Fatty acid profile of phospholipids and sphingomyelin in milk and regulation of sphingomyelin synthesis of mammary glands in cows receiving increasing levels of crushed sunflower seeds

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ABSTRACT

The objective of this study was to investigate the effect of increasing dietary supplementation of crushed sunflower seed (CSS) in the diet of dairy cows on the fatty acid (FA) composition of phospholipids and sphingomyelin in milk, and on mammary transcription of genes that are important for sphingomyelin de novo synthesis. Four groups of 6 cows received diets supplemented with CSS at 0% (control), or 5, 10, or 15% of dry matter for a 5-wk experimental period. Milk samples and mammary biopsies were collected at the end of the experiment. Phospholipid concentration in milk fat decreased linearly with CSS supplementation. Sphingomyelin concentration in milk fat was unaffected by CSS supplementation. Daily yield of phospholipids decreased linearly with CSS supplementation. Daily yield of sphingomyelin was not significantly affected. The CSS supplementation linearly increased the proportion of monounsaturated FA in milk phospholipids. The major isomer incorporated into phospholipids was C18:1 (n-9 *cis*), which showed a linear increase with CSS supplementation. The C22:0 proportion in sphingomyelin increased linearly with CSS supplementation and constituted between 15.2 to 25.4% of total FA in sphingomyelin. However, CSS supplementation linearly decreased C23:0 sphingomyelin. Mammary transcription of serine palmitoyl transferase, long chain subunit 1 and subunit 2, the rate-limiting enzymes in ceramide synthesis, showed a linear decrease with increasing CSS supplementation. In conclusion, the data showed that dietary supplementation of CSS linearly increased the proportion of unsaturated FA and monounsaturated FA in milk phospholipids with no effect on phospholipid concentration. In addition, CSS supplementation

linearly decreased n-3 polyunsaturated fatty acid proportion in sphingomyelin. The results further showed that mammary transcription of important genes for sphingomyelin de novo synthesis is regulated by lipid supplementation.

Key words: mammary lipogenesis, fatty acid composition, sphingomyelin, phospholipid

INTRODUCTION

Dietary phospholipids have been shown to have several potentially health-promoting effects, including inhibition of colorectal carcinogenesis, lowering the prevalence of gastric ulceration, and decreasing intestinal absorption of cholesterol (Spitsberg, 2005; Ward et al., 2006; Lopez, 2011). The inhibitory effect on cholesterol absorption is potentially a result of a physical interaction between phospholipids and cholesterol in the intestine, which decreases cellular uptake of both components (Nyberg et al., 2000; Eckhardt et al., 2002). The interaction between cholesterol and phospholipids is dependent on the fatty acid (FA) composition of phospholipids because long-chain SFA of phospholipids have a stronger interactions with cholesterol than UFA (Lange and Steck, 2008). In accordance with this, animal experiments show that intestinal absorption of cholesterol is better inhibited by saturated phospholipids versus unsaturated phospholipids (Jiang et al., 2001) and long-chain versus medium-chain FA (Noh and Koo, 2004).

Phospholipids are present in the milk fat globule membrane (MFGM) and dairy products rich in MFGM, such as butter milk, are convenient sources of phospholipids for human nutrition (Malmsten et al., 1994). Bovine milk fat contains 0.2 to 1% of phospholipids (Kurtz, 1974) with sphingomyelin constituting approximately 20% of the phospholipids. Sphingomyelin is characterized by a high proportion of long-chain SFA (Noh and Koo, 2004; Sanchez-Juanes et al., 2009; Lopez et al., 2017), which are involved in interactions between sphingomyelin and cholesterol (Cheng et al.,

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2017; Lopez et al., 2018). The strong interactions between sphingomyelin and cholesterol are considered to be fundamental for the formation of lipid rafts [i.e., sphingomyelin and cholesterol-rich sub-membrane domains in cellular membranes that are essential for cell function (Slotte, 1999)]. Lipid rafts were also found in the MFGM (Lopez et al., 2010), indicating that lipid rafts may have a biological role in the hydrolysis and absorption of milk fat in the gut of the offspring.

