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Alterations in blood plasma and milk fatty acid profiles of lactating Holstein cows in response to ruminal infusion of a conjugated linoleic acid mixture

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Abstract — The production of intermediates during hydrogenation of conjugated linoleic acid (CLA) isomers was determined by infusing a CLA mixture (41% *cis*9,*trans*11-18:2 and 44% *trans*10,*cis*12-18:2) into the rumen of lactating cows. Four Holstein cows fed a basal diet were infused for 48 h with doses of 0, 45, 90, or 180 g CLA·d^{-1} into the rumen. Treatments were randomly assigned in a 4 × 4 Latin square with 4-d experimental periods, and a 7-d transition between periods. Milk samples were obtained at –12 and 0 h before infusion, and at 12 h intervals from 0 to 96 h after infusion. Milk yield and DMI were not affected by treatment. Milk fat concentration was 12% lower, causing an 18% decrease in fat yield, when 180 g CLA·d^{-1} was infused. Concentration of *trans*11-18:1 in blood plasma increased in proportion to CLA dose. *Trans*10-18:1 concentration in blood plasma also increased, and was 240% greater when CLA was infused at 180 g·d^{-1}. *Trans*10,*cis*12-18:2 was strictly a function of exogenous CLA input into the rumen, and ranged from 0.2 to 0.7 mg·g^{-1} of total plasma fatty acids. Yields of saturated 6:0 to 16:0 in milk fat decreased by 87 g·d^{-1} when 180 g CLA·d^{-1} was infused. Stearic acid concentration and yield increased by 25 and 6%, but *cis*9-18:1 yield decreased, in response to increasing dose of CLA. Yields of *trans*11-18:1 and *cis*9,*trans*11-18:2 increased in proportion to CLA dose infused. Transfer rates of infused *cis*9,*trans* 11-18:2 or *trans*10,*cis*12-18:2 into milk fat averaged 3% at the highest dose of CLA infused. Milk fat yields of *trans*10-18:1 and *trans*10,*cis*12-18:2 also increased in proportion to CLA input. Lower normalized ratios of *cis*9-18:1 to 18:0 and *cis*9,*trans*11–18:2 to *trans*11-18:1 in milk fat when CLA was infused suggested CLA reduced desaturation in the mammary gland. Results provide additional evidence that enhanced flow of *trans*10-18:1 or *trans*10,*cis*12-18:2 from the rumen may decrease milk fat yield by reducing de novo synthesis and desaturation.

***cis*9,*trans*11-18:2 / *trans*10,*cis*12-18:2 / *trans*10-18:1 / *trans*-vaccenic acid / milk fat**

Résumé — Changements des profils des acides gras dans le plasma sanguin et le lait chez des vaches allaitantes Holstein en réponse à l'infusion ruminale d'un mélange d'acide linoléique conjugué. La production de composés intermédiaires pendant l'hydrogénation des isomères de l'acide linoléique conjugué (CLA) a été déterminée en infusant un mélange de CLA (41 % *cis*9,*trans*11-18:2

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et 44 % *trans*10,*cis*12-18:2) dans le rumen de vaches laitières. Des doses de 0, 45, 90, ou 180 g CLA·j^{-1} ont été infusées pendant 48 h dans le rumen de quatre vaches Holstein alimentées avec une ration complète. Les traitements ont été répartis de façon aléatoire selon un dispositif en carré latin 4 × 4 avec des périodes expérimentales de 4 jours et une transition de 7 jours entre chaque période. Des échantillons de lait ont été prélevés à –12 et 0 h avant l'infusion et à des intervalles de 12 h de 0 à 96 h après l'infusion. La production laitière et l'ingestion de matière sèche n'ont pas été affectées par le traitement. La teneur en matière grasse du lait a été inférieure de 12 %, provoquant une diminution de 18 % de la production de matières grasses, lorsque 180 g CLA·j^{-1} ont été infusés. La concentration du *trans*11-18:1 dans le plasma sanguin a augmenté proportionnellement à la dose de CLA. La concentration plasmatique du *trans*10-18:1 a également augmenté et a été accrue de 240 % lorsque le CLA a été infusé à 180 g·j^{-1}. La concentration du *trans*10,*cis*12-18:2 a été strictement proportionnelle à l'apport de CLA exogène dans le rumen et a varié de 0,2 à 0,7 mg·g^{-1} d'acides gras totaux dans le plasma. Les productions des acides gras saturés de 6:0 à 16:0 dans la matière grasse du lait ont diminué de 87 g·j^{-1} lorsque 180 g de CLA·j^{-1} ont été infusés. La concentration et la production d'acide stéarique ont été accrues de 25 et 6 %, alors que la production du *cis*9-18:1 a diminué, en réponse à l'augmentation des doses de CLA. Les productions du *trans*11-18:1 et du *cis*9,*trans*11-18:2 ont augmenté proportionnellement à la dose de CLA infusée. Les taux de transfert dans la matière grasse du lait du *cis*9,*trans*11-18:2 et du *trans*10,*cis*12-18:2 infusés ont été en moyenne de 3 % à la dose la plus élevée de CLA infusée. Les productions du *trans*10-18:1 et du *trans*10,*cis*12-18:2 ont augmenté dans les mêmes proportions que l'apport de CLA. La diminutions des rapports du *cis*9-18:1 au 18:0 et du *cis*9,*trans*11-18:2 au *trans*11-18:1 dans la matière grasse du lait lorsque le CLA est infusé suggère que le CLA a réduit le processus de désaturation dans la glande mammaire. Les résultats mettent en évidence que l'augmentation du flux du *trans*10-18:1 ou du *trans*10,*cis*12-18:2 à partir du rumen peut diminuer la production de matière grasse du lait en réduisant la synthèse de novo et la désaturation.

***Cis*9,*trans*11-18:2 / *trans*10,*cis*12-18:2 / *trans*10-18:1 / acide *trans*-vaccénique / matière grasse du lait**

1. INTRODUCTION

The *cis*9,*trans*11 isomer of conjugated linoleic acid (CLA), accounts for nearly 90% of total CLA found in milk fat [18]. *Cis*9,*trans*11-18:2 results from isomerization, via *cis*12,*trans*11-isomerase [9, 10], of dietary 18:2n6 by rumen microorganisms during the first step of the biohydrogenation process [8]. Accumulation of *trans*11-18:1 and *cis*9,*trans*11-18:2 in vitro, however, was lower when triglyceride-bound 18:2n6 was the substrate compared with the free fatty acid [15].

