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Effect of unsaturated fatty acids and triglycerides from soybeans on milk fat synthesis and biohydrogenation intermediates in dairy cattle

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ABSTRACT

Increased rumen unsaturated fatty acid (FA) load is a risk factor for milk fat depression. This study evaluated if increasing the amount of unsaturated FA in the diet as triglycerides or free FA affected feed intake, yield of milk and milk components, and feed efficiency. Eighteen Holstein cows (132 ± 75 d in milk) were used in a replicated 3 × 3 Latin square design. Treatments were a control (CON) diet, or 1 of 2 unsaturated FA (UFA) treatments supplemented with either soybean oil (FA present as triglycerides; TAG treatment) or soybean FA distillate (FA present as free FA; FFA treatment). The soybean oil contained a higher concentration of *cis*-9 C18:1 (26.0 vs. 11.8 g/100 g of FA) and lower concentrations of C16:0 (9.6 vs. 15.0 g/100 g of FA) and *cis*-9,*cis*-12 C18:2 (50.5 vs. 59.1 g/100 g of FA) than the soybean FA distillate. The soybean oil and soybean FA distillate were included in the diet at 2% dry matter (DM) to replace soyhulls in the CON diet. Treatment periods were 21 d, with the final 4 d used for sample and data collection. The corn silage- and alfalfa silage-based diets contained 23% forage neutral detergent fiber and 17% crude protein. Total dietary FA were 2.6, 4.2, and 4.3% of diet DM for CON, FFA, and TAG treatments, respectively. Total FA intake was increased 57% for UFA treatments and was similar between FFA and TAG. The intakes of individual FA were similar, with the exception of a 24 g/d lower intake of C16:0 and a 64 g/d greater intake of *cis*-9 C18:1 for the TAG compared with the FFA treatment. Compared with CON, the UFA treatments decreased DM intake (1.0 kg/d) but increased milk yield (2.2 kg/d) and milk lactose concentration and yield. The UFA treatments reduced milk fat concentration, averaging 3.30, 3.18, and 3.11% for CON, FFA, and TAG treatments, respectively. Yield of milk fat, milk protein, and 3.5% fat-corrected milk remained unchanged when comparing CON with the UFA treatments. No differences existed in the yield of milk or milk components between the FFA and TAG treatments. The UFA treatments increased feed efficiency (energy-corrected milk/DM intake), averaging 1.42, 1.53, and 1.48 for CON, FFA, and TAG treatments, respectively. Although milk fat yield was not affected, the UFA treatments decreased the yield of de novo (<16-carbon) synthesized FA (40 g/d) and increased the yield of preformed (>16-carbon) FA (134 g/d). Yield of FA from both sources (16-carbon FA) was reduced by the UFA treatments but to a different extent for FFA versus TAG (72 vs. 100 g/d). An increase was detected in the concentration of *trans*-10 C18:1 and a trend for an increase in *trans*-10,*cis*-12 C18:2 and *trans*-9,*cis*-11 C18:2 for the UFA treatments compared with CON. Under the dietary conditions tested, UFA treatments supplemented at 2% diet DM as either soybean FA distillate or soybean oil increased milk yield but did not effectively cause a reduction in milk fat yield, with preformed FA replacing de novo synthesized FA in milk fat. Further research is required to determine if the response to changes in dietary free and esterified FA concentrations is different in diets that differ in their risk for milk fat depression.
Key words: dairy cow, biohydrogenation, milk fat, unsaturated fatty acid

INTRODUCTION

Feed ingredients vary in the amount and composition of FA that they contribute to dietary FA intake. In a recent study conducted in the Netherlands, approximately 100 samples of corn silage and grass silage were analyzed for FA concentration, with both types of silages varying from approximately 1 to 3% total FA (DM basis; Khan et al., 2012). Grains and by-products also vary in FA concentration, depending on hybrid, processing, and growing conditions (Boufaïed et al., 2003). Notably, distillers grains products vary considerably based on the amount of solubles added back to the grains, with the ratio of grains to solubles being a major contributor to the variation in FA content of the end product (Cao et al., 2009). Limited information exists regarding the total concentration of free FA, and the proportion of total FA present as free FA, in dairy cow diets. However, it has been reported previously that

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harvesting and ensiling of forages can increase the proportion of free FA due to hydrolytic cleavage of esterified FA (Yang and Fujita, 1997; Elgersma et al., 2003; Vanhatalo et al., 2007; Halmemies-Beauchet-Filleau et al., 2013a), whereas suboptimal storage of by-products in humid conditions can also increase the proportion of free FA (Cooke et al., 2007). Dietary ingredients, therefore, not only vary in the amount of total FA that they contribute to the diet but also in their relative proportion of free to esterified FA.

Unsaturated FA are toxic to specific rumen bacteria because they alter cell integrity (Maia et al., 2007). Therefore, rumen bacteria convert (biohydrogenate) unsaturated FA to SFA as a protection mechanism and, consequently, SFA are often considered to be rumen inert. A free carboxyl group is required for the initial isomerase reaction of biohydrogenation to occur (Jenkins, 1993). However, the majority of dietary FA are esterified, and are typically present either as triglycerides or glycolipids in feed ingredients. Upon entering the rumen, esterified FA must first go through the process of hydrolysis, in which the ester bond connecting the FA to the glycerol backbone is cleaved, exposing the carboxyl group and allowing biohydrogenation to proceed. If FA enter the rumen as free FA, they can undergo biohydrogenation immediately without having to go through hydrolysis.

Limited research exists examining whether the amount or proportion of free to esterified FA has an effect on biohydrogenation rates and pathways and whether this could affect milk fat synthesis in the dairy cow. Cooke et al. (2007) reported that increasing the proportion of free FA in cottonseed, while keeping the total FA concentration the same, decreased milk fat concentration when a greater proportion of free FA were fed. Additionally, in vitro work comparing free versus esterified FA found an increase in the amount of unsaturated biohydrogenation intermediates remaining when free FA were added to the culture (Moore et al., 1969; Noble et al., 1974), potentially due to an accumulation of FA that inhibit or change the pathways of biohydrogenation.