The FA composition of bovine milk phospholipids is dependent on breed, diet, fat globule size, and the milk fraction sampled [i.e., whole milk, cream, or MFGM (Fauquant et al., 2005; Lopez et al., 2008)]. Only a few experiments have been conducted to examine the effect of lipid supplementation on FA composition of phospholipids and the relative proportion of individual phospholipids (Palmquist and Schanbacher, 1991; Lopez et al., 2008). These authors reported that increased supplementation of UFA increased the proportion of UFA in milk phospholipids. However, neither the composition of milk sphingomyelin nor mammary transcription of genes involved in sphingomyelin de novo synthesis have been investigated in cows receiving lipid supplements. Given the specific role of sphingomyelin in cellular membranes and their tentative role in the MFGM, it is likely that the FA composition of sphingomyelin in bovine milk is more strictly regulated than that of phospholipids. A strict regulation of the FA composition may have role in transcriptional regulation of enzymes involved in sphingomyelin de novo synthesis, which is mediated by the coordinated action of the several enzymes. Serine palmitoyl transferase, long chain subunit 1 (*SPTLC1*) and subunit 2 (*SPTLC2*), and longevity assurance gene 1 homolog/ceramide synthase 2 (*LASS2*) are involved in the synthesis of ceramide, the backbone of sphingomyelin, and sphingomyelin synthase 2 (*SGMS2*) mediates the phospho-choline transfer to ceramide, ultimately producing sphingomyelin. The UDP-glucose ceramide glycosyltransferase (*UGCG*) mediates the formation of glycosphingolipids from ceramide, and is an alternative endpoint to

sphingomyelin for ceramides. It has been reported that *SPTLC1* and *SPTLC2*, rate-limiting factors in sphingomyelin synthesis, are regulated transcriptionally and post-transcriptionally (Hanada, 2003); however, no data have been reported on the regulatory mechanism of *LASS2*, *SGMS2*, and *UGCG*. The objective of this study was to examine the effect of incremental supplementation of CSS to dairy cows on the FA composition of phospholipids and in particular sphingomyelin, and the mammary transcription of genes involved in sphingomyelin de novo synthesis.

MATERIALS AND METHODS

All procedures were approved by the Danish Animal Experiment Inspectorate and complied with the Danish Ministry of Justice Law no. 1306 and 612 concerning animal experimentation and care of experimental animals (Dyreforsøgsloven).

Experimental Design and Sampling

Twenty-four healthy lactating Holstein Friesian cows (186 ± 20 DIM, yield: 25.3 ± 2.5 kg/d) from the dairy herd at Aarhus University were included in the experiment. In a 1-wk pre-experimental period, they were fed a basal diet (similar to control). On the last day of the pre-experimental period, milk yield was recorded and milk samples analyzed for fat. Cows were blocked according to days in milk within parity, and randomly allocated to 1 of 4 diets for a 5-wk experimental period. Cows were fed either a diet without lipid supplement (control) or diets supplemented with crushed sunflower seed (CSS) at 5, 10, or 15% of DM as a source of UFA. Diet composition is shown in Table 1. To maintain normal rumen pH and function, CSS was not accompanied by linear changes in other ingredients at the 15% CSS level. The decreased inclusion of mineral mix especially at 15% CSS occurs because sunflower contain more minerals than barley. Milk samples and mammary biopsies were obtained at the end of the experiment,

Table 1. Composition of control diet and diets with supplementation of crushed sunflower seeds (CSS; % of DM)

Feedstuff	Diet			
	Control	5% CSS	10% CSS	15% CSS
CSS	—	5.1	10.3	15.7
Soybean meal	14.3	10.5	8.8	13.4
Barley	29.8	28.5	24.6	12.6
Grass clover silage	55.3	55.3	55.8	58.1
Mineral mixture	0.28	0.28	0.17	—
Limestone	0.33	0.33	0.33	0.28

and milk from 2 consecutive milkings were pooled and stored at -20°C until lipid analyses.

Extraction and Analysis of Phospholipids and Sphingomyelin

Di-nonadecyl-phosphatidyl-choline (C19:0 PC) and C17:0 were added as internal standards to milk samples before lipid extraction. Lipids were extracted from milk using the method described by Jensen (2008). Phospholipids were isolated from nonpolar lipids on aminopropyl cartridges (Strata NH2, Phenomenex, Værløse, Denmark) using a slightly modified version of the method of Kaluzny et al. (1985). The cartridges were conditioned in 400 μL of hexane before applying the samples in 200 μL of CHCl_3 . Neutral lipids were eluted with 4 mL of CHCl_3 :2-propanol (2:1), which was followed by elution of FFA with 5 mL of 2% acetic acid in diethylether. Finally, the phospholipids were eluted with 4 mL of MeOH into a new glass. Thirty percent of the phospholipid fraction was *trans*-methylated and analyzed on a GC-flame ionization detector, as described earlier (Werner et al., 2011). In the remaining part of the phospholipid fraction, glycerophospholipids were hydrolyzed by mild alkaline hydrolysis (Jenkins and Hannun, 1999). The sphingomyelin was isolated from the hydrolysate by repeating the aminopropyl-cartridge fractionation, as described above. Hereafter, sphingomyelin was hydrolyzed as described by Avelldano and Horrocks (1983) and FAME was produced from the released FA using the bruno trifluoride (BF_3)-catalyzed procedure described by Werner et al. (2011).