Diet affects the individual profiles of *trans*-18:1 or conjugated 18:2 isomers produced during fermentation. Feeding supplemental soybean oil resulted in greater duodenal flows of *trans*-18:1 with double bonds at positions 6 through 16 [3]. The output of *cis*9,*trans*11-18:2 in effluents from rumen fermenters fed fresh forage ranged from 9 to 23% of total conjugated-18:2 isomer output, and averaged 17% during digestion of a mixed diet plus supplemental 18:2n6 [4, 12]. *Trans*10,*cis*12-18:2 accounted for 7 or 16% of total conjugated isomer output when fresh forage or the mixed diet were the DM input [4, 12]. Outputs of *cis*9,*cis* 11-18:2,*trans*11,*trans*13-18:2, and a mixture of *trans*,*trans*-18:2 isomers were predominant regardless of diet fed [4, 12].

In terms of mammary lipid metabolism, identification of the precursors which give rise to the production of 18:1 and 18:2 isomers with a *trans*10 double bond in the rumen is of interest because these isomers may depress milk fat synthesis [6, 19]. It is well established that production of *trans* 10-18:1 in the rumen is enhanced when high-grain diets containing supplemental oil are fed to dairy cows [6, 19]. However,

it is not clear if *trans*10,*cis*12-18:2 is a required precursor for the formation of *trans*10-18:1. We showed [12] that production of *trans*10-18:1 in rumen fermenters is directly proportional to *cis*9-18:1 input from corn grain, but corn grain also contains substantial amounts of 18:2n6. One way to verify if *trans*10-18:1 can be formed during hydrogenation of *trans*10,*cis*12-18:2 in vivo, is to enhance the availability of the CLA in the rumen by infusing a mixture of CLA which contains substantial amounts of *trans*10,*cis*12-18:2. Our objective was to evaluate the extent of hydrogenation of *cis*9,*trans*11-18:2 and *trans*10,*cis*12-18:2 in response to doses of a CLA mixture infused into the rumen.

2. MATERIALS AND METHODS

2.1. Animals and diets

Four early-lactation primiparous Holstein cows (between 48 and 60 d post-calving) with a rumen cannula were utilized in a 4×4 Latin square design with four 4-d periods to evaluate responses to 0, 45, 90, or 180 g CLA infused continuously into the rumen for 2-d. During infusion, cows were housed in a tie-stall barn and their basal diet was prepared and offered in equal amounts at 14.00 and 02.00 h daily. Feed refusals were removed daily at 12.00 h and 01.00 h and weighed. Daily feed allotment was calculated to allow 5 to 10% feed refusals. Cows were milked each day at 13.00 and 01.00 h.

This basal diet was formulated using Dair4 [22] to meet or exceed nutrient requirements of cows producing 34 kg milk and consuming 19 kg of dry matter daily [14]. The concentrate portion of the diet was mixed in 500 kg batches, stored in sealed plastic containers, and removed as needed to mix with the forage on a daily basis (Tab. I). The experimental protocol was reviewed and approved by the Virginia Polytechnic Institute and State University Animal Care Committee.

2.2. CLA infusion

Conjugated linoleic acid (CLA-90, Natural Lipids, Norway) contained 90% nonesterified CLA, with *cis*9,*trans*11-18:2 (410 $mg \cdot g^{-1}$ total fatty acids) and *trans*10,*cis*12-18:2 (440 $mg \cdot g^{-1}$) being the

Table I. Composition of the basal diet[1].

	$g \cdot kg^{-1}$ DM
Ingredient	
Alfalfa silage	315
Corn silage	136
Orchardgrass hay	70
Ground corn	351
Soybean meal, 48%crude protein	80
SoyPlus[2]	34
Mineral/vitamin mix[3]	7
Limestone	5
Dicalcium phosphate	2
Chemical composition	
NDF	307
ADF	195
Crude protein	171
Total fatty acids	30
	$mg \cdot g^{-1}$ of total fatty acids
12:0	2
14:0	1
16:0	134
*cis*9-16:1	2
18:0	34
*cis*9-18:1	262
18:2n6	499
18:3n3	66

[1] Four samples (collected in each period) of forages and supplements were composited and analyzed in duplicate.
[2] SoyPlus® (West Central Cooperative, Ralston, IA, USA): Crude protein = 483 $g \cdot kg^{-1}$ DM, fatty acids = 48 $g \cdot kg^{-1}$ DM.
[3] Mineral/vitamin mix (Southern States Cooperative, Richmond, VA, USA): salt (38-48 $g \cdot kg^{-1}$), $NaHCO_3$ (180 $g \cdot kg^{-1}$), Ca (145-174 $g \cdot kg^{-1}$), P (65 $g \cdot kg^{-1}$), Cl (58 $g \cdot kg^{-1}$), S (32 $g \cdot kg^{-1}$), Mg (22 $g \cdot kg^{-1}$), K (35 $g \cdot kg^{-1}$), Mn (1 $g \cdot kg^{-1}$), Zn (1 $g \cdot kg^{-1}$), Fe (0.3 $g \cdot kg^{-1}$), Cu (0.1 $g \cdot kg^{-1}$), I (0.02 $g \cdot kg^{-1}$), Co (0.003 $g \cdot kg^{-1}$), Se (0.005 $g \cdot kg^{-1}$), F (0.65 $g \cdot kg^{-1}$), retinyl acetate (0.36 $g \cdot kg^{-1}$), cholecalciferol (0.01 $g \cdot kg^{-1}$), dl-α-tocopherol acetate (0.59 $g \cdot kg^{-1}$).

primary isomers. Concentrations of *cis*9,*cis* 11-18:2,*trans*9,*trans*11+*trans*10,*trans* 12-18:2, and *cis*10,*cis*12-18:2 averaged 18, 20, and 12 mg·g^{-1}.

The CLA mixture (0, 45, 90, or 180 g CLA·d^{-1}) was emulsified in skim milk to ensure a uniform supply of CLA during the 48 h infusion. Emulsions were prepared the day prior to infusion by combining the desired amount of CLA with 0.23 g glycerol (Eastman Kodak Co., Rochester, NY, USA)·g^{-1} CLA and 0.12 g soy lecithin powder (Refined, Alfa®, Ward Hill, MA, USA)·g^{-1} CLA in 972.5 mL skim milk at room temperature. The mixture was homogenized at 12000 rpm for 2 min with a Polytron® PT 10/35 homogenizer (Brinkmann Instruments, Westbury, NY, USA), and checked for the presence of clumps before stirring at medium-to-high speed for 30 min at room temperature. Emulsions were dispensed into 1 L Viaflex® plastic bags (Baxter Corporation, Deerfield, IL, USA) and stored at 4 °C until infusion. Ruminal infusion of CLA began at 14.00 h in each period.

