Effect of incremental levels of sunflower-seed oil in the diet on ruminal lipid metabolism in lactating cows

K. J. Shingfield[1]*, S. Ahvenjärvi[1], V. Toivonen[1], A. Vanhatalo[1,2], P. Huhtanen[1] and J. M. Griinari[2]

[1]*Animal Production Research, MTT Agrifood Research Finland, FIN-31600, Jokioinen, Finland*
[2]*Department of Animal Science, PO Box 28, FIN-00014, University of Helsinki, Helsinki, Finland*

Based on the potential benefits of *cis*-9,*trans*-11-conjugated linoleic acid (CLA) for human health there is interest in developing sustainable nutritional strategies for enhancing the concentration of this fatty acid in ruminant-derived foods. Most evidence to date suggests that endogenous synthesis is the major source of *cis*-9,*trans*-11 in milk fat and ruminal outflow is limited and largely independent of dietary 18 : 2*n*-6 supply. Four lactating cows fitted with a rumen cannula were used in a 4 × 4 Latin square with 14 d experimental periods to examine the effects of sunflower-seed oil (SFO) as a source of 18 : 2*n*-6 on ruminal lipid metabolism. Cows were offered grass silage-based diets supplemented with 0, 250, 500 or 750 g SFO/d. Supplements of SFO had no effect on DM intake, milk fat or protein secretion, but increased linearly ($P < 0.01$) milk yield and milk lactose output and shifted ($P < 0.001$) rumen fermentation towards propionate at the expense of acetate. SFO supplements increased linearly ($P < 0.05$) the flow of 18 : 0, 18 : 1, 18 : 2*n*-6 and total CLA at the omasum and enhanced ruminal *cis*-9-18 : 1, 18 : 2*n*-6 and 18 : 3*n*-3 metabolism. Flows of all-*trans*- (Δ4–16) and *cis*- (Δ9–16) 18 : 1 isomers were elevated, while increases in ruminal CLA outflow were confined to *trans*-8,*trans*-10 and geometric 9,11 and 10,12 isomers. It is concluded that supplementing grass silage-based diets with plant oils rich in 18 : 2*n*-6 enhances ruminal outflow of *trans*-11-18 : 1 and *cis*-9,*trans*-11-CLA in lactating cows.

Sunflower-seed oil: Biohydrogenation: *Trans*-fatty acids: Conjugated linoleic acid: Lactating cows

Conjugated linoleic acid (CLA) is a generic term used to describe one or a mixture of positional and geometric isomers of 18 : 2 fatty acids containing a conjugated double bond. Ruminant fats are the major source of CLA in the human diet[1,2] and a number of experiments with various human cell lines and animal models have provided a body of evidence to suggest that *cis*-9,*trans*-11-CLA, the major isomer in ruminant-derived foods[3], exhibits anti-mutagenic actvity[4,5]. In view of the potential benefits to long-term human health there is considerable interest in developing nutritional strategies to enhance the *cis*-9,*trans*-11-CLA content of ruminant milk and meat.

Cis-9,*trans*-11-CLA is formed in the rumen during metabolism of 18 : 2*n*-6[6] or synthesised endogenously in ruminant tissues from *trans*-11-18 : 1 via stearoyl-CoA desaturase in ruminant tissues[3,7,8]. Studies in lactating cows have shown that increases in milk fat *cis*-9,*trans*-11-CLA content to abomasal infusions of a mixture of fatty acids containing *trans*-11-18 : 1 are equivalent to proportionately 0·21 of the response to *cis*-9,*trans*-11-CLA infusions[9]. These findings indicate that enhancing the formation and accumulation of *cis*-9, *trans*-11-CLA in the rumen represents the biologically most efficient means to increase the concentration of this fatty acid in ruminant foods.

Incubation of 18 : 2*n*-6 with pure strains or mixed cultures of rumen bacteria causes *cis*-9,*trans*-11-CLA to accumulate *in vitro*[10,11]. Detailed studies of ruminal *cis*-9,*trans*-11-CLA formation and outflow *in vivo* are limited. Measurements of fatty acids at the duodenum in ruminants have reported relatively minor changes in the flow of *cis*-9,*trans*-11-CLA to diets containing plant oils rich in 18 : 2*n*-6[12-14]. However, most of these studies have been made in animals offered high-concentrate diets, and there is evidence that the amount of *cis*-9,*trans*-11-CLA leaving the rumen in response to plant oils rich in 18 : 2*n*-6 is greater on diets containing higher proportions of forage[15,16]. Overall, the available evidence suggests that increases in 18 : 2*n*-6 intake on high-forage diets could be used as a nutritional strategy for enhancing the supply of *cis*-9,*trans*-11-CLA available for absorption. To evaluate this hypothesis, ruminal lipid metabolism and the flow of NEFA at the omasum was determined in lactating cows fed a grass silage-based diet (forage:concentrate ratio 60:40, on a DM basis) supplemented with incremental levels of sunflower-seed oil (SFO) rich in 18 : 2*n*-6.

Materials and methods

Animals and experimental design

All experimental procedures were approved by the Animal Experiment Committee of MTT Agrifood Research Finland

in accordance with the 1985 Use of Vertebrates for Scientific Purposes Act 1985. Four rumen-fistulated multiparous Finnish Ayrshire cows 166 (SE 16·2) d postpartum of mean live weight 626 (SE 65·2) kg were used to evalute the effects of supplementing the diet with 0, 250, 500 or 750 g SFO/d on ruminal lipid metabolism according to a 4 × 4 Latin square with 14 d experimental periods. Cows were housed in a metabolism unit in individual stalls with continuous access to water and milked *in situ* at 07.00 and 16.45 hours.

Experimental diets

Cows were offered grass silage supplemented with a cereal-based concentrate (proportionately 0·60 and 0·40 on a DM basis, respectively) at 95 % of *ad libitum* intake measured over a 14 d interval immediately before the start of the experiment. Silage was produced from secondary growths of mixed timothy (*Phleum pratense*) and meadow fescue (*Festuca pratensis*) swards. Grass used to prepare silage was cut and wilted for 6 h before being harvested with a precision-chop forage harvester and treated with a formic acid-based additive (760 g formic acid/kg and 55 g ammonium formate/kg; Kemira Agro Ltd, Helsinki, Finland) applied at a rate of 4·4 litres/t grass.

Concentrate supplements were formulated (g/kg) from rolled barley (522), molassed sugarbeet pulp (250), solvent-extracted rapeseed meal (200) of low glucosinolate content (Raisio Feeds Limited, Raisio, Finland) and a proprietary general-purpose mineral and vitamin supplement (28; Suomen Rehu Limited, Helsinki, Finland). Both the basal diet and SFO were offered as two equal meals at 06.00 and 18.00 hours. Supplements of SFO were mixed with concentrate ingredients just before feeding.

Measurements and sampling

Individual cow intakes were recorded daily, but only measurements collected during the last 5 d of each experimental period were used for statistical analysis. During this period, representative samples of fresh silage and concentrate were composited daily and stored at −20°C. Chemical composition of silage and concentrates was determined using standard procedures[17], while the concentration of indigestible neutral-detergent fibre (iNDF) was determined using three cows over 288 h ruminal incubations[18]. Samples of rumen fluid (*n* 8) were collected on the last day of each period from each cow at 1·5 h intervals starting at 06.00 hours. Following removal, pH was measured and samples were filtered through two layers of cheesecloth. Samples of rumen fluid were stored at −20°C until analysed for volatile fatty acid (VFA) and ammonia-N determinations using standard methods[19].

Digesta flow was assessed using LiCoEDTA, Yb-acetate and Cr-mordanted straw as indigestible markers for liquid, and small and large particles, respectively. Coarsely chopped barley straw was soaked in tap water overnight, rinsed with neutral detergent and labelled with Cr[20]. Cr-mordanted straw containing 79·0 (SE 0·09) mg Cr/g DM was administered (40 g/d) twice daily on top of the rumen mat via the cannula at 12 h intervals starting at 18.00 hours on day 7 of each experimental period. LiCoEDTA was prepared using standard procedures[20] while Yb-acetate was obtained from a commercial source (Dasico A/S, Birkerød, Denmark). LiCoEDTA (12 g) and Yb-acetate (4 g) were dissolved in 6 litres distilled water and infused via separate lines

at 18.00 hours on day 8 into the rumen at a constant rate (4·2 ml/min). Ruminal infusions were made using polyamide tubing (internal diameter 4 mm) that passed through the rumen fistula and a peristaltic pump (Watson-Marlow, High Wycombe, Bucks, UK). Markers were administered to each animal to provide daily doses of 2·9, 1·6 and 1·5 g/d of Cr, Co and Yb, respectively. At the start of marker administration cows received priming doses of CoEDTA, Yb-A and CrEDTA supplying 3·6, 2·5 and 2·2 g Cr, Co and Yb, respectively, to facilitate rapid equilibration of the marker concentrations in the rumen.

Spot samples (500 ml) of digesta entering the omasal canal were collected three times daily at 4 h intervals on day 11 through to day 14 using the omasal sampling device[21] with modifications[22]. Sampling started at 06.00 hours and was advanced 1 h each day to cover a 12 h period that was considered representative of the entire feeding cycle. On each occasion, samples were stored at −20°C immediately after collection. At the end of the study, digesta was thawed at room temperature, composited and separated into large-particle, small-particle and liquid fractions by filtration and centrifugation[22]. Each phase was freeze-dried and stored at −20°C, while subsamples for lipid determinations were stored at −80°C. Each digesta phase was submitted for the determination of DM, organic matter (OM), N and marker concentrations, while neutral-detergent fibre (NDF) and iNDF were measured in the large-particle and small-particle fractions, and VFA and ammonia-N concentrations were assessed in the fluid phase[22]. Based on marker concentrations, the relative proportions of the fluid phase, small-particle and large-particle fractions in true digesta were calculated using the digesta reconstitution technique[23]. Thereafter, appropriate amounts of freeze-dried digesta fractions previously stored at −80°C were weighed to provide a 20 g composite sample and submitted for fatty acid determination.