Altered rumen fermentation can result in shifts from normal biohydrogenation to alternative pathways, producing specific FA intermediates that reduce fat synthesis in the mammary gland resulting in milk fat depression (**MFD**; Bauman et al., 2011). It is well documented that increasing the amount of PUFA contained in the diet can reduce milk fat yield through the production of specific biohydrogenation intermediates (e.g., Griinari et al., 1998; Leonardi et al., 2005). However, it is unclear if a difference exists in the risk for MFD depending on if the increased dietary unsaturated FA are esterified or free FA. Our objective, therefore, was to examine if altering the amount of unsaturated FA in the diet as esterified or free FA affected feed intake and production responses of dairy cows. We hypothesized that free FA fed to lactating dairy cows would be more detrimental than esterified FA for milk fat production because free FA are more rapidly available in the rumen and this could cause a greater shift in biohydrogenation toward pathways that produce FA intermediates that cause MFD.

MATERIALS AND METHODS

Design and Treatments

Experimental procedures were approved by the Institutional Animal Care and Use Committee at Michigan State University. Eighteen mid-lactation (132 ± 75 DIM) Holstein cows (6 primiparous and 12 multiparous) from the Michigan State University Dairy Field Laboratory (East Lansing) were blocked by parity and 3.5% FCM yield and then randomly assigned to treatment sequence in a replicated 3 × 3 Latin square design experiment with 21-d periods.

Treatments consisted of a control (**CON**) diet, and 2 unsaturated FA (**UFA**) treatments supplemented with either soybean oil (FA present as triglycerides; **TAG** treatment) or soybean FA distillate (FA present as free FA; **FFA** treatment). The soybean oil and soybean FA distillate supplements were included in the diet at 2% DM to replace soyhulls in the CON diet. Addition of supplements to diets was based upon weight of lipid, not total FA content, and the supplements were premixed with dried ground corn before inclusion in TMR. The soybean oil (West Central Cooperative, Ralston, IA) contained approximately 84% FA, of which 99% were present as triglycerides, as the source of esterified FA. The soybean FA distillate (Arm & Hammer Animal Nutrition, Ewing, NJ) contained approximately 97% FA, of which 95% were present as free FA, as the source of free FA (Table 1). Although both supplements contained comparable concentrations of total unsaturated FA, differences existed in the concentration of individual FA: soybean oil contained a higher concentration of *cis*-9 C18:1 (26.0 vs. 11.8 g/100 g of FA) and a lower concentration of C16:0 (9.6 vs. 15.0 g/100 g of FA) and *cis*-9,*cis*-12 C18:2 (50.5 vs. 59.1 g/100 g of FA) than the soybean FA distillate (Table 1). The ingredient and nutrient composition of the diets fed as TMR are described in Table 2. All treatment diets contained equal amounts of forages in an approximate 2:1 ratio of corn silage to alfalfa silage and diets were formulated to contain 23% forage NDF and 17% CP, and mineral and vitamins were formulated according to NRC (2001) recommendations. The DM concentration

Table 1. Composition of FA supplements fed during the treatment periods[1]

Item	Soybean FA distillate[2]	Soybean oil[3]
Total FA content	97.0 ± 1.47	83.9 ± 0.47
Free FA (% of FA)	95.3 ± 0.76	0.6 ± 0.13
Esterified FA (% of FA)	4.7 ± 0.76	99.4 ± 0.13
FA (g/100 g of FA)		
14:0	0.1 ± 0.003	0.1 ± 0.002
16:0	15.0 ± 0.87	9.6 ± 0.26
18:0	3.8 ± 0.17	5.3 ± 0.37
cis-9 18:1	11.8 ± 0.15	26.0 ± 0.23
cis-9,*cis*-12 18:2	59.1 ± 0.88	50.5 ± 0.55
cis-9,*cis*-12,*cis*-15 18:3	6.8 ± 0.09	5.5 ± 0.10
Σ Others	3.3 ± 0.08	2.9 ± 0.14

[1]Average (±SD) composition of the supplements based on samples taken before the supplements were blended with corn grain at the start of each treatment period.

[2]Provided by Arm & Hammer Dairy Nutrition (Ewing, NJ).

[3]Provided by West Central Cooperative (Ralston, IA).

was determined twice weekly for forages and diets were adjusted when necessary.

All cows were housed in the same tie-stall throughout the experiment and milked twice daily (0400 and 1500 h). Access to feed was blocked from 0800 to 1000 h to allow for collection of orts and offering feed. Cows were fed 115% of expected intake at 1000 h daily. Water was available ad libitum in each stall and stalls were bedded with sawdust and cleaned twice daily.

Data and Sample Collection

Samples and data were collected during the last 4 d of each treatment period (d 18 to 21). Samples of all diet ingredients (0.5 kg) and orts from each cow (12.5%) were collected daily and composited by period for analysis. Milk yield was recorded and 2 milk samples were collected at each milking. One aliquot was collected in a sealed tube with preservative (bronopol tablet; D&F Control Systems Inc., San Ramon, CA) and stored at 4°C for milk component analysis. The second aliquot was stored without preservative at −20°C until analyzed for FA composition. Body weight was measured on the last 3 d of each period after the morning milking. Three trained investigators determined BCS on a 5-point scale (in 0.25-point increments; Wildman et al., 1982) on the last day of each period.

Sample Analysis

Diet ingredients and orts were dried at 55°C in a forced-air oven for 72 h for DM determination. Dried samples were ground with a Wiley mill (1-mm screen; Arthur H. Thomas Co., Philadelphia, PA). Diet ingredients were analyzed for NDF with heat-stable α-amylase and sodium sulfite (Van Soest et al., 1991), CP (AOAC International, 2000; method 990.03), and starch (Hall,

Table 2. Ingredients and nutrient composition of the treatment diets[1]

Item	Treatment[2]		
	CON	FFA	TAG
Ingredient (% of DM)			
Corn silage	34.9	34.9	34.9
Alfalfa silage	17.9	17.9	17.9
Wheat straw	3.5	3.5	3.5
Ground corn	22.3	22.3	22.3
Soybean meal	15.8	15.8	15.8
Soyhulls	2.0	—	—
Soybean FA distillate[3]	—	2.0	—
Soybean oil[4]	—	—	2.0
Vitamin and mineral mix[5]	2.2	2.2	2.2
Limestone	0.6	0.6	0.6
Sodium bicarbonate	0.7	0.7	0.7
Nutrient composition			
DM (%)	55.3	55.3	55.4
NDF (% of DM)	28.1	27.4	27.5
Forage NDF (% of DM)	23.2	23.1	23.1
Starch (% of DM)	28.7	29.6	29.3
CP (% of DM)	17.2	17.0	17.0
Total FA (% of DM)	2.6	4.2	4.3
16:0	0.34	0.58	0.49
18:0	0.07	0.13	0.12
cis-9 18:1	0.48	0.55	0.89
cis-9,*cis*-12 18:2	1.24	2.18	2.10
cis-9,*cis*-12,*cis*-15 18:3	0.21	0.31	0.31
Free FA (% of FA)	20.6	50.9	16.3
Esterified FA (% of FA)	79.4	49.1	83.7

[1]Average composition of 3 periods of feeding to lactating dairy cows (n = 18).