During infusion, bags containing CLA emulsions were attached to a flat platform on a wrist-action shaker (Burrell Corporation, Pittsburgh, PA, USA) set at low speed. Emulsions were infused via Tygon® tubing (1.6 mm i.d., 0.8 mm wall; Fisher Scientific Co., Pittsburgh, PA, USA) that passed through a Harvard Peristaltic pump (55-1762; Harvard Apparatus, South Natick, MA, USA). Flow from the pump was via Tygon® tubing (3.2 mm i.d., 1.6 mm wall) that passed through the rumen cannula and into the rumen. A perforated Nalgene® plastic bottle (60 mL) was attached to the end of the tubing. The tubing was primed with 15 mL infusate at the start of infusion, and flow rate was set at 41.7 mL·h^{-1}.

2.3. Sampling, measurements, and analysis

Forages and concentrate were sampled during the last day of each experimental period. Samples were dried in a forced-air oven at 60 °C, then stored in sealed plastic containers. Equal amounts of samples from each period were combined to determine chemical composition. In preparation for analyses, dried forages and concentrate were ground first through a 2 mm screen (Thomas-Wiley Laboratory Mill), then through a 1 mm screen in a Cyclotec mill (Tecator 1093, Hoganas, Sweden). Forages and concentrates were analyzed for ADF and NDF [23] and total N [1].

Milk was collected in a stainless steel bucket, weighed, and thoroughly mixed prior to obtaining samples every 12 h from –12 h before infusion through 96 h relative to the start of infusion. A 30 mL aliquot was collected in a 50 mL vial containing Bronopol (milk preservative; D & F Control Systems, San Ramon, CA, USA) immediately after milking. Milk was analyzed for milk fat, protein, lactose, and solids-not-fat (SNF) by infrared analysis with a 4-channel spectrophotometer (Virginia Dairy Herd Improvement Association, VA, USA). An additional aliquot of milk without Bronopol also was collected, then frozen at –20 °C. Subsequently, samples were thawed at room temperature and centrifuged at $10000 \times g$ for 1 h to isolate milk fat.

Blood samples (10 mL) were obtained from the coccygeal artery immediately after the collection of milk samples. After collection, blood was transferred to tubes containing 286 IU heparin in 100 µL of sterile saline and centrifuged at $3000 \times g$ for 15 min for harvesting plasma.

Plasma lipids were extracted with chloroform/methanol (2:1, vol/vol). Fatty acids in forages, concentrate, milk fat, and blood plasma lipids were methylated by in situ transesterification with 0.5N methanolic NaOH followed by 14% boron trifluoride in methanol as described by Park and Goins [17]. Undecenoate (Nu-Check Prep, Elysian, MN, USA) was used as the internal standard. Samples were injected by autosampler into a Hewlett-Packard 5890A gas

chromatograph equipped with a flame ionization detector (Hewlett-Packard, Sunnyvale, CA, USA). Methyl esters of fatty acids were separated on a 100 m × 0.25 mm i.d. fused silica capillary column (CP-Sil 88 Chrompack, Middelburg, The Netherlands). Pure methyl ester standards (Nu-Check Prep, Elysian, MN, USA; Supelco Inc., Bellefonte, PA, USA) were used to identify peaks, and determine correction factors for individual fatty acids.

For fatty acid analysis of milk fat, forage, and concentrate (0.5 μL methyl esters in hexane injected at a 35:1 split ratio) the injector temperature was maintained at 250 °C and the detector temperature was maintained at 255 °C. The initial oven temperature was 70 °C (held for 1 min) and increased 5 °C·min^{-1} to 100 °C (held for 2 min), 10 °C·min^{-1} to 175 °C (held for 40 min), and then increased 5 °C·min^{-1} to a final temperature of 225 °C (held for 15 min). Hydrogen was the carrier gas.

Analysis of blood plasma fatty acids required injection of 2 μL methyl esters (splitless). The injector temperature was maintained at 150 °C and the detector temperature at 255 °C. The purge valve on the GC was closed for 1.5 min after sample injection. The initial column temperature was 40 °C (held for 1.5 min) and increased 40 °C·min^{-1} to 100 °C (held for 10 min), 25 °C·min^{-1} to 175 °C (held for 70 min), and then increased 10 °C·min^{-1} to a final temperature of 220 °C (held for 20 min). Ultra pure helium was the carrier gas.

2.4. Statistical analysis

Data for dry matter and fatty acid intake, milk production and composition, plasma fatty acid profiles, milk fatty acid yields, and normalized ratios of milk fatty acids are reported as Least squares means ± SEM. All data, except plasma fatty acid profiles, were analyzed as a 4 × 4 Latin square with repeated measures using the MIXED procedure of SAS [21]. Observations obtained at –12 and 0 h were averaged and served as a covariate for observations at 12, 24, 36, 48, 60, 72, 84, or 96 h. Main effects in the model included covariate adjustment, cow, period, CLA dose, time, time by CLA dose interaction, and residual error. For plasma fatty acid profiles, main effects in the model included cow, period, CLA dose, and residual error. Linear and quadratic contrasts were used to determine differences due to CLA infusion. Overall differences between treatment means were considered to be significant when $P \leq 0.05$. However, all *P*-values are presented in tables.

3. RESULTS

3.1. Diet composition

Total fatty acid content of the basal diet was 30 g·kg^{-1} (Tab. I). Linoleic acid accounted for 500 mg·g^{-1} of total fatty acids, and *cis*9-18:1 and 18:3n3 for 260 or 70 mg·g^{-1} of total fatty acids. The primary sources of fatty acids were SoyPlus® and ground corn, which provided the majority of supplemental 18:2n6. Forages contributed primarily 18:3n3.

3.2. Fatty acid intake, dry matter intake, and milk production

Estimated intake of total fatty acids increased in proportion with CLA dose due to the combination of the amounts of CLA infused (Tab. II) and variations in dry matter intake (DMI) (Tab. III). Daily DMI and milk yields were not affected by dose of CLA, and averaged 19 or 31 kg·d^{-1} (Tab. III). Because of numerically lower milk yield as the dose of CLA infusion increased, yields of protein, lactose, and SNF in milk also decreased. Milk fat percentage and yield decreased by 13 and 16% due primarily to CLA infusion at 180 g·d^{-1}. At this rate of CLA infusion, concentration of milk fat (Fig. 1A) decreased markedly from

Table II. Estimated daily fatty acid intake by cows infused continuously into the rumen for 2 d with 0, 45, 90, or 180 g·d^{-1} of a conjugated linoleic acid (CLA) mixture[1].