Whole-tract apparent digestibility coefficients were determined by total faecal collection. Faeces were collected over 96 h starting at 18.00 hours on day 11 of each experimental period. Total faeces excreted were weighed, thoroughly mixed, subsampled (5 %, w/w) and stored at −20°C before chemical analysis. Urine was separated from faeces by means of a light harness and flexible tubing attached to the vulva. Faecal DM, OM, NDF, iNDF and N content was determined using the same methods applied to feeds, and marker concentrations were determined as for omasal digesta.

Milk yields of individual cows were recorded daily, while only measurements collected during the last 5 d of each experimental period were analysed statistically. Samples of milk for the measurement of fat, crude protein and lactose were collected from each cow over four consecutive milkings starting at 16.45 hours on day 12 of each experimental period and treated with Bronopol preservative (Valio Limited, Helsinki, Finland). Milk fat, crude protein and lactose were determined by near-IR spectroscopy (Milko-Scan 133B analyser; Foss Electric, Hillerød, Denmark) calibrated using milk samples for which reference measurements had previously been made.

Lipid analysis

Fatty acid methyl esters (FAME) of lipid in SFO and freeze-dried samples of silage and concentrate were prepared in a one-step extraction–transesterification procedure using chloroform[24]

and 2 % (v/v) sulfuric acid in methanol[25]. Feed fatty acid content was determined using tritridecanoin (T-135; Nu-Chek-Prep, Elysian, MN, USA) as an internal standard. Following the addition of internal standard (13 : 0, N-13A; Nu-Chek-Prep), the pH of omasal digesta was adjusted to 2·0 using 2 M-hydrochloric acid. Lipid in omasal digesta was extracted using a mixture (3:2, v/v) of hexane and isopropanol and NEFA were separated from other lipid classes by solid-phase extraction and transesterified to methyl esters by incubation with 1 % (v/v) sulfuric acid in methanol at 50°C for 30 min[25].

Methyl esters were quantified by GLC using a gas chromatograph (6890; Hewlett-Packard, Wilmington, DE, USA) equipped with a flame-ionisation detector, selective quadrupole mass detector (model 5973N; Agilent Technologies Inc., Wilmington, DE, USA) and 100 m fused silica capillary column (CP-SIL 88, Chrompack 7489; Chrompack, Middelburg, The Netherlands) using H_2 as the carrier and fuel gas. Total FAME profile in a 2 μl sample at a split ratio of 1:50 was determined using a temperature gradient program[25]. Isomers of 18 : 1 and 18 : 2 methyl esters were further resolved in a separate analysis under isothermal conditions at 170°C[25]. Peaks were routinely identified using authentic standards (GLC-463 and GLC-606; Nu-Check-Prep). Identification was verified by GC–MS analysis of FAME and 4,4-dimethyloxazoline (DMOX) fatty acid derivatives under an ionisation voltage of 400 eV, using He as the carrier gas and the same temperature gradient used for routine analysis of FAME. Preparation of DMOX fatty acid derivatives from FAME of selected digesta samples (n 4) was in accordance with procedures outlined elsewhere[26]. Electron impact ionisation spectra of DMOX derivatives was used to locate double bonds based on atomic mass unit distances, with an interval of 12 atomic mass units between the most intense peaks of clusters of ions containing n and n-1 carbon atoms being interpreted as cleavage of the double bond between carbon n and $n + 1$ in the fatty acid moiety. Identification was further validated by comparison with an online reference library of DMOX fatty acid derivative electron impact ionisation spectra (http://www.lipidlibrary.co.uk/ms/arch_dm/index.htm). Geometric configuration of polyunsaturated methyl ester double bonds was elucidated based on the elution order of authentic methyl ester standards containing geometric isomers of 9,12–18 : 2 and 9,12,15–18 : 3 (L-8404 and L-6301; Sigma-Aldrich, Helsinki, Finland). A partial gas chromatogram indicating the separation of 18 : 2 methyl esters under isothermal conditions is shown in Fig. 1.

The distribution of CLA isomers in omasal digesta was determined by HPLC using four silver-impregnated silica columns (ChromSpher 5 lipids, 250 × 4·6 mm, 5 μm particle size; Varian Ltd, Walton-on-Thames, Surrey, UK) coupled in series and 0·1 % (v/v) acetonitrile in heptane as the mobile phase[25]. Isomers were identified using an authentic CLA methyl ester standard (O-5632; Sigma-Aldrich) and chemically synthesised *trans*-9,*cis*-11-CLA[27]. Identification was verified by cross-referencing with the elution order reported in the literature[28] using *cis*-9,*trans*-11-CLA as a landmark isomer.

Statistical analysis

Intake, milk production, omasal flow and digestibility data were subjected to ANOVA using the mixed linear model
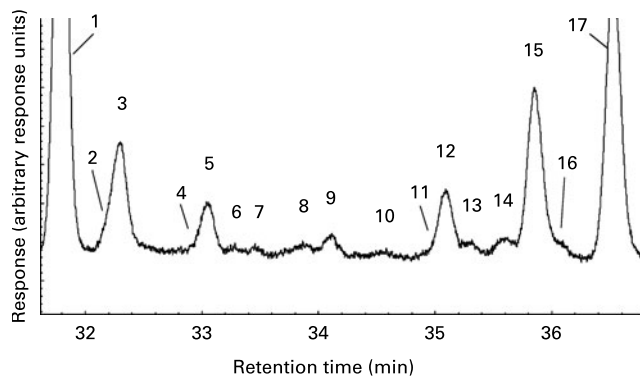

Fig. 1. Partial gas chromatogram indicating the separation of 18:2 isomers obtained under isothermal conditions (170°C) in omasal digesta of cows fed grass silage-based diets supplemented with sunflower-seed oil. Identification was based on electron impact ionisation spectra obtained by GC–MS analysis of fatty acid methyl esters and 4,4-dimethyloxaline derivatives and the elution of an authentic standard (L-8404; Sigma-Aldrich, Helsinki, Finland) containing a mixture of 9,12 geometric 18:2 isomers. Peak identification: 1, *trans*-16-18 : 1; 2, 19 : 0; 3, *cis*-15-18 : 1; 4, *trans*-9,*trans*-14-18 : 2; 5, *trans*-11,*trans*-15-18 : 2; 6, *trans*-10,*trans*-14-18 : 2 (tentative identification); 7, unresolved *trans*-10,*trans*-15-18 : 2 and *trans*-9,*trans*-12-18 : 2; 8, *trans*-11,*trans*-14-18 : 2; 9, methyl-11-cyclohexyl-11 : 0; 10, *trans*-9,*cis*-15-18 : 2; 11, *cis*-9,*trans*-12-18 : 2; 12, *cis*-16-18 : 1; 13, 18 : 2 (double-bond position and geometry undetermined); 14, *trans*-9,*cis*-12-18 : 2; 15, *trans*-11,*cis*-15-18 : 2; 16, *cis*-7-19 : 1; 17, *cis*-9,*cis*-12-18 : 2.

procedure of Statistical Analysis Systems software package version 8.2 (SAS Institute, Inc., Cary, NC, USA) with a model that included the random effect of cow and fixed effects of period and treatment. Sums of squares for treatment effects were further separated using orthogonal contrasts into single degree of freedom comparisons to test for the significance of linear, quadratic and cubic components of the response to experimental treatments.

Measurements of rumen fermentation characteristics were initially subjected to ANOVA for repeated measures using a mixed linear model that evaluated the effects of sampling time, cow, period, treatment and interactions between sampling time and treatment assuming an autoregressive order 1 covariance structure. Treatment and sampling time interactions were not significant ($P < 0.10$) for measured rumen fermentation characteristics and therefore rumen fermentation data were reduced to daily means and analysed using the same statistical model applied to other experimental data. Least-square means with their standard errors are reported and treatment effects were declared significant at $P < 0.05$.

Results

Diet composition

Silage was of high quality both in terms of nutritive value and fermentation characteristics and had the following composition (g/kg DM, unless otherwise stated): DM (g/kg fresh weight), 206; OM, 916; N, 20·3; NDF, 539; iNDF, 105; digestible OM, 650; pH 4·00; lactic acid, 47; acetic acid, 18; water-soluble carbohydrate, 42; soluble N (g/kg total N), 611; ammonium-N (g/kg total N), 72. Concentrate contained (g/kg DM, unless otherwise stated): DM (g/kg fresh weight), 887; OM, 921; N, 26·9; NDF, 214; iNDF, 60·6. Silage contained 16·2 g fatty acids/kg DM with relatively high amounts

(g/kg DM) of 16 : 0 (2·82), 18 : 2*n*-6 (2·89) and 18 : 3*n*-3 (8·41), whilst 16 : 0 (3·55), *cis*-9-18 : 1 (4·55) and 18 : 2*n*-6 (5·88) predominated in concentrates (total fatty acids 16·9 g/kg DM). SFO was rich in 18 : 2*n*-6 (597), but also contained 16 : 0 (56·0), 18 : 0 (39·7) and *cis*-9-18 : 1 (184), with a total fatty acid content of 901 g/kg.

