–20:0) SFA from 62 to 54 g/100 g of FA (Harvatine and Allen, 2006).

The reduction in total SFA concentration with increasing Ca-MUFA inclusion was balanced by an enhancement in concentration of total *cis*- and *trans*-18:1, total CLA, and total nonconjugated 18:2 in the present study. The proportion of *cis*-9 18:1 in milk fat increased with increasing inclusion of Ca-MUFA supplement. This FA is derived from dietary sources, but also from desaturation of 18:0 by mammary Δ^9 -desaturase. One in vivo study estimated that Δ^9 -desaturase was respon-

sible for 59% *cis*-9 18:1 present in milk fat (Mosley and McGuire, 2007). Supplementation with Ca-MUFA also increased the concentration of minor *cis*-18:1 isomers (*cis*-11 18:1 and *cis*-13 18:1), which are probably biohydrogenation intermediates of dietary PUFA. Concentration of *cis*-13 18:1 was enhanced during in vitro incubation of 18:2 n-6 with rumen bacteria (Jouany et al., 2007), and rumen fluid concentration of *cis*-11 18:1 and *cis*-13 18:1 was higher in cows supplemented with canola or soybean oil than in cows fed a control diet (Loor et al., 2002).

Supplement inclusion increased all *trans*-18:1 isomers measured, which are intermediates of rumen biohydrogenation of PUFA and MUFA. In vitro and in vivo studies have established that *trans*-11 18:1, usually the most abundant *trans*-18:1 in milk fat, is a common intermediate of 18:2 n-6 and 18:3 n-3 metabolism in the rumen (Harfoot and Hazlewood, 1997; Palmquist et al., 2005). In vitro studies have also demonstrated that *cis*-9 18:1 is converted to a range of *trans*-18:1 isomers during incubation with rumen-derived microorganisms including *trans*-7, *trans*-9, *trans*-10, and *trans*-12 18:1 (Mosley et al., 2002; McKain et al., 2010). Almost all *trans*-9 18:1 produced during 24 h of in vitro incubation derived from *cis*-9 18:1 (Mosley et al., 2002). In addition, *trans*-9 18:1 has been shown to further isomerize during in vitro incubation with rumen bacteria to a range of positional *trans* 18:1 isomers (Proell et al., 2002). Furthermore, it is thought that altered rumen fermentation with certain diets results in a shift in biohydrogenation pathway for 18:2 n-6, leading to accumulation of *trans*-10 18:1 (Bauman and Grinari, 2001). The intermediate in this pathway (*trans*-10, *cis*-12 CLA) is thought to be involved in milk fat depression, and abomasal infusion of this isomer has resulted in significant reductions in milk fat concentration (Baumgard et al., 2000; Lock et al., 2007). An increased concentration was observed in this isomer together with several other CLA isomers, such as *trans*-7, *cis*-9 and *cis*-9, *trans*-11 CLA. The increases in these latter isomers suggest increased availability of *trans*-7 18:1 and *trans*-11 18:1 at the mammary gland, and subsequent mammary desaturation (Piperova et al., 2002). In vitro incubation of 18:2 n-6 has also been found to enhance concentrations of *trans*-8, *trans*-10 CLA and *trans*-9, *trans*-11 CLA (Jouany et al., 2007). These CLA isomers were enhanced in milk in the current study with increasing supplement inclusion (consistent with overall dietary 18:2 n-6 content), providing further evidence of some rumen exposure to the supplement FA.

The increase in nonconjugated 18:2 isomers with Ca-MUFA inclusion also reflects increased rumen exposure to 18:2 n-6. Concentrations of *trans*-9, *cis*-12 18:2 and *cis*-9, *trans*-12 18:2 were found to be greater during in

vitro incubation of 18:2 n-6 with rumen fluid (Jouany et al., 2007), and studies have shown supplementing dairy cows with oils rich in 18:2 n-6 enhances rumen *cis*-9, *trans*-12 concentration (Loor et al., 2002).

Replacing SFA with *cis*-MUFA in milk fat offers a successful means of improving the health quality of milk. A modeling exercise demonstrated that if milk fat SFA and *cis*-MUFA content was altered from 70 and 20 g/100 g of FA to 55 and 32 g/100 g of FA, respectively, this would result in a mean reduction in risk of coronary heart disease event in 11 EU member states by almost 2% (UK reduction in risk of 1.67%; Givens, 2008). Considering the cost of CVD in the EU through direct and indirect healthcare costs, a reduction in risk of this magnitude is substantial. However, this model did not consider the negative effects of any TFA that may be enhanced in milk fat following manipulation of milk FA profile. At current intake levels, ruminant-derived TFA are thought not to have negative effects on human health (Mozaffarian et al., 2006), and as such, are not included in public health drives to reduce *trans* fat intake (Faculty of Public Health, 2010). In addition, the different isomer profile of ruminant-derived compared with industrial TFA may result in differing responses in certain CVD risk factors (Motard-Bélanger et al., 2008). However, food labeling for TFA is now mandatory in the United States, Denmark, and Canada, with calls for compulsory labeling in the United Kingdom (Clarke and Lewington, 2006). Until such labels distinguish between the 2 sources of TFA, efforts should be made to keep any increase in concentration in milk and dairy products to a minimum. This could include rumen protection of dietary unsaturated FA, so research should be conducted into further development of such products.

CONCLUSIONS

The main objectives of this study were to measure the effect of feeding increasing amounts of a high-C18:1 calcium salt supplement on feed intake, milk yield or composition, and FA composition. Overall, the Ca-MUFA preparation used to protect unsaturated FA from rumen metabolism was not entirely successful, but the product was very effective at reducing the SFA (by 27%) and increasing *cis*-MUFA (by 55%) concentrations of milk fat. No effect on milk yield was noted, but fat and protein concentrations were both reduced, which may suggest inhibition of fat synthesis and some modification of the rumen environment, or reflect the slight changes in milk yield observed. Fat reduction (and decreased concentration of de novo-synthesized SFA) may also have been partially due to unsaturated FA from the Ca-MUFA being protected from rumen

biohydrogenation. But evidence of biohydrogenation of unsaturated FA also exists, in that milk 18:0, *trans*-18:1, CLA, and nonconjugated 18:2 were all enriched in milk fat from cows fed diets supplemented with increasing amounts of Ca-MUFA. This indicates that some of the unsaturated FA in the Ca-MUFA supplement were exposed to rumen microorganisms.

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