Mammary Biopsies

On the last day of experiment (d 28) and after milking, the left front quarter of the udder was shaved, and an incision site without visual blood vessels was localized and anaesthetized subcutaneously with 2 mL of Xylocain (20 mg/mL lidocaine, AstraZeneca, Albertslund, Denmark) and cleaned with Hibitane (70% alcohol solution 1:10, Sigma-Aldrich, St. Louis, MO). Biopsies were taken with an automatic biopsy instrument (Manan Pro-Mag 2.2, Manan Medical Products Inc., Wheeling, IL) loaded with a 14 gauge $\times$ 10 cm needle (Manan ACN II, Manan Medical Products Inc.). Approximately 20 mg of tissue was collected per sampling, and a maximum of 2 samples was taken per cow. Tissue was snap frozen in liquid nitrogen and stored in Cryotubes (Nunc GmbH & Co. KG, Langenselbold, Germany) at -80°C until further analysis as described by Sorensen et al. (2006).

Real-Time PCR

The relative mRNA abundance was determined according to Sorensen et al. (2006) by quantitative PCR using gene-specific primers (DNA Technology, Aarhus, Denmark) and various commercial chemicals to detect mRNA abundance (using either locked nucleic acid fluorescent probes from Exiqon, Værum, Denmark, or fluorescent TaqMan probes purchased from Applied Biosystems, Nærum, Denmark) were used. Fold change was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method under consideration of PCR efficiency for each assay. Cycle threshold (**Ct**) is the number of PCR cycles required for the fluorescent signal to cross the threshold (i.e., exceeds background level). Three endogenous controls [glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), ubiquitously expressed transcript (*UXT*), and beta actin (*ACTB*)] were tested by normalizing against the RNA content and all were found to be suitable housekeeping genes as they were not affected by the diets ($P = 0.49$, $P = 0.93$, and $P = 0.84$, respectively). Thus, the mean Ct of the 3 housekeeping genes were subsequently used to normalize all target genes. All samples were analyzed in duplicates for each gene except for endogenous control genes, which were analyzed in triplicates. Primer and probe sequences are listed in Table 2. No signal was detected in samples of pure water or bovine DNA.

Statistical Analysis

Statistical analysis was performed in SAS software (version 9.4, SAS Institute Inc., Cary, NC). The statistical model was a general linear model:

$$Y_{ijk} = \mu + T_i + B_j + E_{ijk},$$

where Y_{ijk} is the dependent variable, μ is the overall mean, and the model includes the fixed effects of treatment (T_i) and random effects of block (B_j), and E_{ijk} is the random error. In analysis of production data, the model further included pre-experimental production data as covariates. A polynomial contrast analysis was employed to test for linear, quadratic, and cubic effects. The polynomial contrasts should be interpreted with caution because the linear inclusion of CSS was not accompanied by linear changes in other ingredients at the 15% CSS level. All statistics on mRNA abundance were performed at the ΔCt level, whereas the relative mRNA abundance (mean, lower and upper 95% CI) was calculated using the $2^{-\Delta\Delta\text{Ct}}$ transformation. Significance was declared at $P < 0.05$.

RESULTS

DMI and Milk Yield

Dry matter intake was 18.4, 17.3, 14.5, and 14.8 kg/d in control and 5, 10, and 15% CSS, respectively, and was decreased linearly ($P < 0.01$) with supplementation of CSS. As well, daily milk yield was decreased linearly ($P < 0.01$) with CSS supplementation (i.e., 29.0, 27.6, 20.9, and 20.9 kg/d for control and 5, 10, and 15% CSS, respectively). The proportion of UFA in milk was 27.5, 32.5, 40.2, and 40.30 (g/100 g of milk FA) in control and 5, 10, and 15% CSS, respectively, and increased linearly ($P < 0.01$) with CSS supplementation.

Phospholipid and Sphingomyelin Yield and Concentration

Milk fat concentration increased linearly with CSS supplementation ($P < 0.01$; Table 3). Phospholipid yield decreased linearly due to a decrease in milk yield ($P < 0.01$). Sphingomyelin concentration increased linearly with CSS supplementation ($P = 0.04$), whereas sphingomyelin yield was not significantly affected ($P = 0.06$). Concentration of phospholipid in milk fat linearly decreased with increasing CSS supplementation ($P < 0.01$). However, concentration of sphingomyelin in milk fat was not affected. Proportion of sphingomyelin

in phospholipids linearly increased with CSS supplementation ($P < 0.01$).