Animal performance

Supplements of SFO decreased linearly ($P < 0.001$) silage DM intake, causing a reduction in NDF, N and 18 : 3*n*-3 ingestion, but had no effect on total DM and OM intake (Table 1). Incremental amounts of SFO in the diet resulted in linear increases ($P < 0.001$) in 14 : 0, 16 : 0, *cis*-9-16 : 1, 18 : 0, *cis*-9-18 : 1, *cis*-11-18 : 1, 18 : 2*n*-6, 20 : 0, 22 : 0, 24 : 0 and total fatty acid intake (Table 1). Supplementing the diet with SFO was associated with a linear increase ($P < 0.01$) in milk yield and lactose secretion, but had no effect ($P > 0.05$) on milk fat and protein output, due to significant ($P < 0.001$) reductions in milk protein content (Table 2).

Rumen fermentation

Inclusion of SFO in the diet had no effect on rumen pH or VFA content, but tended ($P = 0.09$) to decrease rumen ammonia-N concentrations (Table 3). Supplements of SFO modified rumen fermentation patterns, causing a shift towards propionate at the expense of acetate (Table 3). Effects on rumen fermentation characteristics were relatively minor in response to 250 or 500 g SFO/d with the main changes occurring to diets supplemented with 750 g SFO/d.

Nutrient flow entering the omasal canal

Increasing levels of SFO in the diet had no effect on OM, N or non-ammonia-N flow, but altered that of NDF and potentially digestible NDF in a quadratic or cubic manner. Flow of OM, NDF and potentially digestible NDF was lower ($P < 0.05$) for diets supplemented with 500 compared with 750 g SFO/d, and the amounts of non-ammonia-N and NDF entering the omasum were reduced ($P < 0.05$) on diets supplemented with 500 g SFO/d compared with the control diet (Table 4). Incremental amounts of SFO decreased linearly ($P < 0.05$) the flow of VFA and increased linearly ($P < 0.001$) the amount of fatty acids at the omasum (Table 4). Fatty acid flow at the omasum exceeded dietary intake for all diets, with an indicated net synthesis of 131, 160, 125 and 233 g/d on diets supplemented with 0, 250, 500 and 750 g SFO/d, respectively. Addition of SFO in the diet modified the flow of fatty acids entering the omasal canal, responses characterised by linear or quadratic increases ($P < 0.05$) in the flow of 12 : 0, 14 : 0, 16 : 0, 18 : 0, 10-keto-18 : 0, 18 : 1, 18 : 2, CLA and long-chain SFA (20 : 0–30 : 0), and a decrease in 18 : 3*n*-3 at the omasum (Table 4). Flows of *cis*- (Δ9–16) and *trans*-(Δ4–16) 18 : 1 leaving the rumen were all increased ($P < 0.05$) in a linear dose-dependent manner to SFO (Table 5). *Trans*-11 was the predominant 18 : 1 isomer in

Table 1. Effect of incremental levels of sunflower-seed oil in the diet on nutrient intake

(Mean values with their standard errors)

| Nutrient intake (g/d) | Sunflower-seed oil (g/d) | | | | SEM† | P* |
	0	250	500	750		
Silage DM	8783	8417	7897	7770	135·3	0·001
Total DM	15 123	15 007	14 737	14 859	135·2	0·130
OM	13 884	13 799	13 570	13 710	125·1	0·228
NDF	6089	5899	5622	5535	71·0	<0·001
PdNDF	4782	4622	4402	4332	57·2	<0·001
N	349	342	331	327	2·8	<0·001
12 : 0	0·8	0·8	0·7	0·7	0·01	0·002
14 : 0	1·2	1·3	1·4	1·5	0·01	<0·001
16 : 0	50·0	62·8	75·3	88·9	0·38	<0·001
cis-9-16 : 1	2·2	2·3	2·4	2·5	0·02	<0·001
18 : 0	4·3	14·1	23·9	33·8	0·04	<0·001
cis-9-18 : 1	36·3	82·1	128	174	0·1	<0·001
cis-11-18 : 1	7·9	9·4	10·8	12·4	0·02	<0·001
18 : 2*n*-6	66·0	214	361	510	0·5	<0·001
18 : 3*n*-3	83·7	79·8	75·7	76·0	1·48	0·006
20 : 0	1·9	2·5	3·0	3·7	0·02	<0·001
cis-11-20 : 1	0·9	1·2	1·5	1·8	<0·01	<0·001
22 : 0	3·1	4·7	6·4	8·1	0·04	<0·001
24 : 0	2·5	3·0	3·4	4·0	0·03	<0·001
Summary						
Σ Saturates	14·4	27·1	39·7	52·6	0·14	<0·001
Σ Monounsaturates	45·6	93·2	141	189	0·1	<0·001
Σ Polyunsaturates	151	296	440	590	1·97	<0·001
Σ Fatty acids	264	481	698	923	2·6	<0·001

OM, organic matter; NDF, neutral-detergent fibre; pdNDF, potentially digestible neutral-detergent fibre.

* Significance of linear responses to sunflower-seed oil in the diet. Quadratic and cubic responses to sunflower-seed oil were not significant ($P > 0.05$).

† SEM for *n* 16 measurements; error degrees of freedom 6.

Table 2. Effect of incremental levels of sunflower-seed oil in the diet on milk yield and composition

(Mean values with their standard errors)

	Sunflower-seed oil (g/d)				SEM†	P*
	0	250	500	750		
Yield						
Milk (kg/d)	16·8	17·0	17·4	18·8	0·37	0·007
Fat (g/d)	818	844	857	856	18·7	0·178
Protein (g/d)	562	563	550	586	9·7	0·222
Lactose (g/d)	818	830	853	924	19·2	0·008
Concentration						
Fat (g/kg)	48·8	49·5	49·0	45·7	0·94	0·056
Protein (g/kg)	33·6	33·2	31·5	31·3	0·27	<0·001
Lactose (g/kg)	48·9	49·1	49·0	49·3	0·21	0·306
Urea (mmol/l)	2·78	2·66	2·86	2·41	0·164	0·259

* Significance of linear responses to sunflower-seed oil in the diet. Quadratic and cubic responses to sunflower-seed oil were not significant ($P > 0.05$).
† SEM for n 16 measurements; error degrees of freedom 6.

Table 3. Effect of incremental levels of sunflower-seed oil in the diet on rumen fermentation characteristics

(Mean values with their standard errors)

	Sunflower-seed oil (g/d)				SEM†	P*		
	0	250	500	750		L	Q	C
pH	6·50	6·39	6·40	6·54	0·097	0·781	0·248	0·970
Ammonia-N (mmol/l)	5·89	6·07	5·98	4·63	0·426	0·088	0·125	0·628
Volatile fatty acids (mmol/l)	104	102	103	98	3·5	0·334	0·683	0·710
Molar proportions (mmol/mol)								
Acetate	670	670	667	646	2·3	<0·001	0·003	0·180
Propionate	164	165	167	190	3·7	0·003	0·022	0·259
Butyrate	132	132	134	129	3·9	0·715	0·555	0·561
Isobutyrate	8·63	8·51	8·38	8·86	0·222	0·586	0·226	0·568
Valerate	9·49	9·09	9·02	9·86	0·145	0·157	0·005	0·409
Isovalerate	11·1	11·5	11·0	13·4	0·71	0·094	0·200	0·267
Caproate	4·13	3·70	3·23	3·09	0·220	0·011	0·541	0·712
Molar ratio								
Acetate:propionate	4·10	4·08	4·01	3·41	0·081	0·001	0·012	0·218
(Acetate + butyrate):propionate	4·90	4·88	4·82	4·09	0·112	0·003	0·020	0·247

* Significance of linear (L), quadratic (Q) and cubic (C) responses to sunflower-seed oil in the diet.
† SEM for n 16 measurements; error degrees of freedom 6.

omasal digesta, accounting for 49, 45, 46 and 56 % of total *trans*-18 : 1, in cows fed 0, 250, 500 and 750 g SFO/d, respectively.

Supplements of SFO increased linearly ($P < 0.05$) omasal 18 : 2*n*-6- and *trans*-9,*cis*-12-18 : 2 flow, had no effect ($P > 0.05$) on *trans*-11,*trans*-15-18 : 2, and reduced in a quadratic manner ($P < 0.05$) the amount of *trans*-11,*cis*-15-18 : 2 at the omasum (Table 6). Furthermore, SFO supplements increased linearly ($P < 0.01$) ruminal *cis*-9,*cis*-11-CLA, *cis*-9,*trans*-11-CLA, *trans*-9,*trans*-11-CLA, *cis*-10,*cis*-12-CLA, *trans*-10,*cis*-12-CLA, *trans*-10,*trans*-12-CLA and *trans*-8,*trans*-10-CLA outflow, but had no effect on the flow of other CLA isomers at the omasum (Table 6). On all diets, *cis*-9,*trans*-11 was the major conjugated 18 : 2 isomer entering the omasal canal, accounting for 66, 78, 83 and 80 % of total CLA flow, in cows fed 0, 250, 500 and 750 g SFO/d, respectively. Omasal digesta was devoid of *trans*-7,*cis*-9-CLA, *trans*-9,*cis*-11-CLA and *cis*-10,*trans*-12-CLA.

Supplements of SFO also altered the flow of branched-chain and odd-chain fatty acids at the omasum, responses characterised by linear or quadratic increases ($P < 0.05$) in the amount of 11-cyclohexyl-11 : 0, 15 : 0, 15 : 0 *anteiso*, 3,7,11,15-tetramethyl-16 : 0, 23 : 0, 27 : 0 and 29 : 0 at the omasum (Table 7). Flows of unsaturated fatty acids at the omasum were lower than their respective intake, while increasing amounts of SFO in the diet increased linearly ($P < 0.05$) the extent of ruminal *cis*-9-18 : 1, 18 : 2*n*-6 and 18 : 3*n*-3 metabolism (Table 8).