[2]Treatments contained 2% soyhulls (control, CON), 2% soybean FA distillate (FFA treatment), or 2% soybean oil (TAG treatment).

[3]Provided by Arm & Hammer Dairy Nutrition (Ewing, NJ).

[4]Provided by West Central Cooperative (Ralston, IA).

[5]Vitamin and mineral mix contained 34.1% dry ground shell corn, 25.6% white salt, 21.8% calcium carbonate, 9.1% Biofos (The Mosaic Co., Plymouth, MN), 3.9% magnesium oxide, 2% soybean oil, and <1% of each of the following: manganese sulfate, zinc sulfate, ferrous sulfate, copper sulfate, iodine, cobalt carbonate, vitamin E, vitamin A, vitamin D, and selenium.

2009) by Cumberland Valley Analytical Services Inc. (Hagerstown, MD). Total FA concentration of feed ingredients was determined using a modification of the one-step transesterification method developed by Sukhija and Palmquist (1988) as described by Lock et al. (2013).

Additional FA analysis of feed ingredients determined the proportion of individual FA either in free or esterified fractions. One gram of dried, ground sample was combined with 1 mL of internal standard (1 mg/mL of toluene). Internal standards used were *cis*-10 C17:1 for free FA and tritridecanoin for triglycerides, which were used to determine FA yields. Total lipids were extracted with chloroform and methanol using a modified method of Folch et al. (1957), dried under nitrogen gas, and reconstituted in 0.5 mL of chloroform. Solid-phase extraction of lipid fractions was performed using a modified method of Agren et al. (1992). A vacuum manifold fitted with aminopropyl (N2) solid-phase extraction columns (1 g/mL; HyperSep Amino SPE Column, Thermo Scientific, Bellefonte, PA) was used to separate the free FA fraction, a combined triglyceride/cholesterol ester fraction, and phospholipid fraction of each sample. The column was loaded with sample and flushed with 4 mL of chloroform:isopropanol solution (2:1) to separate and remove the triglyceride fraction. Columns were subsequently flushed with 6 mL of chloroform:methanol:acetic acid (100:2:2) to separate and remove the free FA fraction. To separate and remove the phospholipid fraction, columns were flushed with 6 mL of methanol:chloroform:water solution (10:5:4). The phospholipid fraction was dried using 2% sodium chloride solution and dissolved in chloroform. The phospholipid and triglyceride fractions were subsequently combined into a single esterified fraction and all fractions were dried under N_2 gas and reconstituted in 0.5 mL of toluene before methylation.

Fatty acid methyl esters for both fractions were prepared with a modified 2-step transmethylation procedure as described by Jenkins (2010). In brief, 1.0 mL of sodium methoxide (0.5 *M* solution in methanol) was added to each sample, vortexed, and incubated at 50°C for 10 min. After the samples had cooled, 1.5 mL of a 5% methanolic HCl solution was added, samples vortexed, and incubated at 80°C for 10 min. Samples were cooled and neutralized with a 6% potassium carbonate solution. The FAME were extracted with hexane and filtered through charcoal and silica. Hexane was evaporated under nitrogen gas and FAME were weighed and then reconstituted in hexane to obtain a 1% solution.

Feed FAME were determined on a GC-2010 Plus gas chromatograph (Shimadzu Corp., Kyoto, Japan) equipped with a split injector (1:100 split ratio) and a flame ionization detector using a CP8827 WCOT fused silica column (30 m × 0.32 mm i.d. × 0.25-μm film thickness; Varian Inc., Lake Forest, CA). Analytical conditions and FAME standards were the same as described previously for FAME analyses of feeds (Lock et al., 2013).

Individual milk samples were analyzed for fat, true protein, and lactose concentration by mid-infrared spectroscopy (AOAC International, 2000; method 972.160) by the Michigan Herd Improvement Association (Universal Lab Services, Lansing MI). Yields of 3.5% FCM, ECM (NRC, 2001), and milk components were calculated using milk yield and component concentrations for each milking, summed for a daily total, and averaged for each collection period. Milk samples used for analysis of FA composition were composited based on milk fat yield (d 18 to 21 of each period). Lipids were extracted, methylated, and FAME composition determined by GLC, according to the methods described by Lock et al. (2013). Quantification of FA composition covered approximately 70 FA in the range C4:0 to C24:0. Yield of individual FA (g/d) in milk fat were calculated by using milk fat yield and FA concentration to determine yield on a mass basis using the molecular weight of each FA while correcting for glycerol content and other milk lipid classes (Piantoni et al., 2013).

Statistical Analysis

All data were analyzed using the fit model procedure of JMP software (version 8; SAS Institute Inc., Cary, NC). Data were analyzed using the following model:

$$Y_{ijkl} = \mu + S_i + C(S)_{ij} + P_k + T_l + e_{ijkl},$$

where Y_{ijkl} = the dependent variable, μ = the overall mean, S_i = fixed effect of square (i = 1 to 6), $C(S)_{ij}$ = random effect of cow nested within square (j = 1 to 18), P_k = fixed effect of period (k = 1 to 3), T_l = fixed effect of treatment (l = 1 to 3), and e_{ijkl} = the residual error.

The interaction between period and treatment was initially included in the model and removed because $P > 0.20$ for all variables. Two preplanned, orthogonal contrasts were used to evaluate the effects of added FA to diets [CON vs. UFA; 1/2 (FFA+TAG)] and the effects of free FA versus esterified FA (FFA vs. TAG). Contrasts were declared significant at $P \leq 0.05$ and trends were declared at $P \leq 0.10$.