	CLA (g·d^{-1})					Effect[2]		
	0	45	90	180	SEM	CLA	L	Q
Fatty acid	g·d^{-1}							
14:0	0.8	0.8	0.8	0.8	0.04	0.33	0.67	0.54
16:0	73.6	78.7	74.4	73.6	3.4	0.33	0.67	0.55
*cis*9-16:1	1.1	1.2	1.1	1.1	0.03	0.33	0.67	0.56
18:0	18.0	23.1	26.2	23.8	0.8	0.01	0.01	0.29
*cis*9-18:1	137.3	146.2	138.6	137.3	7.0	0.39	0.89	0.48
18:2 isomers								
*cis*9,*cis*12	264.4	282.8	267.1	264.6	13.8	0.32	0.66	0.44
*cis*9,*trans*11	0.0	20.4	40.8	81.5	...	...	...	...
*trans*10,*cis*12	0.0	21.8	43.7	87.5	...	...	...	...
*cis*9,*cis*11	0.0	0.8	1.5	3.0	...	...	...	...
*cis*10,*cis*12	0.0	0.5	1.0	2.0	...	...	...	...
trans,*trans* [3]	0.0	0.9	1.9	3.8	...	...	...	...
other	0.0	0.6	1.1	2.2	...	...	...	...
18:3n3	19.8	20.5	19.4	19.5	1.0	0.33	0.67	0.45
Total	558.8	605.1	599.2	651.2	19.1	0.01	0.01	0.82

[1] Values are the average of means obtained during CLA infusion, and include the daily amount of CLA isomers infused.
[2] Overall effect due to CLA, and linear (L) or quadratic (Q) effects of CLA dose.
[3] *trans*9,*trans*11 + *trans*10,*trans*12.

36 through 84 h, and remained below pre-infusion levels by 96 h.

3.3. Blood plasma fatty acid profiles

Overall, total plasma fatty acid concentrations did not differ due to CLA dose and averaged 1132 μg·mL^{-1} at the end of the 48-h ruminal infusion (Tab. IV). Among 18:1 isomers derived from hydrogenation of exogenous *cis*9,*trans*11-18:2 and *trans*10,*cis*12-18:2, concentrations of *trans*10-18:1 and *trans*11-18:1 increased in proportion to dose of CLA infused. Compared with basal (0 g CLA·d^{-1}), however, the extent of the increase was greater for *trans*10-18:1 (+225%) than *trans*11-18:1 (+68%) when 180 g CLA·d^{-1} were infused. Concentrations of *trans*10,*cis*12-18:2 also were proportional to CLA dose, and averaged 0, 0.2, 0.5, or 0.7 μg·mL^{-1} when 0, 22, 44, or 88 g *trans*10,*cis*12-CLA·d^{-1} were infused. Although not statistically significant, the concentration of *cis*9,*trans*11-18:2 was 33% (0.5 μg·mL^{-1}) greater due to infusion of 180 g CLA·d^{-1} compared with basal levels.

3.4. Milk fatty acid yields

Total milk fatty acid yield during the 96 h period relative to basal levels decreased 13% when the 180 g CLA·d^{-1} dose was infused for 48 h (Tab. V). The decrease was primarily due to a 22% reduction in yields of saturated fatty acids with 6 to 16 carbons. Concentration of these fatty acids in response to 180 g CLA·d^{-1} infused decreased linearly

Table III. Dry matter intake (DMI) and milk production, composition, and component yields by cows infused continuously into the rumen for 2 d with 0, 45, 90, or 180 g·d^{-1} of a conjugated linoleic acid (CLA) mixture[1].

	CLA (g·d^{-1})					Effect[2]		
	0	45	90	180	SEM	CLA	L	Q
Item	kg·d^{-1}							
DMI	18.6	19.4	18.4	18.4	0.8	0.33	0.67	0.45
Milk yield	32.0	31.4	31.6	30.7	0.9	0.08	0.06	0.54
Composition	%							
Fat	3.53	3.61	3.57	3.11	0.10	0.01	0.02	0.01
Protein	2.82	2.80	2.83	2.80	0.05	0.44	0.50	0.56
Lactose	4.68	4.65	4.66	4.64	0.04	0.03	0.01	0.85
SNF	8.25	8.19	8.24	8.19	0.07	0.07	0.03	0.90
Yield	kg·d^{-1}							
Fat	1.14	1.12	1.14	0.95	0.04	0.01	0.01	0.01
Protein	0.90	0.88	0.90	0.84	0.02	0.01	0.01	0.13
Lactose	1.50	1.46	1.48	1.41	0.04	0.02	0.01	0.31
SNF	2.64	2.58	2.62	2.46	0.05	0.02	0.01	0.24

[1] Values are the average of means obtained every 12 h from 12 through 96 h after the start of infusions.
[2] Overall effect due to CLA, and linear (L) or quadratic (Q) effects of CLA dose.

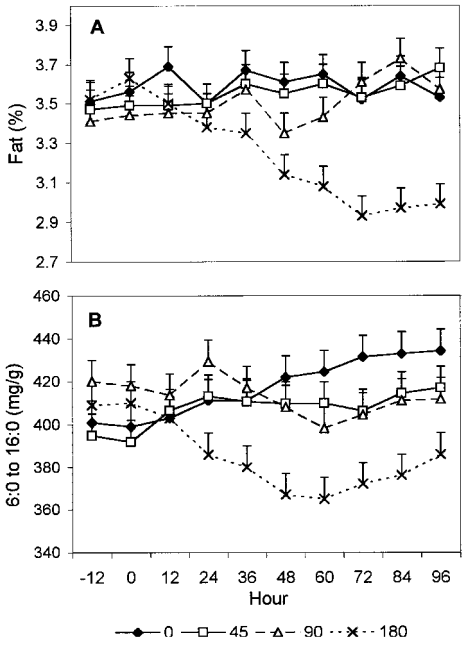

Figure 1. Milk fat percentage (**A**) and concentration (**B**) of saturated 6:0 to 16:0 fatty acids in milk fat from cows infused continuously into the rumen for 2 d with 0, 45, 90, or 180 g·d^{-1} of a conjugated linoleic acid (CLA) mixture. Values are means plus pooled SEM for four cows at each 12 h milking interval. Infusion of 180 g CLA·d^{-1} decreased ($P < 0.05$) milk fat percentage and concentrations of saturated 6:0 to 16:0 fatty acids in milk fat.

from 12 through 60 h (Fig. 1B) and, similar to milk fat percentage, did not return to pre-infusion levels by 96 h. The yield of 18:0, however, was greater due to infusion of CLA. A marked increase in concentration of 18:0 (Fig. 2A), was observed from 12 to 60 h of 180 g CLA·d^{-1} infusion. In contrast, yield of *cis*9-18:1 (derived in part from 18:0

Table IV. Fatty acid concentrations in blood plasma from cows infused continuously into the rumen for 2 d with 0, 45, 90, or 180 g·d^{-1} of a conjugated linoleic acid (CLA) mixture[1].