Nutrient digestibility

Inclusion of SFO in the diet had no effect ($P > 0.05$) on apparent ruminal and total-tract OM and N digestibility coefficients or on the relative proportion of nutrient digestion occurring in the rumen and intestines, but decreased in a quadratic manner ($P < 0.05$) ruminal digestibility of potentially digestible NDF and whole-tract NDF digestibility (Table 9).

Discussion

Animal performance

Plant oils rich in PUFA typically depress DM intake in lactating cows, an effect that has been attributed to the effects

Table 4. Effect of incremental levels of sunflower-seed oil in the diet on the flow of nutrients at the omasum

(Mean values with their standard errors)

Flow (g/d)	Sunflower-seed oil (g/d)					P*		
	0	250	500	750	SEM†	L	Q	C
DM	10 009	10 122	9309	10 435	291·5	0·733	0·133	0·070
OM	7568	7732	7082	8077	234·3	0·435	0·126	0·057
NDF	2658	2673	2328	2809	92·4	0·796	0·045	0·029
PdNDF	1494	1482	1309	1663	60·6	0·264	0·023	0·044
N	356	350	325	345	8·1	0·157	0·162	0·133
NAN	353	345	319	341	8·6	0·155	0·139	0·137
VFA	718	691	618	626	31·6	0·048	0·592	0·409
Fatty acids								
12:0	0·37	0·39	0·35	0·51	0·023	0·008	0·029	0·043
14:0	1·2	1·5	1·4	2·1	0·16	0·010	0·227	0·232
16:0	46·5	59·8	68·3	94·2	4·31	<0·001	0·196	0·298
18:0	237	408	514	672	40·5	<0·001	0·878	0·539
10-keto-18:0	0·73	0·63	0·77	3·97	0·346	<0·001	0·003	0·118
18:1 *cis*	11·6	20·4	29·0	43·4	3·73	<0·001	0·483	0·734
18:1 *trans*	30·3	67·2	118	226	16·5	<0·001	0·074	0·575
Σ 18:1	41·9	87·5	147	269	20·2	<0·001	0·105	0·602
Σ 18:2‡	8·8	11·4	11·7	16·1	1·44	0·012	0·613	0·332
Σ CLA	3·0	6·3	11·4	15·3	1·86	0·002	0·863	0·727
18:3n-3	1·5	1·2	1·0	0·9	0·04	<0·001	0·054	0·605
cis-9,*trans*-11,*cis*-15-18:3	0·32	0·27	0·31	0·26	0·05	0·534	0·994	0·425
20:0	4·0	5·0	5·3	6·6	0·37	0·002	0·741	0·368
cis-11-20:1	0·4	0·6	0·6	0·9	0·07	0·005	0·363	0·250
22:0	3·0	5·0	6·4	9·0	0·50	<0·001	0·642	0·459
cis-15-22:1	0·10	0·08	0·08	0·09	0·023	0·418	0·139	0·319
24:0	3·2	3·7	3·9	4·9	0·23	0·002	0·337	0·363
26:0	12·6	15·0	15·0	17·5	1·10	0·024	0·950	0·329
28:0	7·2	9·0	9·2	10·8	0·65	0·009	0·819	0·352
30:0	3·3	4·0	4·1	4·7	0·25	0·008	0·849	0·283
Summary								
Σ Saturates	336	531	649	850	47·8	<0·001	0·947	0·480
Σ Monounsaturates	45·6	91·1	150	274	20·3	<0·001	0·103	0·595
Σ Polyunsaturates	13·6	19·2	24·0	32·0	3·15	0·005	0·709	0·794
Σ Fatty acids	395	641	823	1156	61·0	<0·001	0·501	0·459

OM, organic matter; NDF, neutral-detergent fibre; pdNDF, potentially digestible neutral-detergent fibre; NAN, non-ammonia-N; VFA: volatile fatty acid; CLA, conjugated linoleic acid.
* Significance of linear (L), quadratic (Q) and cubic (C) responses to sunflower-seed oil in the diet.
† SEM for *n* 16 measurements; error degrees of freedom 6.
‡ Sum of 18:2 fatty acids excluding isomers of CLA.

of unsaturated fatty acids on rumen fermentation and ruminal OM digestion coupled with a tendency for plant lipids to shift the site of nutrient digestion from the rumen to the intestines[29,30]. Even though SFO at the highest level of inclusion in this experiment decreased ruminal digestion of potentially digestible NDF, DM intake was not altered with no evidence of an increase in the proportion of nutrient digestion occurring in the intestine. Previous studies have also shown that plant oils rich in 18:2n-6 have no adverse effect on DM intake of diets containing proportionately 0·60 forage DM when incorporated at rates of between 37 and 60 g/kg diet DM[31,32].

Supplementing the diet with up to 50 g SFO/kg diet DM had no effect on milk fat yield consistent with the previous findings in cows fed high-forage diets containing 37 g SFO/kg diet DM[31]. In contrast, other studies have shown that milk fat synthesis is decreased in response to 50 g SFO/kg diet DM supplements[33] or 60 g safflower-seed oil/kg diet DM[32], with the extent of diet-induced milk-fat depression being dependent on the relative proportions of starch and fibre in the diet[33]. In the present study, SFO supplements increased linearly the flow of *trans*-10,*cis*-12-CLA and *trans*-10-18:1 at the omasum from 0·09 to 0·40 and 1·3 to 20·6 g/d, respectively, but had no effect

on milk fat synthesis. *Trans*-10,*cis*-12-CLA is a known inhibitor of milk fat synthesis in the lactating cow[34], but the marginal increases in omasal flow of this biohydrogenation intermediate in response to 750 g SFO/d would be predicted[35] to induce minimal reductions (4·3 %) in milk fat yield. Furthermore, milk fat secretion was also independent of increases in ruminal *trans*-10-18:1 outflow consistent with direct evidence based on abomasal infusions that this biohydrogenation intermediate has no role in the regulation of milk fat synthesis in lactating cows[36]. Overall, milk fat responses to SFO in cows fed grass silage-based diets are consistent with the view that alterations in ruminal lipid metabolism characterised as a shift towards *trans*-10-18:1 at the expense of *trans*-11-18:1 are a prerequisite for diet-induced milk-fat depression when plant oils are fed[34,37].

Rumen fermentation

Supplementation of SFO had no effect on rumen pH, but at the highest level of inclusion was associated with a shift in rumen fermentation towards propionate at the expense of acetate, consistent with responses to soyabean oil reported in

Table 5. Effect of incremental levels of sunflower-seed oil in the diet on the flow of 18 : 1 fatty acids at the omasum

(Mean values with their standard errors)

	Sunflower-seed oil (g/d)				SEM†	P*
	0	250	500	750		
cis-9-18 : 1	7·2	12·7	17·0	26·4	2·44	0·001
cis-11-18 : 1	2·0	2·4	2·8	4·2	0·28	0·001
cis-12-18 : 1	0·6	3·2	6·6	9·2	0·91	<0·001
cis-13-18 : 1	0·3	0·3	0·6	1·0	0·17	0·014
cis-15-18 : 1	1·0	1·2	1·4	1·7	0·11	0·005
cis-16-18 : 1	0·4	0·5	0·6	0·7	0·05	0·005
trans-4-18 : 1	0·3	0·6	1·1	1·9	0·15	<0·001
trans-5-18 : 1	0·3	0·5	0·7	1·3	0·11	<0·001
trans-6- + 7- + 8-18 : 1	1·3	3·3	6·1	10·5	0·75	<0·001
trans-9-18 : 1	0·8	2·2	4·2	7·0	0·48	<0·001
trans-10-18 : 1	1·3	4·1	8·6	20·6	4·26	0·017
trans-11-18 : 1	14·9	30·2	54·7	126·2	11·94	<0·001
trans-12-18 : 1	1·7	4·9	8·7	12·9	0·87	<0·001
trans-13- + 14-18 : 1	4·5	10·5	16·9	22·6	1·36	<0·001
trans-15-18 : 1	2·3	5·1	7·9	10·7	0·66	<0·001
trans-16-18 : 1‡	2·9	5·7	9·0	12·3	0·88	<0·001

* Significance of linear responses to sunflower-seed oil in the diet. Quadratic and cubic responses to sunflower-seed oil were not significant ($P > 0.05$).
† SEM for n 16 measurements; error degrees of freedom 6.
‡ Contains cis-14-18 : 1 as a minor component.