RESULTS

Dietary NDF, forage NDF, starch, and CP remained similar across all treatments, whereas total dietary FA were 2.6, 4.2, and 4.3% of diet DM for CON, FFA, and

Table 3. Fatty acid composition of free and esterified fractions of treatment diets[1]

FA (g/100 g of total FA)	Treatment[2]					
	CON		FFA		TAG	
	Free	Esterified	Free	Esterified	Free	Esterified
14:0	0.4	0.4	0.2	0.4	0.2	0.2
16:0	19.4	13.7	18.1	14.0	16.4	11.9
18:0	2.8	2.6	3.9	2.7	3.5	3.8
cis-9 18:1	21.6	23.0	13.6	20.2	21.0	23.9
cis-9,*cis*-12 18:2	42.4	49.3	53.8	51.0	47.5	50.3
cis-9,*cis*-12,*cis*-15 18:3	8.4	5.6	7.1	6.1	7.4	5.6
22:0	0.3	0.3	0.4	0.3	0.3	0.3
24:0	0.2	0.2	0.2	0.2	0.2	0.1
Σ Others	3.8	4.7	2.6	4.8	3.2	3.6
Σ SFA	23.2	17.2	22.7	17.7	20.5	16.4
Σ RUFAL[3]	72.5	77.9	74.5	77.3	75.9	79.8

[1]Average composition of 3 periods of feeding to lactating dairy cows (n = 18).

[2]Treatments contained 2% soyhulls (control, CON), 2% soybean FA distillate (FFA treatment), or 2% soybean oil (TAG treatment).

[3]Rumen unsaturated FA load: (*cis*-9 18:1 + *cis*-9,*cis*-12 18:2 + *cis*-9,*cis*-12,*cis*-15 18:3).

TAG treatments, respectively (Table 2). The supplements used in this study provided a marked contrast as sources for free versus esterified FA, with both supplements having similar total unsaturated FA concentration, although as noted earlier, differences did exist between the supplements for individual FA concentrations (Table 1). Using these supplements, we were able to achieve differences in the proportion of FA in the treatment diets as free or esterified FA. As a proportion of total dietary FA, CON, FFA, and TAG treatments contained 21, 51, and 16% free FA, respectively (Table 2). The majority of the 21% free FA in CON came from alfalfa and corn silages (data not shown). The FA composition of the diets when separated into free and esterified fractions was similar, with most of the dietary FA being unsaturated 18-carbon FA for both fractions (Table 3). Due to the low concentration of *cis*-9 C18:1 in the soybean FA distillate (Table 1), the concentration of this FA was lower in the free versus esterified FA fraction in the FFA treatment (Table 3). Total FA intake was increased by 57% for both UFA treatments, with no difference between FFA and TAG treatments ($P = 0.21$; Table 4). As expected, the intake of all individual FA increased for the UFA treatments compared with CON (all $P < 0.001$). No difference existed in the intake of C18:0, *cis*-9,*cis*-12 C18:2, or *cis*-9,*cis*-12,*cis*-15 C18:3 between FFA and TAG treatments (all $P \geq 0.20$; Table 4). However, we observed a 24 g/d lower intake of C16:0 and a 64 g/d higher intake of *cis*-9 C18:1 for the TAG compared with the FFA treatment (both $P < 0.001$; Table 4).

We observed a decrease in DMI of 1 kg/d, for the UFA treatments compared with CON ($P = 0.02$; Table 5), with no difference between FFA and TAG ($P = 0.74$).

Table 4. Intake of FA (g/d) for cows fed treatment diets (n = 18)

FA intake[1] (g/d)	Treatment[2]			SEM	*P*-value[3]	
	CON	FFA	TAG		CON vs. UFA	FFA vs. TAG
16:0	93.9	154	130	4.15	<0.001	<0.001
18:0	18.2	33.5	31.9	1.61	<0.001	0.34
cis-9 18:1	129	172	236	5.49	<0.001	<0.001
cis-9,*cis*-12 18:2	342	577	561	16.0	<0.001	0.20
cis-9,*cis*-12,*cis*-15 18:3	56.6	83.0	82.5	2.36	<0.001	0.79
Total FA	716	1,108	1,136	31.4	<0.001	0.21

[1]Calculated using DMI for individual cows and the FA composition of the respective diet during each treatment period.

[2]Treatments contained 2% soyhulls (control, CON), 2% soybean FA distillate (FFA treatment), or 2% soybean oil (TAG treatment).

[3]*P*-values associated with contrasts: CON vs. UFA = *P*-value associated with differences between control and the average of FFA and TAG treatments; FFA vs. TAG = *P*-value associated with differences between FFA and TAG treatments.

Milk yield was 2.2 kg/d greater for UFA treatments than CON ($P = 0.004$), with no difference between FFA and TAG ($P = 0.47$). The yield of milk fat was not affected by treatment; however, the concentration of fat decreased with UFA treatments ($P = 0.05$), with no difference between FFA and TAG ($P = 0.35$). Similarly, we observed no effect of treatments on the yield of milk protein, but milk protein concentration was lower for the UFA treatments ($P < 0.001$). Further, milk protein concentration was lower for the FFA compared with the TAG treatment ($P = 0.01$). Milk lactose yield and concentration were increased by the UFA treatments compared with CON ($P = 0.001$ and $P = 0.01$, respectively). We observed no effect of treatments on the yields of 3.5% FCM and ECM ($P = 0.15$ and $P = 0.14$, respectively). Because of the decrease in DMI with the UFA treatments, we observed a 6% improvement in feed efficiency (ECM/DMI) compared with CON ($P = 0.01$), with no difference between FFA and TAG ($P = 0.16$). We did not observe any differences in BW or BCS across treatments ($P > 0.14$).

Milk FA are derived from 2 sources: <16-carbon FA from de novo synthesis in the mammary gland and >16-carbon FA originating from extraction from plasma. Mixed-source FA (C16:0 and *cis*-9 C16:1) can originate from de novo synthesis in the mammary gland and extraction from plasma. The UFA treatments decreased the concentration of de novo synthesized milk FA (Figure 1A) through a reduction in the concentration of C8:0 to C14:0 (all $P < 0.01$; Table 6). The concentration of 16-carbon FA was also lower for the UFA treatments ($P < 0.001$; Figure 1A). However, we observed an increase in the concentration of preformed FA with the UFA treatments compared with CON ($P < 0.001$; Figure 1A) because the concentrations of all 18-carbon FA were increased ($P < 0.10$; Table 6). Of note, we observed an increase in the concentration of *trans*-10 C18:1 ($P = 0.004$) and a trend for an increase in *trans*-10,*cis*-12 C18:2 ($P = 0.06$) and *trans*-9,*cis*-11 C18:2 ($P = 0.07$) for the UFA treatments compared with CON (Table 6).