	CLA (g·d^{-1})					Effect[2]		
	0	45	90	180	SEM	CLA	L	Q
Fatty acid	µg·mL^{-1}							
14:0	4.6	4.1	5.3	4.1	0.4	0.26	0.25	0.57
*cis*9-14:1	2.1	1.1	2.6	1.6	1.3	0.66	0.29	0.86
16:0	115	111	134	100	17	0.40	0.25	0.74
*cis*9-16:1	16	12	17	12	2	0.22	0.17	0.85
*trans*9-16:1	2.2	1.4	2.4	1.9	0.4	0.37	0.62	0.70
18:0	132	120	151	117	18	0.27	0.43	0.39
Cis 18:1								
9	101	91	112	91	12	0.55	0.53	0.63
11	4.5	4.0	5.0	3.8	0.5	0.26	0.28	0.45
12	4.1	3.3	4.9	3.9	0.5	0.13	0.68	0.82
13	0.9	0.6	1.1	0.7	0.1	0.01	0.05	0.16
15	0.8	0.8	1.1	0.8	0.1	0.03	0.52	0.09
Trans 18:1								
6, 7, 8	0.3	0.3	0.4	0.6	0.2	0.49	0.22	0.60
9	0.3	0.3	0.4	0.4	0.1	0.48	0.35	0.77
10	0.4	0.6	0.9	1.3	0.2	0.02	0.01	0.58
11	3.1	3.2	4.4	5.2	0.4	0.04	0.02	0.46
12	1.1	1.0	1.4	1.2	0.2	0.37	0.85	0.70
13, 14	1.5	1.4	2.1	1.8	0.2	0.15	0.29	0.68
16	0.7	0.7	1.1	0.8	0.2	0.05	0.25	0.16
Isolated 18:2								
*cis*9,*cis*12	652	540	686	543	90.1	0.32	0.26	0.81
*trans*9,*trans*12	0.2	0.1	0.2	0.2	0.1	0.40	0.70	0.47
*trans*9,*cis*12	1.5	1.8	1.3	1.7	0.3	0.59	0.59	0.79
*trans*11,*cis*15	1.5	1.0	1.5	0.9	0.1	0.01	0.01	0.36
Conjugated 18:2								
*cis*9,*trans*11	1.5	1.2	2.0	2.0	0.3	0.23	0.25	0.61
*trans*10,*cis*12	0.0	0.2	0.5	0.7	0.02	0.01	0.01	0.92
18:3n3	59	48	62	47	7.3	0.31	0.23	0.77
20:3n3	18	15	21	15	4.7	0.17	0.31	0.49
20:4n6	32	26	35	26	7.0	0.35	0.31	0.79
20:5n3	16	13	18	14	4.3	0.38	0.50	0.98
Total	1205	1016	1287	1021	164	0.32	0.30	0.75

[1] Values are the average of means obtained at the end of 48 h of infusion.
[2] Overall effect due to CLA, and linear (L) or quadratic (Q) effects of CLA dose.

Table V. Milk fatty acid yields by cows infused continuously into the rumen for 2 d with 0, 45, 90, or 180 g·d^{-1} of a conjugated linoleic acid (CLA) mixture[1].

	CLA (g·d^{-1})					Effect[2]		
	0	45	90	180	SEM	CLA	L	Q
Fatty acid	g·d^{-1}							
4:0	48.2	49.4	46.8	43.0	2.0	0.01	0.01	0.02
6:0	23.6	23.4	23.6	19.0	0.6	0.01	0.01	0.01
8:0	11.6	11.2	11.6	8.6	0.2	0.01	0.01	0.01
10:0	21.6	20.0	21.4	15.0	0.6	0.01	0.01	0.01
12:0	22.6	21.0	22.4	16.2	0.6	0.01	0.01	0.01
14:0	86.6	83.0	82.8	65.6	1.1	0.01	0.01	0.01
*cis*9-14:1	7.2	6.4	6.6	5.2	0.2	0.01	0.01	0.04
16:0	234.2	228.8	222.4	185.6	5.2	0.01	0.01	0.01
6:0 to16:0	399.4	385.2	384.6	312.4	8.4	0.01	0.01	0.01
*cis*9-16:1	18.4	18.2	17.4	14.4	0.5	0.01	0.01	0.01
*trans*9-16:1	4.2	4.4	4.2	3.8	0.2	0.03	0.07	0.02
18:0	113.6	127.8	120.6	120.6	4.1	0.01	0.04	0.01
Cis 18:1								
9	266.2	276.2	271.2	243.4	6.2	0.01	0.01	0.01
11	10.0	10.2	10.0	8.8	0.3	0.01	0.01	0.01
12	6.6	6.2	6.2	6.2	0.2	0.67	0.28	0.61
13	4.2	4.2	3.8	3.4	0.3	0.17	0.07	0.43
15	2.2	2.4	2.2	2.2	0.2	0.65	0.52	0.72
Trans 18:1								
6, 7, 8	2.2	2.4	2.4	2.4	0.04	0.01	0.01	0.14
9	1.6	1.8	1.8	1.8	0.05	0.05	0.01	0.37
10	2.2	2.6	2.8	3.8	0.1	0.01	0.01	0.01
11	8.2	8.6	8.8	10.2	0.1	0.01	0.01	0.07
12	2.0	2.2	2.4	2.8	0.04	0.01	0.01	0.26
13, 14	4.0	4.8	5.4	5.6	0.1	0.01	0.01	0.27
16	2.8	2.8	3.0	3.0	0.1	0.21	0.08	0.70
Isolated 18:2								
*cis*9,*cis*12	25.8	26.8	25.2	23.0	0.3	0.01	0.01	0.01
*trans*9,*trans*12	0.2	0.2	0.2	0.2	0.02	0.16	0.34	0.79
*trans*11,*cis*15	1.2	1.2	1.2	0.8	0.02	0.01	0.13	0.02
Conjugated 18:2								
*cis*9,*trans*11	6.2	6.0	6.4	6.6	0.1	0.04	0.04	0.31
*trans*10,*cis*12	0.0	0.2	0.6	1.0	0.02	0.01	0.01	0.13
18:3n3	4.4	4.6	4.4	3.8	0.04	0.01	0.01	0.01
20:3n3	0.6	0.8	0.8	0.6	0.03	0.28	0.69	0.06
20:4n6	1.4	1.4	1.6	1.2	0.03	0.04	0.38	0.01
20:5n3	0.1	0.1	0.1	0.1	0.02	0.16	0.03	0.97
Total	947.0	964.4	940.0	825.0	15.0	0.01	0.01	0.01