Table 6. Effect of incremental levels of sunflower-seed oil in the diet on the flow of 18 : 2 fatty acids at the omasum

(Mean values with their standard errors)

Flow (mg/d)	Sunflower-seed oil (g/d)				SEM†	P*	
	0	250	500	750		L	Q
cis-9,cis-12-18 : 2	5526	8675	8715	12 543	1349·0	0·013	0·810
trans-9,cis-12-18 : 2	0·0	24	99	110	11·8	<0·001	0·597
trans-11,cis-15-18 : 2	2306	1828	1613	1794	130·7	0·024	0·045
trans-11,trans-14-18 : 2	147	124	142	188	15·6	0·089	0·073
trans-11,trans-15-18 : 2	536	502	491	599	50·0	0·463	0·205
cis-9,cis-11-CLA	26	50	144	153	27·5	0·009	0·784
cis-10,cis-12-CLA	7	26	44	69	13·4	0·041	0·409
cis-9,trans-11-CLA	1927	4768	9228	11 571	1533·5	0·003	0·877
cis-12,trans-14-CLA	36	14	17	23	8·2	0·366	0·132
trans-10,cis-12-CLA	84	144	182	396	83·0	0·040	0·387
trans-11,cis-13-CLA	334	237	143	322	105·4	0·795	0·238
trans-13,cis-15-CLA	39	37	38	48	8·9	0·501	0·510
trans-8,trans-10-CLA	5·0	26	37	36	5·2	0·004	0·075
trans-9,trans-11-CLA	184	444	930	1675	174·8	<0·001	0·214
trans-10,trans-12-CLA	30	131	226	424	63·6	0·004	0·477
trans-11,trans-13-CLA	221	241	210	337	69·8	0·348	0·470
trans-12,trans-14-CLA	132	139	121	218	42·5	0·251	0·326
trans-13,trans-15-CLA	14	18	16	18	4·4	0·728	0·822

CLA, conjugated linoleic acid.
* Significance of linear (L) and quadratic (Q) responses to sunflower-seed oil in the diet. Cubic responses to sunflower-seed oil in the diet were not significant ($P > 0.05$).
† SEM for n 16 measurements; error degrees of freedom 6.

non-lactating cows fed hay-based diets[30]. In other studies, supplements of plant oils rich in 18 : 2n-6 have been reported to have no effect on rumen VFA profiles in cows fed maize silage-based diets[31] or increase the molar proportions of propionate, but reduce that of butyrate in grain-fed cattle[38]. The present data indicate that in addition to the composition of the basal diet, the amount of supplemental lipid is also an important determinant of the extent of changes in rumen fermentation patterns that can be expected when plant oils are fed.

Rumen lipid metabolism

Measurements of ruminal metabolism of dietary unsaturated fatty acids are typically based on the comparison of intake with fatty acid flow at the duodenum. Fatty acids entering the duodenum are derived from NEFA adhered to food particles and microbial cells, lipids in rumen bacteria and protozoa, desquamated endothelial cells and bile[39,40]. Sampling at the omasum has the advantage of reducing the contribution of

Table 7. Effect of incremental levels of sunflower-seed oil in the diet on the flow of odd-chain and branched-chain fatty acids at the omasum

(Mean values with their standard errors)

| | Sunflower-seed oil (g/d) | | | | | P* | |
Flow (g/d)	0	250	500	750	SEM†	L	Q
11-Cyclohexyl-11 : 0	0·25	0·25	0·30	0·35	0·029	0·039	0·419
13 : 0 *iso*	0·11	0·10	0·09	0·15	0·017	0·225	0·133
13 : 0 *anteiso*	0·07	0·08	0·05	0·11	0·019	0·442	0·297
14 : 0 *iso*	0·32	0·29	0·95	0·35	0·350	0·648	0·446
15 : 0	2·9	3·1	3·1	4·0	0·19	0·011	0·144
15 : 0 *iso*	0·70	0·65	0·63	0·93	0·063	0·053	0·034
15 : 0 *anteiso*	1·15	1·08	1·09	1·63	0·060	0·002	0·002
16 : 0 *iso*	0·57	0·56	0·53	0·67	0·054	0·313	0·220
3,7,11,15-Tetramethyl-16 : 0	4·6	5·2	5·9	6·9	0·53	0·018	0·699
17 : 0	2·6	2·6	2·5	2·9	0·18	0·296	0·362
17 : 0 *iso*	1·6	1·6	1·6	2·0	0·13	0·138	0·255
17 : 0 *anteiso*	2·9	2·3	1·8	2·1	0·17	0·009	0·037
18 : 0 *iso*	0·27	0·30	0·26	0·28	0·023	0·858	0·938
23 : 0	0·79	0·82	0·82	0·97	0·036	0·015	0·193
cis-11-23 : 1	0·20	0·19	0·17	0·16	0·030	0·345	0·984
25 : 0	0·34	0·41	0·37	0·43	0·025	0·106	0·858
27 : 0	0·49	0·83	0·80	0·94	0·097	0·022	0·338
29 : 0	0·38	0·57	0·50	0·58	0·044	0·035	0·258

* Significance of linear (L) and quadratic (Q) responses to sunflower-seed oil in the diet. Cubic responses to sunflower-seed oil in the diet were not significant ($P > 0·05$).
† SEM for *n* 16 measurements; error degrees of freedom 6.

Table 8. Effect of incremental levels of sunflower-seed oil in the diet on apparent ruminal biohydrogenation of unsaturated fatty acids

(Mean values with their standard errors)

| | Sunflower-seed oil (g/d) | | | | | P* | |
Biohydrogenation (%)	0	250	500	750	SEM†	L	Q
cis-9-18 : 1	80·2	84·5	86·7	84·9	0·96	0·009	0·019
18 : 2*n*-6	91·7	95·9	97·6	97·5	0·19	<0·001	<0·001
18 : 3*n*-3	98·2	98·5	98·7	98·8	0·06	<0·001	0·194

* Significance of linear (L) and quadratic (Q) responses to sunflower-seed oil in the diet. Cubic responses to sunflower-seed oil in the diet were not significant ($P > 0·05$).
† SEM for *n* 16 measurements; error degrees of freedom 6.

endogenous secretions in digesta and therefore provides a more direct assessment of ruminal fatty acid outflow. In the present experiment, the effects of SFO in the diet on ruminal lipid metabolism were assessed based on the flow of NEFA at the omasum using procedures optimised for the methylation of this fraction[25], which was considered more important to understanding the effects of SFO on the formation of specific biohydrogenation intermediates, rather than using alternative transesterification procedures for the methylation of total fatty acids that increase the risk of isomerisation of unsaturated fatty acids or synthesis of methoxy-artifacts[41].

Comparison of fatty acid intake and flows of NEFA at the omasum indicated a net synthesis of fatty acids in the rumen on all diets. These measurements are consistent with minimal or insignificant metabolism of long-chain fatty acids by rumen microbes or absorption of fatty acids across the rumen epithelium or omasum[39] with the implication that SFO in the diet had no apparent inhibitory effects on microbial *de novo* fatty acid synthesis. Based on an extensive evaluation of 113 mean treatment values of fatty acid flow at the

duodenum, proportionately 0·80 of ingested fatty acids were reported to be recovered, with evidence that the extent of negative balances was associated with diets containing more than 50 g fatty acids/kg DM[40,42] with no clear association with dietary lipid composition. More recent studies have estimated net losses[43] or increases in fatty acids at the duodenum in cows fed diets containing SFO[13,31]. Furthermore, fatty acid flows at the omasum have also been shown to exceed fatty acid ingestion in cows fed diets containing soyabean oil[44]. Discrepancies in estimates of fatty acid synthesis in the rumen in response to plant oils rich in 18 : 2*n*-6 are difficult to reconcile, but must to some extent reflect differences in experimental techniques used to estimate post-ruminal DM flow and the fatty acid content of feed ingredients and digesta.

Supplements of SFO modified ruminal lipid metabolism causing an increase in the flow of 18 : 0, *cis*-18 : 1, *trans*-18 : 1, 18 : 2, CLA and total fatty acids at the omasum. Previous studies have shown that incubation of increasing amounts of soyabean oil containing 56·5 g 18 : 2*n*-6/100 g fatty acids with mixed rumen microbes reduced the rate of

Table 9. Effect of incremental levels of sunflower-seed oil in the diet on rumen and whole tract apparent digestibility coefficients

(Mean values with their standard errors)

Digestibility coefficient	Sunflower-seed oil (g/d)				SEM†	P*	
	0	250	500	750		L	Q
Rumen							
DM	0·338	0·329	0·371	0·302	0·0184	0·451	0·150
OM	0·507	0·494	0·527	0·459	0·0144	0·138	0·110
NDF	0·562	0·549	0·582	0·496	0·0167	0·070	0·068
pdNDF	0·687	0·681	0·702	0·619	0·0134	0·022	0·028
N	−0·018	−0·015	0·025	−0·051	0·0206	0·553	0·099
Whole tract							
DM	0·686	0·693	0·696	0·684	0·0026	0·906	0·009
OM	0·714	0·722	0·725	0·711	0·0024	0·617	0·004
NDF	0·650	0·658	0·651	0·621	0·0079	0·038	0·050
pdNDF	0·778	0·794	0·781	0·752	0·0157	0·245	0·204
N	0·634	0·627	0·640	0·626	0·0060	0·676	0·548
Proportion occurring in the rumen							
DM	0·493	0·473	0·533	0·444	0·0281	0·511	0·266
OM	0·710	0·683	0·726	0·648	0·0215	0·189	0·285
NDF	0·866	0·833	0·896	0·804	0·0323	0·434	0·395
pdNDF	0·885	0·859	0·900	0·834	0·0332	0·481	0·569

OM, organic matter; NDF, neutral-detergent fibre; pdNDF, potentially digestible neutral-detergent fibre.
* Significance of linear (L) and quadratic (Q) responses to sunflower-seed oil in the diet. Cubic responses to sunflower-seed oil in the diet were not significant ($P > 0.05$).
† SEM for n 16 measurements; error degrees of freedom 6.

lipolysis and extent of 18 : 2n-6 metabolism *in vitro*[45]. Even though the flow of TAG, phospholipids, sphingolipids and sterol lipids at the omasum were not determined, a mean marginal recovery of C$_{18}$ fatty acids in SFO as non-esterified 18 : 0 at the omasum of 73·3 %, coupled with increased accumulation of 18 : 1 and 18 : 2 biohydrogenation intermediates, indicate that biohydrogenation rather than lipolysis is rate limiting for ruminal metabolism of SFO unsaturated fatty acids *in vivo*.