Similar to the results for milk FA on a concentration basis, the yield of de novo synthesized and 16-carbon FA were reduced by the UFA treatments (40 and 86 g, respectively; both $P < 0.001$), with the yield of 16-carbon FA greater for FFA than for TAG ($P = 0.03$; Figure 1B). Preformed FA were increased by the UFA treatments (134 g; $P < 0.001$), with no differences between FFA and TAG treatments (Figure 1B). The UFA treatments increased the yield of all 18-carbon milk FA (Table 7). As a consequence of the shift in sources of milk FA, no significant change occurred in overall milk fat yield. However, individual FA associated with MFD were affected by the UFA treatments, as demonstrated by an increased yield of *trans*-10 C18:1 ($P < 0.001$) and *trans*-9,*cis*-11 C18:2 ($P = 0.05$) in milk fat, and a trend for an increased yield of *trans*-10,*cis*-12 C18:2 ($P = 0.07$; Table 7). We observed no differences in these

Table 5. Dry matter intake, milk production and composition, feed efficiency (ECM/DMI), BW, and BCS for cows fed treatment diets (n = 18)

	Treatment[1]				*P*-value[2]	
Variable	CON	FFA	TAG	SEM	CON vs. UFA	FFA vs. TAG
DMI (kg/d)	27.6	26.5	26.7	0.63	0.02	0.74
Milk yield (kg/d)						
Milk	39.5	42.0	41.4	0.94	0.004	0.47
3.5% FCM[3]	38.2	39.9	38.8	1.26	0.15	0.22
ECM[4]	38.7	40.2	39.4	1.14	0.14	0.32
Milk composition						
Fat (kg/d)	1.30	1.34	1.29	0.06	0.70	0.22
Fat (%)	3.30	3.18	3.11	0.12	0.05	0.35
Protein (kg/d)	1.25	1.28	1.29	0.03	0.17	0.83
Protein (%)	3.16	3.06	3.12	0.04	<0.001	0.01
Lactose (kg/d)	1.88	2.03	2.00	0.06	0.001	0.45
Lactose (%)	4.77	4.83	4.82	0.07	0.01	0.63
ECM/DMI	1.42	1.53	1.48	0.05	0.01	0.16
BW	709	705	706	20.9	0.16	0.78
BCS	3.00	2.96	2.93	0.13	0.14	0.42

[1]Treatments contained 2% soyhulls (control, CON), 2% soybean FA distillate (FFA treatment), or 2% soybean oil (TAG treatment).

[2]*P*-values associated with contrasts: CON vs. UFA = *P*-value associated with differences between control and the average of FFA and TAG treatments; FFA vs. TAG = *P*-value associated with differences between FFA and TAG treatments.

[3]3.5% FCM = (0.4324 × kg of milk) + (16.216 × kg of milk fat).

[4]ECM = (0.327 × kg of milk) + (12.95 × kg of milk fat) + (7.20 × kg of milk protein) (Tyrrell and Reid, 1965).

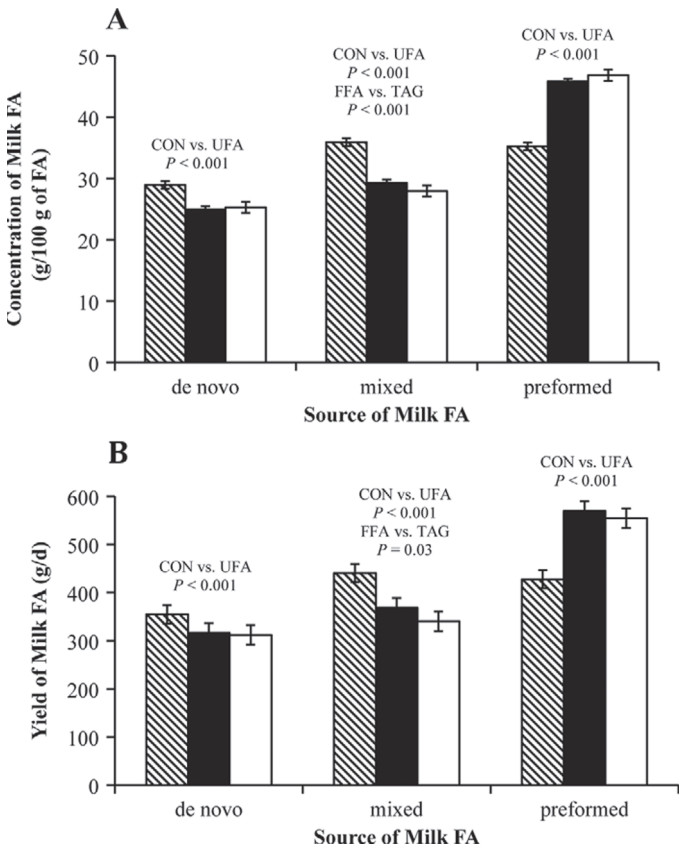

Figure 1. Concentration (panel A) and yield (panel B) of milk FA by source for cows (n = 18) fed treatment diets. The hashed bar represents the control (CON; 2% soyhulls) treatment, the solid bar represents the FFA treatment (2% soybean FA distillate), and the white bar represents the TAG treatment (2% soybean oil). De novo = milk FA <16 carbons in length; mixed = milk FA 16 carbons in length; preformed = milk FA >16 carbons in length. *P*-values associated with contrasts: CON vs. UFA = *P*-value associated with differences between CON and the average of FFA and TAG treatments; FFA vs. TAG = *P*-value associated with differences between FFA and TAG treatments. Error bars represent the standard error of the mean.

biohydrogenation intermediates between FFA and TAG treatments ($P > 0.10$), except for a trend for the TAG treatment to increase *trans*-9,*cis*-11 C18:2 compared with the FFA treatment ($P = 0.07$; Table 7).