[1] Values are the average of means obtained every 12 h from 12 through 96 h after the start of infusions.
[2] Overall effect due to CLA, and linear (L) or quadratic (Q) effects of CLA dose.

desaturation) decreased when the dose of CLA was 180 g. Compared with the control infusion, yields of *cis*9,*trans*11-18:2 were only 6% greater when 90 or 180 g CLA were infused. Supplemental CLA was the primary source of *trans*10,*cis*12-18:2 in the rumen, and resulted in yields of 0.2, 0.6, and 1 g·d^{-1} when 22, 44, or 88 g *trans*10,*cis*12-CLA·d^{-1}, respectively, were infused. Greater yields of this CLA corresponded with its gradual incorporation into milk fat. Concentrations of *trans*10,*cis*12-18:2 (Fig. 2B) increased from non-detectable levels at –12 or 0 h before infusion to 0.5, 1.1, or 2.2 mg·g^{-1} at 60 h of due to infusion of 22, 44, or 88 g *trans*10, *cis*12-18:2·d^{-1}. All doses of CLA increased the yields of most *trans*-18:1 isomers compared with the control infusion without CLA. *Trans*10-18:1 concentration (Fig. 2C), in particular, increased gradually from 12 through 60 h of CLA infusion, but the response was more pronounced due to 180 g CLA.

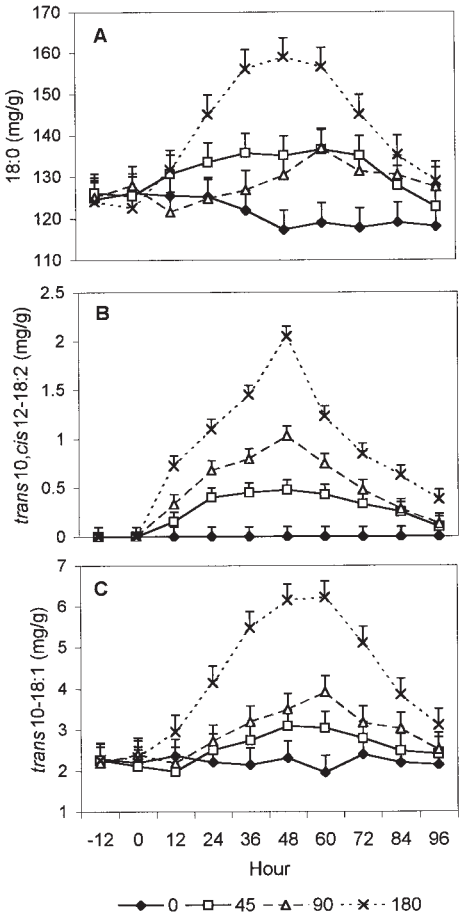

Figure 2. Concentrations of 18:0 (**A**), *trans*10,*cis*12-18:2 (**B**), and *trans*10-18:1 (**C**) in milk fat from cows infused continuously into the rumen for 2 d with 0, 45, 90, or 180 g·d^{-1} of a conjugated linoleic acid (CLA) mixture. Values are means plus pooled SEM for four cows at each 12 h milking interval. Incremental infusion of CLA resulted in a linear increase ($P < 0.05$) in concentrations of 18:0, *trans*10,*cis*12-18:2, and *trans*10-18:1 in milk fat.

3.5. Normalized ratios of milk fatty acids

Normalized ratios (mg·g^{-1} product/ [mg·g^{-1} substrate + mg·g^{-1} product]) [16] were estimated to assess the relative extent of desaturation of specific fatty acids during milk fat synthesis in response to elevated amounts of *trans*10,*cis*12-18:2 or *cis*9,*trans* 11-18:2 [5, 12]. The ratios of *cis*9-14:1 to 14:0 (mg·g^{-1} *cis*9-14:1/[mg·g^{-1} 14:0 + mg·g^{-1} *cis*9-14:1]), *cis*9-18:1 to 18:0, and *cis*9,*trans* 11-18:2 to *trans*11-18:1 decreased due to CLA infusion, primarily at the 180 g dose (Tab. VI).

4. DISCUSSION

Our experiment evaluated the quantitative significance of ruminal availability of *cis*9,*trans*11-18:2 and *trans*10,*cis*12-18:2 on their secretion in milk fat. Plasma fatty acid profiles and milk fatty acid yields were used to assess changes in the production of hydrogenation intermediates. Milk fatty acid data also provided the means to evaluate apparent changes in lipogenesis and desaturation in the mammary gland due to exogenous CLA. Daily DMI or milk yield were not affected by CLA dose.

Table VI. Normalized ratios[1] of fatty acids in milk fat from cows infused continuously into the rumen for 2 d with 0, 45, 90, or 180 g·d^{-1} of a conjugated linoleic acid (CLA) mixture[2].

	CLA (g·d^{-1})					Effect[3]		
	0	45	90	180	SEM	CLA	L	Q
Ratio								
14:1 ⇒ 14:0	0.079	0.072	0.074	0.073	0.002	0.04	0.03	0.12
16:1 ⇒ 16:0	0.072	0.072	0.072	0.072	0.004	0.96	0.74	0.79
18:1 ⇒ 18:0	0.70	0.68	0.69	0.66	0.003	0.01	0.01	0.90
*cis*9,*trans*11-18:2 ⇒ *trans*11-18:1	0.43	0.41	0.42	0.39	0.004	0.01	0.01	0.94
20:4n6 ⇒ 18:2n6	0.049	0.053	0.058	0.049	0.003	0.11	0.83	0.03
20:5n3 ⇒ 18:3n3	0.059	0.054	0.054	0.046	0.004	0.38	0.10	0.79

[1] Normalized ratio = mg·g^{-1} product/[mg·g^{-1} substrate + mg·g^{-1} product].
[2] Values are the average of means obtained every 12 h from 12 through 96 h after the start of infusions.
[3] Overall effect due to CLA, and linear (L) or quadratic (Q) effects of CLA dose.