Incubation of trilinolein[46] or soyabean oil[45] with strained rumen contents has shown that biohydrogenation of 18 : 2n-6 is slower and less complete as the concentration of added lipid substrate increases. In contrast, supplementing the diet with incremental amounts of SFO in the present experiment was associated with linear increases in apparent ruminal *cis*-9-18 : 1, 18 : 2n-6 and 18 : 3n-3 metabolism from 80·2, 91·7 and 98·2 % to 84·9, 97·5 and 98·8 %, respectively. However, these estimates are more apparent than true, because they are based on NEFA and the contribution of esterified lipids to total fatty acid flow at the omasum is not accounted for. Ruminal 18 : 2n-6 and 18 : 3n-3 biohydrogenation based on measurements of fatty acid flow at the duodenum varies between 70 and 95 % and 85 and 100 %, respectively[40]. Even though ruminal biohydrogenation of dietary unsaturated fatty acids is overestimated based on the flow of NEFA at the omasum, the relative changes to incremental amounts of SFO in the diet can be considered more reliable, since TAG are extensively hydrolysed in the rumen[6,40,42] and plant oils in the diet enhance the incorporation of unsaturated fatty acids as NEFA in cytoplasmic droplets, but have no effect on diacylglycerol, phospholipid or sterol ester content of rumen bacteria[47]. Extensive evaluation of published studies implied that the amount or source of 18 : 2n-6 in the diet has little influence on the extent of ruminal 18 : 2n-6 biohydrogenation *in vivo*[40]. However, evidence from this and earlier studies[13,16] tends to suggest that ruminal 18 : 2n-6

metabolism is more extensive when the amount of SFO in the diet is increased.

Measurements of fatty acid flow at the omasum in response to incremental amounts of SFO in the diet supports earlier *in vitro* and *in vivo* studies[6] demonstrating that the major pathway of ruminal 18 : 2n-6 biohydrogenation involves the formation of *cis*-9,*trans*-11-CLA and *trans*-11-18 : 1 as major intermediates with 18 : 0 being the final endproduct. Increases in ruminal outflow of *trans*-11-18 : 1 to SFO were approximately ten-fold higher compared with *cis*-9,*trans*-11-CLA. Differences in the flow of specific biohydrogenation intermediates indicate that the metabolism of *cis*-9,*trans*-11-CLA to *trans*-11-18 : 1 occurs at a faster rate than the conversion of *trans*-11-18 : 1 to 18 : 0 and that the final reduction of *trans*-18 : 1 intermediates is rate limiting for complete ruminal biohydrogenation of non-esterified 18 : 2n-6 *in vivo*.

Inclusion of SFO in the diet as a source of 18 : 2n-6 enhanced linearly the flow of *cis*-9,*trans*-11-CLA leaving the rumen. A mean marginal increase in the amount of *cis*-9,*trans*-11-CLA leaving the rumen of 198 mg *cis*-9,*trans*-11-CLA/g SFO per kg diet DM is markedly higher than corresponding values of 11·0 and 39·8 based on measurements of fatty acids at the duodenum in steers[16] or lactating cows[43] fed high-concentrate diets. Several factors including amount of oil supplement, composition of the basal diet[3], sampling site, choice of marker system[18] and procedures for handling, storage and analysis of digesta lipids[41] may contribute to variation in post-ruminal *cis*-9,*trans*-11-CLA outflow to increases in dietary 18 : 2n-6 supply. Studies in sheep have shown that increases in the proportion of forage in the diet enhance *cis*-9,*trans*-11-CLA flow at the duodenum when supplements of soyabean oil are fed[15]. In contrast, increases in the forage:concentrate ratio of diets containing SFO from 12:88 to 36:64 were reported to have no effect on the flow of *cis*-9,*trans*-11-CLA at the duodenum in

steers[16]. The present measurements indicated that *cis*-9,*trans*-11-CLA was the most abundant isomer in digesta, accounting for between 67 and 83 % of total CLA, consistent with previous determinations of the CLA isomer profile of omasal digesta[25]. Analysis of lipids in duodenal digesta have shown that the relative abundance of *cis*-9,*trans*-11-CLA is considerably lower, accounting for between 14 and 31 % of total CLA content[12,48,49]. It is possible that *cis-trans* and *trans-cis* isomers of CLA entering the omasal canal are further metabolised during the passage of digesta into the duodenum. However, previous studies have reported estimates of ruminal biohydrogenation based on the collection of omasal digesta samples[44] consistent with those derived from fatty acid flows at the duodenum[40]. No direct comparisons of sampling sites have been made, but the available evidence does not support extensive metabolism of fatty acids during transit from the omasum to duodenum. Further studies are required to establish the role of the omasum and abomasum on the supply of fatty acids available for absorption in ruminants. There is also clear evidence that the choice of catalyst used for the transesterification of lipids can alter the isomer profile and amount of CLA recovered in digesta[41]. It is therefore probable that at least part of the variation in the distribution of CLA isomers and changes in post-ruminal *cis*-9,*trans*-11-CLA flow to 18 : 2*n*-6 in the diet may arise from differences in the methods used for the extraction and transesterification of digesta lipids.

Data from the present experiment also confirmed *trans*-10,*cis*-12-CLA as a minor intermediate of ruminal 18 : 2*n*-6 metabolism on grass silage-based diets. Previous studies have also shown that the flow of *trans*-10,*cis*-12-CLA at the duodenum is increased in response to plant oils rich in 18 : 2*n*-6[12,41], with evidence that ruminal formation of *trans*-10,*cis*-12-CLA is further promoted when supplements containing 18 : 2*n*-6 are included in high-concentrate diets[15,16]. Supplements of SFO also enhanced the flow of *trans*-8,*trans*-10 and several other geometric isomers of 9,11- and 10,12-CLA at the omasum. Recent studies have also shown that *cis*-9,*cis*-11-CLA, *trans*-9,*cis*-11- and *trans*-9,*trans*-11-CLA are intermediates of 18 : 2*n*-6 metabolism

in vitro[50,51]. Furthermore, abomasal infusion studies in lactating cows have established that *trans*-9,*trans*-11-CLA[52] and *trans*-10,*trans*-12-CLA[53] alter milk fatty acid composition and decrease milk fat desaturase indices demonstrating the potential physiological importance of intermediates produced during ruminal 18 : 2*n*-6 metabolism.

Omasal flow of other CLA and non-conjugated 18 : 2 isomers were decreased or independent of SFO in the diet. The occurrence of these fatty acids can be attributed to 18 : 3*n*-3 metabolism in the rumen and serve to highlight the diversity of specific fatty acid intermediates formed during ruminal biohydrogenation of dietary unsaturated fatty acids. Previous studies have also shown that one or more of these biohydrogenation intermediates accumulates during incubation of 18 : 3*n*-3 with mixed rumen microbes or pure cultures[6,50] or are enhanced in duodenal digesta in response to diets containing linseed oil as a source of 18 : 3*n*-3[43,49].

Dietary inclusion of SFO increased the flow of *cis*- (Δ9−16) and *trans*- (Δ4−16) 18 : 1 isomers at the omasum. With the exception of *trans*-11-18 : 1[54], formation of 18 : 1 metabolites during the metabolism of dietary unsaturated fatty acids is not well established. In attempting to identify the origins of 18 : 1 biohydrogenation intermediates, it is important to recognise that SFO contains significant amounts of *cis*-9-18 : 1, and that the rates of *trans*-18 : 1 reduction to 18 : 0 vary according to the position of the double bond, being higher for *trans* (Δ8−10) isomers than *trans* (Δ5−7) or *trans* (Δ11−13)[54]. Further work has also shown that metabolism of *cis*-9-18 : 1 *in vitro* results in the production of *trans*-18 : 1 isomers with double bonds from Δ4−16[55] with evidence that once *trans*-18 : 1 intermediates accumulate further conversion to a number of positional isomers may occur[56]. Nevertheless, through consideration of the flow of 18 : 2 fatty acids at the omasum and probable reduction products coupled with the changes in the profile of 18 : 1 fatty acids in omasal digesta to SFO supplementation, it is possible to deduce that metabolism of 18 : 2*n*-6 in the rumen involves the formation of *cis*- (Δ10−12) and *trans*- (Δ8−12) 18 : 1 intermediates *in vivo* (Fig. 2). The present data confirm and extend earlier studies investigating the formation of fatty acid metabolites liberated during
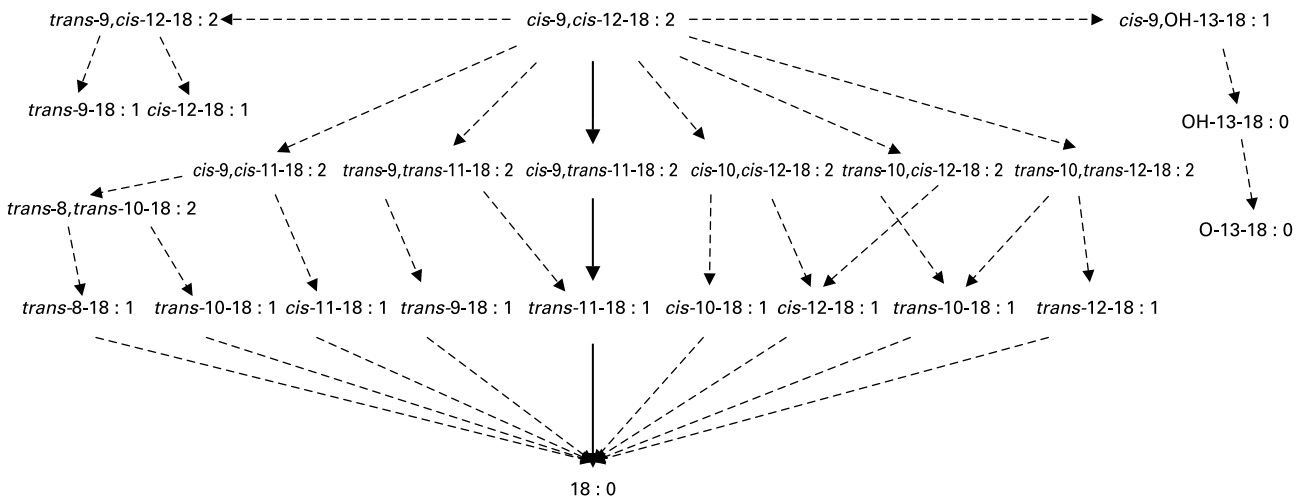

Fig. 2. Putative pathways of 18 : 2*n*-6 metabolism in the rumen. ⟶, Production of major biohydrogenation intermediates of 18 : 2*n*-6; ⇢, formation of minor fatty acid metabolites liberated during ruminal biohydrogenation of 18 : 2*n*-6.

in vitro incubations of 18 : 2*n*-6 with pure cultures or mixed rumen microbes[6,50,51,57].