DISCUSSION

The amount of FA in feed ingredients is influenced by several factors (e.g., species, growing conditions, and stage of harvest; Boufaïed et al., 2003), with the proportion of free FA increased by the action of plant lipases during harvest and storage (Yang and Fujita, 1997; Elgersma et al., 2003; Vanhatalo et al., 2007; Halmemies-Beauchet-Filleau et al., 2013a). For example, in fresh perennial ryegrass, 98% of the FA was present as esterified FA but after ensiling, 27 to 73% of the total FA consisted of free FA (Elgersma et al., 2003). The variation that exists in the relative proportion of free to esterified FA in feed ingredients highlights the need to determine whether differences between the 2 sources of FA in diets affect production responses of dairy cattle. Unsaturated dietary free FA can undergo biohydrogenation immediately and have instant interaction with rumen microbial cells, whereas esterified FA must first undergo hydrolysis before entering the free FA pool. Previous research using an in vitro model to examine rumen metabolism of esterified and free FA suggested that when free FA are introduced directly into rumen cultures, the initial high concentration of free C18:2 inhibits some steps in the biohydrogenation process (Moore et al., 1969) and (or) reduces the rate of biohydrogenation (Noble et al., 1974), thereby increasing concentrations of biohydrogenation intermediates. In the current study, we used soybean FA distillate and soybean oil as sources of free and esterified FA, respectively, which allowed for dietary treatments with different proportions of free and esterified FA. Although we based the supplementation of soybean FA distillate and soybean oil upon weight of lipid and not total FA content, we were able meet our objective of providing similar amounts of FA as either free or esterified FA.

Although the concentration of total unsaturated FA was comparable in both supplements, the soybean oil contained a higher concentration of *cis*-9 C18:1 and lower concentrations of C16:0 and *cis*-9,*cis*-12 C18:2 compared with the soybean FA distillate. This resulted in similar daily intakes of dietary FA, with the exception of a 24 g/d lower intake of C16:0 and a 64 g/d higher intake of *cis*-9 C18:1 for the TAG compared with the FFA treatment. Importantly, daily intake of *cis*-9,*cis*-12 C18:2 was not different between the FFA and TAG treatments. Recent work comparing the effect of increasing dietary concentrations of *cis*-9 C18:1 and *cis*-9,*cis*-12 C18:2 on MFD indicated that *cis*-9,*cis*-12 C18:2 has a greater potential to reduce milk fat concentration and yield compared with *cis*-9 C18:1 (He et al., 2012). However, it remains to be determined whether maintaining *cis*-9 C18:1 between these treatments would have altered our results. The 64 g/d increased intake of *cis*-9 C18:1 for TAG was a result of the higher concentration of *cis*-9 C18:1 in the soybean oil versus soybean FA distillate. Reasons for the differences in the FA profile of the soybean oil and soybean FA distillate are unclear, but could simply be attributed to differences in individual sources of soybeans used to make both supplements or incomplete hydrolysis of individual FA to produce the distillate, or both. It is interesting to note, however, that there is evidence from studies with fresh and ensiled perennial ryegrass (Elgersma et al., 2003); fresh, dried, or ensiled timothy and meadow fescue (Halmemies-Beauchet-Filleau et al., 2013a); grass

Table 6. Milk FA concentration of cows fed treatment diets (n = 18)

Selected individual FA[1](g/100 g of total FA)	Treatment[2]			SEM	*P*-value[3]	
	CON	FFA	TAG		CON vs. UFA	FFA vs. TAG
4:0	2.89	3.06	2.99	0.08	0.06	0.34
6:0	2.13	2.04	2.02	0.07	0.09	0.81
8:0	1.35	1.21	1.22	0.05	0.001	0.79
10:0	3.68	2.96	3.02	0.14	<0.001	0.51
12:0	4.55	3.43	3.54	0.15	<0.001	0.23
14:0	13.2	11.4	11.5	0.26	<0.001	0.49
cis-9 14:1	1.07	0.86	0.95	0.06	<0.001	0.01
16:0	34.3	28.0	26.6	0.55	<0.001	<0.001
cis-9 16:1	1.62	1.27	1.29	0.06	<0.001	0.51
18:0	7.29	10.24	10.18	0.26	<0.001	0.76
trans-4 18:1	0.011	0.023	0.028	0.001	<0.001	<0.001
trans-5 18:1	0.008	0.019	0.023	0.001	<0.001	<0.001
trans-6–8 18:1	0.21	0.39	0.48	0.02	<0.001	0.002
trans-9 18:1	0.17	0.32	0.36	0.01	<0.001	0.001
trans-10 18:1	0.35	0.82	1.24	0.21	0.004	0.11
trans-11 18:1	0.62	1.54	1.70	0.07	<0.001	0.04
trans-12 18:1	0.32	0.66	0.69	0.02	<0.001	0.13
cis-9 18:1	16.3	20.2	20.6	0.55	<0.001	0.29
cis-11 18:1	0.47	0.51	0.52	0.03	0.03	0.66
cis-9,*cis*-12 18:2	2.23	3.14	2.99	0.08	<0.001	0.03
cis-9,*trans*-11 18:2	0.35	0.79	0.83	0.04	<0.001	0.28
trans-9,*cis*-11 18:2	<0.001	0.004	0.012	0.003	0.07	0.07
trans-10,*cis*-12 18:2	<0.001	0.003	0.005	0.002	0.06	0.44
cis-9,*cis*-12,*cis*-15 18:3	0.37	0.44	0.45	0.01	<0.001	0.07

[1]A total of approximately 70 individual FA were quantified and used for calculations (summation by concentrations). Only select FA are reported in the table.

[2]Treatments contained 2% soyhulls (control, CON), 2% soybean FA distillate (FFA treatment), or 2% soybean oil (TAG treatment).

[3]*P*-values associated with contrasts: CON vs. UFA = *P*-value associated with differences between control and the average of FFA and TAG treatments; FFA vs. TAG = *P*-value associated with differences between FFA and TAG treatments.

and red clover silage (Halmemies-Beauchet-Filleau et al., 2013b); and corn silage [A. L. Lock (Michigan State University, East Lansing) and T. C. Jenkins (Clemson University, Clemson, SC), unpublished data], suggesting that the FA profile of esterified and free FA fractions from these plants may differ. For example, in grass silage and red clover silage, differences existed in the concentrations of unsaturated FA between free FA, phospholipid, and neutral lipid fractions (Halmemies-Beauchet-Filleau et al., 2013b). Further research is warranted to determine possible reasons for differences in these fractions across plants and factors that may alter the concentration and profile of free FA in feedstuffs. For experimental purposes, the ability to blend other oils (or distillates) into treatments to correct imbalance in the FA profile of starting FA supplements should be considered for future studies.