Despite CLA infusion, the concentration of *cis*9,*trans*11-18:2 in blood plasma did not increase significantly. Concentration of *trans*11-18:1 in plasma, however, increased with each increment of CLA infused (Tab. IV). Yields of *trans*11-18:1 and *cis*9,*trans*11-18:2 in milk fat increased in proportion to CLA dose (Tab. V). Thus, the greatest (3%) apparent transfer efficency [(g·d^{-1} *trans*11-18:1 + g·d^{-1} *cis*9,*trans*11-18:2 in milk fat)/g infused *cis*9,*trans*11-18:2] of infused *cis*9,*trans*11-18:2 into milk fat was obtained when 180 g CLA were infused. Greater availability of exogenous *cis*9,*trans*11-18:2 in the rumen may have overcome the capacity for microbes to hydrogenate it completely. Polan et al. [20], first noted that hydrogenation of 18:2n6 to 18:0 in strained rumen fluid decreased linearly as the concentration of 18:2n6 substrate in the incubation increased. Isomers of 18:1, however, accumulated up to the point where concentration of 18:2n6 in the medium was 3-fold greater than basal. When 18:2n6 concentration was 8-fold greater than basal, hydrogenation was only 12% [20]. A recent study confirmed that *Butirivibrio fibrisolvens* A38 produced significant amounts of *cis*9,*trans*11-18:2 when the concentration of 18:2n6 was high enough to inhibit hydrogenation of 18:1 isomers to 18:0 [11]. Because *trans*11-18:1 could be desaturated to *cis*9,*trans*11-18:2 in the mammary gland [5], it also could serve as an alternate source for endogenous synthesis of *cis*9,*trans*11-18:2. However, the lower ratio of *cis*9,*trans*11-18:2 to *trans*11-18:1 (Tab. VI) in response to 180 g CLA suggests that desaturation of rumen-derived *trans*11-18:1 ⇒ *cis*9,*trans*11-18:2 was inhibited, possibly by greater uptake of *trans*10,*cis*12-18:2 [5, 12].

*Trans*12-18:1 and *trans*13/14-18:1 yields in milk fat increased in proportion to CLA dose. Exogenous *cis*9,*trans*11-18:2 and *trans*10,*cis*12-18:2 accounted for 85% of total CLA isomers infused, and it could be possible that *trans*12-18:1 and *trans*13/14-18:1 were derived from the isomerization of end products which accumulated during hydrogenation. *Trans*-18:1 isomers produced during hydrogenation studies with *B. fibrisolvens* reflected in part the double bond positions of the substrates. Thus, hydrogenation of 18:2n6 led to production of *trans*11-18:1, primarily, but *trans*9-18:1 also accumulated [8]. However, incubating a

mixture of *cis*9,*trans*11-18:2 (39% of total fatty acids), *trans*10,*cis*12-18:2 (3%), and *cis*8,*trans*10-18:2 (54%) resulted in accumulation of *trans*8-18:1 (28% of total fatty acids recovered), *trans*9-18:1 (7%), *trans* 10-18:1 (10%), *trans*11-18:1 (46%), and *trans*12-18:1 (9%) [8]. Isomerization of the *cis*8- double bond followed by hydrogenation of the *trans*10 double bond in *cis*8,*trans*10-18:2 may have led to substantial accumulation of *trans*8-18:1. In contrast, hydrogenation of the *cis*9 double bond in *cis*9,*trans*11-18:2 seemed to be primarily responsible for accumulation of *trans*11-18:1. The position of a *cis* double bond in a CLA molecule could be a factor determining the profile of *trans*-18:1 isomers produced in the rumen. Although *B. fibrisolvens* accounts for a large number of total rumen bacteria, numerous isomers also can be produced during hydrogenation of unsaturated fatty acids by other strains of bacteria [7] suggesting microorganisms may possess isomerases other than *cis*12,*trans*11-isomerase [9].

Infused CLA was the major source of *trans*10,*cis*12-18:2 in blood plasma or milk fat. However, concentrations of *trans*10-18:1 and *trans*10,*cis*12-18:2 in plasma and yields in milk fat increased in proportion to CLA infused. The greater response in *trans*10-18:1 (Fig. 2C) resulted from partial hydrogenation of exogenous *trans*10, *cis*12-18:2, shown in vitro by Kepler et al. [8], as availability of the CLA in the rumen increased. Similar to *cis*9,*trans*11-18:2, however, availability of *trans*10,*cis*12-18:2 was large enough to prevent complete hydrogenation (Fig. 2B). The apparent transfer efficiency [($g \cdot d^{-1}$ *trans*10-18:1 + $g \cdot d^{-1}$ *trans*10,*cis*12-18:2 in milk fat)/g infused *trans*10,*cis*12-18:2] of infused *trans*10,*cis* 12-18:2 into milk fat during the 96 h period was highest (3%) when 180 g CLA were infused.

Milk fat percentage and yield decreased significantly when 180 g CLA were infused relative to basal levels. Yields of saturated 6:0 to 16:0 in milk fat also decreased. Responses were a function of lower concentrations of milk fat or 6:0 to 16:0 fatty acids (Fig. 1A,B), as the concentrations of *trans*10,*cis*12-18:2 or *trans*10-18:1 in milk fat increased (Fig. 2B,C). The overall effect, was a reduction in total fatty acid yields (Tab. V). Lower fat concentration and yields of short and medium-chain fatty acids were previously observed when the concentrations of *trans*10-18:1 [6, 19] or *trans*10, *cis*12-18:2 [2, 12] in milk fat increased. The reduction in milk fat percentage due to *trans*10-18:1 and *trans*10,*cis*12-18:2 was directly proportional to lower fatty acid synthase and acetyl-CoA carboxylase activities in mammary tissue [19]. Based on the level reported to decrease milk fat synthesis [2, 12], however, *trans*10,*cis*12-18:2 appears to be a more potent inhibitor than *trans*10-18:1. The greater yields of *trans*10-18:1 and *trans*10,*cis*12-18:2 observed at the 180 g CLA dose, were proportional to lower milk fat percentage, lower milk fat yield, and reduced yields of short and medium-chain fatty acids.

Opposite to the response for medium-chain fatty acids, yield of 18:0 in milk fat increased with each dose of CLA infused. The temporal nature of the increase in 18:0 concentration in milk fat (Fig. 2A) in response to all doses of CLA, suggests hydrogenation of supplemental CLA may have increased availability of 18:0 for desaturation in the mammary gland. Despite greater 18:0 concentration and yield, however, the yield of *cis*9-18:1 (a product of 18:0 desaturation) was markedly lower (19% of the reduction in total fatty acid yield) when 180 g CLA was infused (Tab. V). The lower ratio of *cis*9-18:1 to 18:0 suggested that desaturation of 18:0 to *cis*9-18:1 in response to infusion with 180 g CLA, was impaired. Ratios of fatty acid pairs affected by Δ^9 desaturase activity have been previously used to estimate the potential effect of exogenous fatty acids on desaturation. Inhibiting the activity of Δ^9 desaturase, by infusing sterculic acid into the abomasum, decreased the ratios of *cis*9-14:1 to 14:0,

*cis*9-18:1 to 18:0, or *cis*9,*trans*11-18:2 to *trans*11-18:1 in milk fat [5]. An increase in *trans*10,*cis*12-18:2 concentration in milk fat (by infusing the isomer into the abomasum) also decreased the above ratios [2, 12]. In the present study, ratios were lower when CLA was infused at the rate of 180 $g \cdot d^{-1}$ (Tab. VI). Overall, results confirmed that greater availability of *trans*10,*cis*12-18:2 could decrease lipogenesis and desaturation of long-chain fatty acids in the mammary gland.