Inclusion of plant oils rich in 18 : 2*n*-6 is a well-established nutritional strategy for enhancing milk fat *cis*-9,*trans*-11-CLA content[3,33]. Supplementing grass silage-based diets with SFO enhanced ruminal *cis*-9,*trans*-11-CLA and *trans*-11-18 : 1 outflow with no evidence of a shift in biohydrogenation towards *trans*-10-18 : 1 at the expense of *trans*-11-18 : 1 that can occur on high-starch rations based on maize silage or low-forage diets[33]. These observations appear to account for the relatively high enrichment of milk fat *cis*-9,*trans*-11-CLA content reported over an extended period of time in cows fed mixed forage diets containing 60 g safflower-seed oil/kg diet DM[32].

SFO in the diet increased the flows of 15 : 0 *iso*, 15 : 0 *anteiso* and 17 : 0 *anteiso*, but had no effect on ruminal outflow of other odd-branched fatty acids. Since SFO was devoid of these fatty acids, alterations in the flow of specific odd-branched-chain fatty acids can be attributed to the effects of additional lipid in the diet on microbial *de novo* fatty acid synthesis. Odd-branched-chain fatty acids incorporated into bacterial lipids are derived from the elongation of branched-chain Co-A precursors. Leucine and isoleucine are deaminated and decarboxylated to yield isovaleryl-Co A and 2-methylbutyryl Co A that serve as substrates for microbial synthesis of *iso* and *anteiso* fatty acids, respectively[58]. Variations in the relative abundance of odd-branched-chain fatty acids of rumen bacteria are considered to be more dependent on the specificity of inherent fatty acid synthetase rather than the availability of Co-A precursors[58]. A number of studies have established that the fatty acid profiles of rumen bacteria are species specific[6,58,59], such that measurements of odd-branched-chain fatty acids in digesta or milk may reflect changes in ruminal microbial populations. No direct measurements of microbial community were made in the present study, but the changes in the flow of several odd-branched-chain fatty acids at the omasum may imply that SFO induced alterations in the relative abundance of certain bacterial populations. It appears reasonable to assume that populations of rumen bacteria most sensitive to the toxic effects of 18 : 2*n*-6 would be decreased in response to incremental amounts of SFO in the diet.

Conclusions

Supplementing grass silage diets with SFO had no effect on DM intake, milk fat or protein yield, but increased milk yield and at the highest level of inclusion shifted rumen fermentation towards propionate at the expense of acetate. SFO altered ruminal lipid metabolism, leading to dose-dependent increases in the flow of 18 : 0, *trans*-18 : 1, *cis*-18 : 1, 18 : 2*n*-6 and several positional and geometric isomers of CLA at the omasum. *Cis*-9,*trans*-11 was the most abundant isomer of CLA while *trans*-11 was quantitatively the most important 18 : 1 biohydrogenation intermediate in omasal digesta. It is concluded that supplementing grass silage-based diets with plant oils rich in 18 : 2*n*-6 increases ruminal outflow of *trans*-11-18 : 1 and *cis*-9,*trans*-11-CLA in lactating cows.

Acknowledgements

References

1. Lawson RE, Moss AR & Givens DI (2001) The role of dairy products in supplying conjugated linoleic acid to man's diet: a review. *Nutr Res Rev* **14**, 153–172.
2. Ritzenthaler KL, McGuire MK, Falen R, Shultz TD, Dasgupta N & McGuire MA (2001) Estimation of conjugated linoleic acid intake by written dietary assessment methodologies underestimates actual intake evaluated by food duplicate methodology. *J Nutr* **131**, 1548–1554.
3. Palmquist DL, Lock AL, Shingfield KJ & Bauman DE (2005) Biosynthesis of conjugated linoleic acid in ruminants and humans. In *Advances in Food and Nutrition Research*, vol. 50, pp. 179–217 [SL Taylor, editor]. San Diego, CA: Elsevier Academic Press.
4. Wahle KW, Heys SD & Rotondo D (2004) Conjugated linoleic acids: are they beneficial or detrimental to health? *Prog Lipid Res* **43**, 553–587.
5. Yaqoob P, Tricon S, Burdge GC & Calder PC (2006) Conjugated linoleic acids (CLAs) and health. In *Improving the Fat Content of Foods*, pp. 182–209 [CM Williams and J Buttriss, editors]. Cambridge: Woodhead Publishing Ltd.
6. Harfoot CG & Hazlewood GP (1988) Lipid metabolism in the rumen. In *The Rumen Microbial Ecosystem*, pp. 285–322 [PN Hobson, editor]. London: Elsevier Science.
7. Griinari JM, Corl BA, Lacy SH, Chouinard PY, Nurmela KV & Bauman DE (2000) Conjugated linoleic acid is synthesized endogenously in lactating dairy cows by Δ(9)-desaturase. *J Nutr* **130**, 2285–2291.
8. Mosley EE, Shafii B, Moate PJ & McGuire MA (2006) *Cis*-9, *trans*-11 conjugated linoleic acid is synthesized directly from vaccenic acid in lactating dairy cattle. *J Nutr* **136**, 570–575.
9. Shingfield KJ, Ahvenjärvi S, Toivonen V, Vanhatalo A & Huhtanen P (2007) Transfer of absorbed *cis*-9, *trans*-11 conjugated linoleic acid into milk is biologically more efficient than endogenous synthesis from absorbed vaccenic acid in the lactating cow. *J Nutr* **137**, 1154–1160.
10. Kepler CR, Hirons KP, McNeill JJ & Tove SB (1966) Intermediates and products of the biohydrogenation of linoleic acid by Butyrivibrio fibrisolvens. *J Biol Chem* **241**, 1350–1354.
11. Kemp P, White RW & Lander DJ (1975) The hydrogenation of unsaturated fatty acids by five bacterial isolates from the sheep rumen, including a new species. *J Gen Microbiol* **90**, 100–114.
12. Duckett SK, Andrae JG & Owens FN (2002) Effect of high-oil corn or added corn oil on ruminal biohydrogenation of fatty acids and conjugated linoleic acid formation in beef steers fed finishing diets. *J Anim Sci* **80**, 3353–3360.
13. Lock AL & Garnsworthy PC (2002) Independent effects of dietary linoleic and linolenic fatty acids on the conjugated linoleic acid content of cows' milk. *Anim Sci* **74**, 163–176.
14. Kucuk O, Hess BW & Rule DC (2004) Soybean oil supplementation of a high-concentrate diet does not affect site and extent of organic matter, starch, neutral detergent fiber, or nitrogen digestion, but influences both ruminal metabolism and intestinal flow of fatty acids in limit-fed lambs. *J Anim Sci* **82**, 2985–2994.
15. Kucuk O, Hess BW, Ludden PA & Rule DC (2001) Effect of forage:concentrate ratio on ruminal digestion and duodenal flow of fatty acids in ewes. *J Anim Sci* **79**, 2233–2240.
16. Sackmann JR, Duckett SK, Gillis MH, Realini CE, Parks AH & Eggelston RB (2003) Effects of forage and sunflower oil levels on ruminal biohydrogenation of fatty acids and conjugated

linoleic acid formation in beef steers fed finishing diets. *J Anim Sci* **81**, 3174–3181.

17. Shingfield KJ, Jaakkola S & Huhtanen P (2001) Effects of level of nitrogen fertiliser application and various nitrogenous supplements on milk production and nitrogen utilization of dairy cows fed grass silage-based diets. *Anim Sci* **73**, 541–554.

18. Ahvenjärvi S, Vanhatalo A, Shingfield KJ & Huhtanen P (2003) Determination of digesta flow entering the omasal canal of dairy cows using different marker systems. *Br J Nutr* **90**, 41–52.

19. Shingfield KJ, Jaakkola S & Huhtanen P (2002) Effect of forage conservation method, concentrate level and propylene glycol on diet digestibility, rumen fermentation, blood metabolite concentrations and nutrient utilization of dairy cows. *Anim Feed Sci Technol* **97**, 1–21.

20. Udén P, Colucci PE & Van Soest PJ (1980) Investigation of chromium, cerium and cobalt as markers in digesta. Rate of passage studies. *J Sci Food Agric* **31**, 625–632.