The UFA treatments reduced DMI, as has previously been reported for unsaturated FA supplements (Allen, 2000; Harvatine and Allen, 2005). The reduction in DMI has been attributed to the hypophagic effect of unsaturated FA, causing satiety signals that decrease meal size, meal frequency, or a combination of the two (Allen, 2000). Abomasal infusions of soybean free FA reduced DMI to a greater extent than abomasal infusions of soybean oil triglycerides (Litherland et al., 2005). However, in our study, because the unsaturated FA from the supplements in the FFA and TAG treatments were fed rather than bypassing the rumen, both supplements would primarily result in free FA reaching the duodenum, which likely explains why we saw no difference between the FFA and TAG treatments. The reduction in milk protein concentration with the UFA treatments could have been the result of lowered DMI. The reduction in DMI would suggest less MP available for milk protein synthesis (Weiss et al., 2011) or because yield did not change, this could also be due to dilution of milk protein, thus changing concentration but not yield.

The observed reduction in DMI and increased yields of milk and milk lactose when cows received the UFA treatments resulted in increased feed efficiency (ECM/DMI). The reduction in DMI and increase in milk yield is similar to the results of a meta-analysis on fat supplements in lactating dairy cattle (Rabiee et al., 2012). The increase in yield of milk and lactose is possibly due to the glucose-sparing effects of FA. The supplemental FA could have been used as an energy source for tis-

Table 7. Milk FA yield of cows fed treatment diets (n = 18)

	Treatment[2]				*P*-value[3]	
Selected individual FA[1] (g/d)	CON	FFA	TAG	SEM	CON vs. UFA	FFA vs. TAG
4:0	35.3	38.8	36.8	2.2	0.08	0.22
6:0	26.1	26.0	25.2	1.7	0.63	0.51
8:0	16.6	15.5	15.4	1.1	0.07	0.84
10:0	45.3	37.8	38.0	2.7	<0.001	0.89
12:0	56.0	43.7	44.1	3.3	<0.001	0.79
14:0	162	144	141	7.7	<0.001	0.65
cis-9 14:1	13.1	10.8	11.1	0.7	<0.001	0.33
16:0	421	353	325	20.1	<0.001	0.02
cis-9 16:1	19.7	15.9	15.1	0.8	<0.001	0.12
18:0	88.9	129	124	7.0	<0.001	0.36
trans-4 18:1	0.14	0.29	0.34	0.02	<0.001	0.003
trans-5 18:1	0.10	0.24	0.27	0.01	<0.001	0.003
trans-6–8 18:1	2.52	4.82	5.51	0.16	<0.001	<0.001
trans-9 18:1	2.09	3.89	4.25	0.13	<0.001	0.01
trans-10 18:1	4.24	9.57	12.29	1.47	<0.001	0.14
trans-11 18:1	7.57	19.1	20.9	1.24	<0.001	0.14
trans-12 18:1	3.84	8.13	8.17	0.29	<0.001	0.92
cis-9 18:1	198	250	241	7.33	<0.001	0.22
cis-9,*cis*-12 18:2	27.1	39.1	35.4	1.2	<0.001	0.003
cis-9,*trans*-11 18:2	4.25	9.69	9.75	0.42	<0.001	0.89
trans-9,*cis*-11 18:2	0.01	0.04	0.10	0.03	0.05	0.07
trans-10,*cis*-12 18:2	<0.01	0.03	0.04	0.02	0.07	0.57
cis-9,*cis*-12,*cis*-15 18:3	4.52	5.42	5.34	0.18	<0.001	0.67

[1]A total of approximately 70 individual FA were quantified and used for calculations (summation by yield). Only select FA are reported in the table.

[2]Treatments contained 2% soyhulls (control, CON), 2% soybean FA distillate (FFA treatment), or 2% soybean oil (TAG treatment).

[3]*P*-values associated with contrasts: CON vs. UFA = *P*-value associated with differences between control and the average of FFA and TAG treatments; FFA vs. TAG = *P*-value associated with differences between FFA and TAG treatments.

sues as well as precursors for preformed FA in milk fat. The reduction in de novo synthesis would reduce the requirement of NADPH from the pentose phosphate pathway, which is produced by glucose oxidation (Bauman and Davis, 1975). Therefore, reducing de novo synthesis in the mammary gland has the potential to reduce the glucose demand for fat synthesis. If glucose supply were limiting, this "spared" glucose could then be used by other tissues or for lactose synthesis in the mammary gland, the osmotic regulator of milk, resulting in increased milk yield.

Grummer (1991) reported that an inverse relationship exists between the amount of fat supplemented in the diet and the concentration of C4:0 to C14:0 in milk fat. As more dietary fat is added, the proportion of de novo synthesized milk FA decreases, whereas the proportion of preformed milk FA increases. In a meta-analysis examining the relationship between milk FA yield and duodenal flows of FA, Glasser et al. (2008) reported that increasing the duodenal supply of 18-carbon FA causes a quadratic increase in 18-carbon milk FA yield, with a subsequent linear reduction in de novo synthesized milk FA yield. However, the meta-analysis also indicated that a severe reduction in the synthesis of de novo milk FA could limit, or even prevent, any increase in 18-carbon milk FA yield (Glasser et al., 2008), as would typically be observed during severe MFD. On an FA yield basis, the substitution effect of preformed for de novo milk FA was recently reported by He and Armentano (2011) and He et al. (2012), who noted that the reduction in yield of de novo milk FA was often compensated for by an increase in the yield of preformed milk FA when fat supplements were fed. Classically, MFD represents a depression of milk fat yield with no change in the yield of milk and other milk components (Bauman et al., 2011), and typically causes a reduction in both de novo and preformed milk FA yield because of the coordinated regulation of key enzymes associated with lipid synthesis in the mammary gland (Peterson et al., 2003; Harvatine and Bauman, 2011).