5. CONCLUSIONS

*Trans*10,*cis*12-18:2 was not detected in blood plasma or milk fat unless the CLA mixture was infused, suggesting it is not a major intermediate of 18:2n6 hydrogenation under normal rumen conditions. *Trans*10-18:1, however, was detected and yield of *trans*10-18:1 was proportional to the amount of CLA mixture infused. Thus, under basal conditions in the rumen, *trans*10-18:1 may arise primarily from isomerization of *cis*9-18:1 to *trans*10-18:1 (T.C. Jenkins, personal communication) [13] rather than isomerization/hydrogenation of 18:2n6. Due to high susceptibility for hydrogenation, the production of *trans*10,*cis*12-18:2 in the normal rumen environment must be at least 22 $g \cdot d^{-1}$ before it is detectable in blood plasma and milk fat. Furthermore, production must range between 44 and 88 $g \cdot d^{-1}$ to potentially reduce de novo synthesis and desaturation in the mammary gland. Overall, results strengthen the view that 18:1 or 18:2 isomers with a *trans*10-double bond may be involved in milk fat depression.

REFERENCES

[1] AOAC, Official Methods of Analysis, 15th ed., AOAC, Arlington, VA, 1990.

[2] Baumgard L., Corl B., Dwyer D., Saebo A., Bauman D., Identification of the conjugated linoleic acid isomer that inhibits milk fat synthesis, Am. J. Physiol. 278 (2000) R179–R184.

[3] Bickerstaffe R., Noakes D., Annison E., Quantitative aspects of fatty acid biohydrogenation, absorption and transfer into milk fat in the lactating goat, with special reference to the *cis*- and *trans*-isomers of octadecenoate and linoleate, Biochem. J. 130 (1972) 607–617.

[4] Fellner V., Sauer F., Kramer J., Effect of nigericin, monensin, and tetronasin on biohydrogenation in continuous flow-through ruminal fermenters, J. Dairy Sci. 80 (1997) 921–928.

[5] Griinari J., Corl B., Lacy S., Chouinard P., Nurmela K., Bauman D., Conjugated linoleic acid is synthesized endogenously in lactating dairy cows by delta(9)-desaturase, J. Nutr. 130 (2000) 2285–2291.

[6] Griinari J., Dwyer D., McGuire M., Bauman D., Palmquist D., Nurmela K., *Trans*-octadecenoic acid and milk fat depression in lactating dairy cows, J. Dairy Sci. 81 (1998) 1251–1261.

[7] Kemp P., White R., Lander D., The hydrogenation of unsaturated fatty acids by five bacterial isolates from the sheep rumen, including a new species, J. Gen. Microbiol. 90 (1975) 100–114.

[8] Kepler C., Hirons K., McNeill J., Tove S., Intermediates and products of the biohydrogenation of linoleic acid by *Butyrivibrio fibrisolvens*, J. Biol. Chem. 241 (1966) 1350–1354.

[9] Kepler C., Tucker W., Tove S., Biohydrogenation of unsaturated fatty acids. IV. Substrate specificity and inhibition of linoleate delta-*cis*12,delta-*trans*11-isomerase from *Butyrivibrio fibrisolvens*, J. Biol. Chem. 246 (1970) 2765–2771.

[10] Kepler C., Tove S., Biohydrogenation of unsaturated fatty acids. III. Purification and properties of a linoleate delta-12-*cis*,delta11-*trans*-isomerase from *Butyrivibrio fibrisolvens*, J. Biol. Chem. 242 (1967) 5686–5692.

[11] Kim Y., Liu R., Bond D., Russell J., Effect of linoleic acid concentration on conjugated linoleic acid production by *Butyrivibrio fibrisolvens* A38, Appl. Environ. Microbiol. 66 (2000) 5226–5230.

[12] Loor J., Alterations in mammary gland synthesis and secretion of fatty acids in response to *trans* isomers of octadecenoic acid or conjugated linoleic acid isomers, Ph.D. Dissertation, Virginia Tech (2001).

[13] Mortimer C., Niehaus W. Jr., Enzymatic isomerization of oleic acid to *trans*-10 octadecenoic acid, Biochem. Biophys. Res. Commun. 49 (1972) 1650–1656.

[14] NRC, Nutrient Requirements of Dairy Cattle, 6th rev. ed., Natl. Acad. Sci., Washington, DC, 1989.

[15] Noble R., Moore J., Harfoot C., Observations on the pattern on biohydrogenation of esterified and unesterified linoleic acid in the rumen, Brit. J. Nutr. 31 (1974) 99–108.

[16] Palmquist D., Santora J., Endogenous synthesis of rumenic acid in rodents and humans, in: Yurawecz M.P., Mossoba M.M., Kramer J.K.G., Pariza M.W., Nelson G.J. (Eds.), Advances in Conjugated Linoleic Acid Research, Vol. 1, AOCS Press, Champaign, IL, 1999, pp. 201–214.

[17] Park P., Goins R., In situ preparation of fatty acid methyl esters for analysis of fatty acid composition in foods, J. Food Sci. 59 (1994) 1262–1266.

[18] Parodi P., Conjugated linoleic acid: the early years, in: Yurawecz M.P., Mossoba M.M., Kramer J.K.G., Pariza M.W., Nelson G.J. (Eds.), Advances in Conjugated Linoleic Acid Research, Vol. 1, AOCS Press, Champaign, IL, 1999, pp. 1–11.

[19] Piperova L., Teter B., Bruckental I., Sampugna J., Mills S., Yurawecz M., Fritsche J., Ku K., Erdman R., Mammary lipogenic enzyme activity, *trans* fatty acids and conjugated linoleic acids are related in lactating dairy cows fed a milk fat-depressing diet, J. Nutr. 130 (2000) 2568–2574.

[20] Polan C., McNeill J., Tove S., Biohydrogenation of unsaturated fatty acids by rumen bacteria, J. Bacteriol. 88 (1964) 1056–1064.

[21] SAS ®, Version 8 Edition, SAS Inst., Inc., Cary, NC, 2000.

[22] Stallings C., Kroll G., Kelley J., McGilliard M., A computer ration evaluation program for heifers, dry cows, and lactating cows, J. Dairy Sci. 68 (1985) 1015–1019.

[23] Van Soest P., Robertson J., Lewis B., Methods of dietary fiber, neutral detergent fiber, and nonstarch polysacharides in relation to animal nutrition, J. Dairy Sci. 74 (1991) 3583–3597.