21. Huhtanen P, Brotz PG & Satter LD (1997) Omasal sampling technique for assessing fermentative digestion in the forestomach of dairy cows. *J Anim Sci* **75**, 1380–1392.

22. Ahvenjärvi S, Vanhatalo A, Huhtanen P & Varvikko T (2000) Determination of reticulo-rumen and whole-stomach digestion in lactating cows by omasal canal or duodenal sampling. *Br J Nutr* **83**, 67–77.

23. France J & Siddons RC (1986) Determination of digesta flow by continuous marker infusion. *J Theor Biol* **121**, 105–119.

24. Sukhija PS & Palmquist DL (1988) Rapid method for determination of total fatty acid content and composition of feedstuffs and feces. *J Agric Food Chem* **36**, 1202–1206.

25. Shingfield KJ, Ahvenjärvi S, Toivonen V, Arölä A, Nurmela KVV, Huhtanen P & Griinari JM (2003) Effect of dietary fish oil on biohydrogenation of fatty acids and milk fatty acid content in cows. *Anim Sci* **77**, 165–179.

26. Shingfield KJ, Reynolds CK, Hervás G, Griinari JM, Grandison AS & Beever DE (2006) Examination of the persistency of milk fatty acid composition responses to fish oil and sunflower oil in the diet of dairy cows. *J Dairy Sci* **89**, 714–732.

27. Shingfield KJ, Reynolds CK, Lupoli B, Toivonen V, Yurawecz MP, Delmonte P, Griinari JM, Grandison AS & Beever DE (2005) Effect of forage type and proportion of concentrate in the diet on milk fatty acid composition in cows fed sunflower oil and fish oil. *Anim Sci* **80**, 225–238.

28. Delmonte P, Kataoka A, Corl BA, Bauman DE & Yurawecz MP (2005) Relative retention order of all isomers of *cis/trans* conjugated linoleic acid FAME from the 6,8- to 13, 15-positions using silver ion HPLC with two elution systems. *Lipids* **40**, 509–514.

29. Jenkins TC (1993) Lipid metabolism in the rumen. *J Dairy Sci* **76**, 3851–3863.

30. Bateman HG & Jenkins TC (1998) Influence of soybean oil in high fiber diets fed to nonlactating cows on ruminal unsaturated fatty acids and nutrient digestibility. *J Dairy Sci* **81**, 2451–2458.

31. Kalscheur KF, Teter BB, Piperova LS & Erdman RA (1997) Effect of fat source on duodenal flow of *trans*-$C_{18:1}$ fatty acids and milk fat production in dairy cows. *J Dairy Sci* **80**, 2115–2126.

32. Bell JA, Griinari JM & Kennelly JJ (2006) Effect of safflower oil, flaxseed oil, monensin, and vitamin E on concentration of conjugated linoleic acid in bovine milk fat. *J Dairy Sci* **89**, 733–748.

33. Roy A, Ferlay A, Shingfield KJ & Chilliard Y (2006) Examination of the persistency of milk fatty acid composition responses to plant oils in cows given different basal diets, with particular emphasis on *trans*-C18:1 fatty acids and isomers of conjugated linoleic acid. *Anim Sci* **82**, 479–492.

34. Bauman DE & Griinari JM (2003) Nutritional regulation of milk fat synthesis. *Ann Rev Nutr* **23**, 203–227.

35. deVeth MJ, Griinari JM, Pfeiffer AM & Bauman DE (2004) Effect of CLA on milk fat synthesis in dairy cows: comparison of inhibition by methyl esters and free fatty acids, and relationships among studies. *Lipids* **39**, 365–372.

36. Lock AL, Tyburczy C, Dwyer DA, Harvatine KJ, Destaillats F, Mouloungui Z, Candy L & Bauman DE (2007) *Trans*-10 octadecenoic acid does not reduce milk fat synthesis in dairy cows. *J Nutr* **137**, 71–76.

37. Shingfield KJ & Griinari JM (2007) Role of biohydrogenation intermediates in milk fat depression. *Eur J Lipid Sci Technol* **109**, 799–816.

38. Hristov AN, Kennington LR, McGuire MA & Hunt CW (2005) Effect of diets containing linoleic acid- or oleic acid-rich oils on ruminal fermentation and nutrient digestibility, and performance and fatty acid composition of adipose and muscle tissues of finishing cattle. *J Anim Sci* **83**, 1312–1321.

39. Noble RC (1981) Digestion, transport and absorption of lipids. In *Lipid Metabolism in Ruminant Animals*, pp. 57–93 [WW Christie, editor]. Oxford, UK: Pergamon Press Ltd.

40. Doreau M & Ferlay A (1994) Digestion and utilisation of fatty acids by ruminants. *Anim Feed Sci Technol* **45**, 379–396.

41. Kramer JKG, Fellner V, Dugan MER, Sauer FD, Mossoba MM & Yurawecz MP (1997) Evaluating acid and base catalysts in the methylation of milk and rumen fatty acids with special emphasis on conjugated dienes and total *trans* fatty acids. *Lipids* **32**, 1219–1228.

42. Doreau M & Chilliard Y (1997) Digestion and metabolism of dietary fat in farm animals. *Br J Nutr* **78**, S15–S35.

43. Loor JJ, Ueda K, Ferlay A, Chilliard Y & Doreau M (2005) Intestinal flow and digestibility of *trans* fatty acids and conjugated linoleic acids (CLA) in dairy cows fed a high-concentrate diet supplemented with fish oil, linseed oil, or sunflower oil. *Anim Feed Sci Technol* **119**, 203–225.

44. Lundy FP III, Block E, Bridges WC, Bertrand JA & Jenkins TC (2004) Ruminal biohydrogenation in Holstein cows fed soybean fatty acids as amides or calcium salts. *J Dairy Sci* **87**, 1038–1046.

45. Beam TM, Jenkins TC, Moate PJ, Kohn RA & Palmquist DL (2000) Effects of amount and source of fat on the rates of lipolysis and biohydrogenation of fatty acids in ruminal contents. *J Dairy Sci* **83**, 2564–2573.

46. Noble RC, Moore JH & Harfoot CG (1974) Observations on the pattern on biohydrogenation of esterified and unesterified linoleic acid in the rumen. *Br J Nutr* **31**, 99–108.

47. Bauchart D, Legay-Carmier F, Doreau M & Gaillard B (1990) Lipid metabolism of liquid-associated and solid-adherent bacteria in rumen contents of dairy cows offered lipid-supplemented diets. *Br J Nutr* **63**, 563–578.

48. Piperova LS, Sampugna J, Teter BB, Kalscheur KF, Yurawecz MP, Ku Y, Morehouse KM & Erdman RA (2002) Duodenal and milk *trans* octadecanoic acid and conjugated linoleic acid (CLA) isomers indicate that postabsorptive synthesis is the predominant source of *cis*-9-containing CLA in lactating dairy cows. *J Nutr* **132**, 1235–1241.

49. Loor JJ, Ueda K, Ferlay A, Chilliard Y & Doreau M (2004) Biohydrogenation, duodenal flow, and intestinal digestibility of *trans* fatty acids and conjugated linoleic acids in response to dietary forage: concentrate ratio and linseed oil in dairy cows. *J Dairy Sci* **84**, 2472–2485.

50. Wąsowska I, Maia M, Niedźwiedzka KM, Czauderna M, Ramalho Ribeiro JMC, Devillard E, Shingfield KJ & Wallace RJ (2006) Influence of fish oil on ruminal biohydrogenation of C18 unsaturated fatty acids. *Br J Nutr* **95**, 1199–1211.

51. Wallace RJ, McKain N, Shingfield KJ & Devillard E (2007) Isomers of conjugated linoleic acids are synthesized via different mechanisms in ruminal digesta and bacteria. *J Lipid Res* **48**, 2247–2254.

52. Perfield JW II, Lock AL, Griinari JM, Sæbø A, Delmonte P, Dwyer DA & Bauman DE (2007) *Trans*-9, *cis*-11 conjugated linoleic acid (CLA) reduces milk fat synthesis in lactating dairy cows. *J Dairy Sci* **90**, 2211–2218.

53. Sæbø A, Sæbø P-C, Griinari JM & Shingfield KJ (2005) Effect of abomasal infusions of geometric isomers of 10,12 conjugated linoleic acid on milk fat synthesis in dairy cows. *Lipids* **40**, 823–832.

54. Kemp P, Lander DJ & Gunstone FD (1984) The hydrogenation of some *cis*- and *trans*-octadecenoic acids to stearic acid by a rumen *Fusocillus* sp. *Br J Nutr* **52**, 165–170.

55. Mosley EE, Powell GL, Riley MB & Jenkins TC (2002) Microbial biohydrogenation of oleic acid to *trans* isomers *in vitro*. *J Lipid Res* **43**, 290–296.

56. Proell JM, Mosley EE, Powell GL & Jenkins TC (2002) Isomerization of stable isotopically labeled elaidic acid to *cis* and *trans* monoenes by ruminal microbes. *J Lipid Res* **43**, 2072–2076.

57. Hudson JA, Morvan B & Joblin KN (1998) Hydration of linoleic acid by bacteria isolated from ruminants. *FEMS Microbiol Lett* **169**, 277–282.

58. Vlaeminck B, Fievez V, Cabrita ARJ, Fonseca AJM & Dewhurst RJ (2006) Factors affecting odd- and branched-chain fatty acids in milk: a review. *Anim Feed Sci Technol* **131**, 389–417.

59. Harfoot CG (1981) Lipid metabolism in the rumen. In *Lipid Metabolism in Ruminant Animals*, pp. 21–55 [WW Christie, editor]. Oxford, UK: Pergamon Press Ltd.