In our study, we observed a reduction in the yield of de novo synthesized milk FA that was compensated for by an increase in the yield of milk FA originating from preformed sources. The increase in the yield and concentration of preformed FA in milk fat was presum-

ably because of the increase in the amount of dietary long-chain FA available to the mammary gland and, as discussed previously, the substitution effect of preformed FA for de novo milk FA is well established (e.g., Grummer, 1991; Glasser et al., 2008). When considering these results, we believe that 2 possible interpretations exist, both of which warrant consideration. One interpretation is that because we did not detect any change in overall milk fat yield, we did not observe what would typically be considered as diet-induced MFD. An alternative interpretation focuses on the overall effect of the UFA treatments shifting biohydrogenation pathways or rates, or both, as evident by the increase in biohydrogenation intermediates associated with MFD. The UFA treatments increased the concentration and yield of *trans*-10 C18:1, with a trend for increased concentrations and yields of *trans*-9,*cis*-11 C18:2 and *trans*-10,*cis*-12 C18:2 in milk fat. Although a reduction in milk fat concentration occurred, the increased uptake of these biohydrogenation intermediates by the mammary gland (and incorporation into milk fat) did not result in an overall reduction in milk fat yield. However, the reduction in de novo synthesized milk FA could have been directly caused by increases in biohydrogenation intermediates associated with MFD, suggesting that under the dietary conditions tested, perhaps we observed what could be referred to as subclinical MFD. It is possible that increases in specific biohydrogenation intermediates were sufficient to reduce de novo synthesis in the mammary gland, which is typically inhibited to a greater extent in normal MFD situations than preformed milk FA (Bauman et al., 2011). If the concentration of dietary UFA were increased further, we might have expected to see a greater reduction in de novo synthesized FA as well as a reduction in preformed milk FA, resulting in an overall reduction in milk fat yield.

As a result of the increase in biohydrogenation intermediates associated with MFD, it appears that the negative effect of the increased dietary unsaturated FA with the UFA treatments was due to effects in the rumen. If we could remove or reduce the negative effect of unsaturated FA on rumen FA metabolism, would increasing unsaturated FA have positive effects on milk fat yield by increasing their use for milk fat synthesis without detrimental effects on de novo FA synthesis? Previously, chemical treatment of oilseeds containing unsaturated FA has been used to reduce the negative effect of unsaturated FA on rumen fermentation. Formaldehyde-treated oilseeds resulted in increases in milk fat yield (Ashes et al., 1992; Petit, 2003), mostly through an increase in 18-carbon unsaturated FA yield and minimal changes in de novo FA yield (Ashes et al., 1992), indicating that minimizing adverse effects on rumen fermentation may have positive effects on milk fat yield. However, when unsaturated FA were infused in the abomasum of dairy cows, bypassing the effects of rumen fermentation, the proportion of unsaturated 18-carbon milk FA increased, whereas the proportion of C16:0 was reduced without affecting total milk fat yield, indicating that other mechanisms in the mammary gland may exist that regulate milk FA synthesis. However, it should be noted that DMI and milk yield were also reduced in this study (Drackley et al., 1992). Interestingly, supplementing a C16:0-enriched fat supplement at 2% of diet DM increased the secretion of 16-carbon FA into milk fat while maintaining both de novo and preformed milk FA secretion, thereby increasing milk fat yield (Lock et al., 2013). Clearly, further research is required to improve our understanding of the effect of specific FA on the balance between the secretion of de novo and preformed FA to maximize milk fat synthesis.

Our study compared a mostly esterified FA (triglycerides) with a mostly free FA supplement to evaluate the effects on rumen metabolism and production responses. Contrary to previous research using cottonseed containing increasing amounts of free FA (Sullivan et al., 2004; Cooke et al., 2007), we observed no differences between the FFA and TAG treatments for milk fat concentration and yield, or the concentration of specific milk FA associated with MFD. Results from in vitro studies indicate that increased free FA results in an accumulation of biohydrogenation intermediates, whereas esterified FA are more completely biohydrogenated to SFA (Moore et al., 1969; Noble et al., 1974). We expected to see a reduction in milk fat concentration and yield when feeding free FA compared with esterified FA due to a more rapid availability of unsaturated FA in the rumen leading to altered rumen biohydrogenation. However, we observed no indication that the FFA treatment caused an increase in biohydrogenation intermediates associated with MFD or reduced milk fat yield to a greater extent than the TAG treatment. However, because we used FA supplements as our source of free and esterified FA, these results should not be extrapolated to conclude that other feeds with elevated concentrations of free FA will not cause MFD. Rather, our results suggest that when MFD occurs in these situations, it probably is not the free FA per se, but may be any number of other issues that develop with poorly fermented and stored feeds.

Rico and Harvatine (2013) reported that by 11 d following the feeding of a milk fat-depressing diet, milk fat yield from cows was not different from the control. Thus, our 21-d periods with sampling starting at d 18 should have been sufficient time for the rumen to adjust to a diet change. The study was balanced for carryover

effects and because no treatment by period interactions existed, no detectable carryover effects existed between treatments. We used soybean-based fats because of the availability of both a free FA distillate and oil containing mostly esterified FA from soybeans. However, we recognize that corn is a more common source of FA for dairy cattle. The FA profile of corn and soybean oil is very similar, with most FA being *cis*-9,*cis*-12 C18:2; therefore, we would expect similar results if the trial were conducted with corn-based FA supplements. Although some differences existed in the FA profile of the supplements, notably *cis*-9 C18:1, their use allowed us to substantially alter the proportion of FA present in the diet either as free or esterified FA. As a result of the low milk fat content in CON, milk fat concentration for all treatments was lower than anticipated. A meta-analysis by Hollmann et al. (2011) indicated that the milk fat concentration of cows fed control diets affects the response to supplementing distillers grains. Studies containing control cows with a milk fat concentration >3.6% tended to respond more negatively to added distillers grains, whereas studies containing control cows with a milk fat concentration <3.6% tended to respond positively for milk fat concentration with added distillers grains (Hollmann et al., 2011). It remains to be determined if control cows starting at a higher concentration of milk fat would have responded similarly to the UFA supplements used in the current study.

CONCLUSIONS

Unsaturated FA supplements, fed as either free or esterified soybean FA reduced DMI and increased milk yield, but had no effect on the yield of milk fat. Although indicators of an altered rumen environment with the UFA treatments were detected, evidenced by the increased concentration and yield of biohydrogenation intermediates associated with MFD in milk fat, we did not observe a reduction in milk fat yield because the increase in preformed FA compensated for the reduction in de novo and 16-carbon milk FA. No differences in yields of milk or components between the FFA and TAG treatments were detected; specifically, we observed no differences in the yield of biohydrogenation intermediates associated with MFD between the 2 UFA treatments, contrary to what has been observed in vitro and with by-products varying in the proportion of free and esterified FA. It remains to be determined if testing with cows starting with a higher milk fat concentration or using diets that differ in their risk of MFD, or both, may have produced differences between free and esterified FA. Further research on the regulation of preformed and de novo synthesized milk FA in the mammary gland is needed to develop strategies to maximize potential income from milk components.

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