FA Composition of Phospholipids

Proportion of C16:0 incorporated in phospholipids decreased linearly with CSS supplementation ($P < 0.01$; Table 4). Proportion of MUFA in phospholipids was increased linearly by CSS supplementation ($P < 0.01$). The major FA contributing to the increase was C18:1 (n-9 *cis*), which increased linearly in response to CSS supplementation ($P < 0.01$), but C20:1 (n-9 *cis*; $P < 0.01$), C18:1 (n-9 *trans*; $P < 0.01$), and C18:1 (*trans*-11; $P < 0.01$) also increased linearly. Although C18:0 proportion in phospholipids showed a quadratic effect with CSS supplementation ($P = 0.03$), the proportion of SFA in phospholipids decreased linearly ($P < 0.01$). The linear decrease in SFA caused a linear decreased proportion of C16:0, C15:0, C14:0, and C12:0 ($P < 0.01$). The proportion of C18:2 (n-6 *cis*) showed a cubic effect with CSS supplementation ($P = 0.04$).

FA Composition of Sphingomyelin

Proportion of C23:0 in sphingomyelin was decreased linearly by CSS supplementation ($P < 0.01$; Table 5). The C22:0 proportion in sphingomyelin showed a quadratic effect with CSS supplementation ($P = 0.03$).

Table 2. Primer and probe design used for real-time PCR

Gene ¹	Primer/probe	Sequence (5' to 3')
<i>LASS2</i>	Forward	TCATGGCTCTGCACGACTCT
	Reverse	CCATCCCGCGTAGTTAAACATC
	Probe	TACCTGCTAGAGTCAGCCA
<i>SPTLC1</i>	Forward	GGAACCTTGTGGACCTAGAGGATTTT
	Reverse	TGCCAGGCGGTCTTCTAAA
	Probe	CACATTTGATGTGCACTTG
<i>SPTLC2</i>	Forward	CTTGTTAGCAAAGGTTGCCTGATT
	Reverse	GGCTCCCAGAACCAGTGAAG
	Probe	TGAGTGATGAACTGAACC
<i>SGMS2</i>	Forward	CCGGTGCTGGAATGC
	Reverse	AGCCGTAGAATCCGCTGTACTT
	Probe	TGCTCCAAAGCTC
<i>UGCG</i>	Forward	CAGAATGATCAGGTGGACCAAAA
	Reverse	TGGCTCGCAAATTATTGTAGCA
	Probe	TGCGAATTAACATGCTTC
<i>ACTB</i>	Forward	ACCCAGATCATGTTTCGAGACCTT
	Reverse	CTGTATGCCTCTGGCCGCACCA
	Probe	TCACCGGAGTCCATCACGAT
<i>GAPDH</i>	Forward	GTCCGAGTGAACGGATTG
	Reverse	CAATGTCCACTTTGCCAGAGTTAA
	Probe	CGCCTGGTCAACAGGGCTGCT
<i>UXT</i>	Forward	GATTTGGGCTGTAACTTCTTCGTT
	Reverse	GGGCCACATAGATCCGTGAA
	Probe	ACACAGTGGTCCCAGACA

¹Longevity assurance gene 1 homolog (*LASS2*), serine-palmitoyl transferase, long chain subunit 1 (*SPTLC1*), serine-palmitoyl transferase, long chain subunit 2 (*SPTLC2*), sphingomyelin synthase 2 (*SGMS2*), UDP-glucose ceramide glucosyltransferase (*UGCG*), beta actin (*ACTB*), glyceraldehyde 3-phosphate (*GAPDH*), ubiquitously expressed transcript (*UXT*).

Table 3. Concentration and yield of phospholipids and sphingomyelin in milk from cows fed diets with increasing levels of crushed sunflower seeds (CSS)

Item	Treatment ¹				SEM	P-value for polynomial contrast		
	Control	5% CSS	10% CSS	15% CSS		Linear	Quadratic	Cubic
Fat, g/100 mL	3.34	3.75	3.92	4.61	0.23	<0.01	0.07	0.97
Phospholipids, % of milk fat	1.47	1.53	1.02	0.93	0.07	<0.01	0.07	0.81
Sphingomyelin, % of milk fat	0.59	0.58	0.56	0.49	0.49	0.13	0.58	0.98
Phospholipid concentration, g/L	0.49	0.43	0.49	0.43	0.02	0.06	0.06	0.56
Phospholipid yield, g/d	13.4	11.8	9.2	8.7	0.5	<0.01	0.16	0.13
Sphingomyelin concentration, g/L	0.21	0.22	0.21	0.23	0.005	0.04	0.57	0.36
Sphingomyelin yield, g/d	6.02	6.00	4.96	4.66	0.3	0.06	0.06	0.36
Sphingomyelin in phospholipids, %	41.1	50.9	56.3	53.4	3.3	0.01	0.07	0.81

¹Cows were fed a diet without lipid supplement (control) or diets supplemented with CSS at 5, 10, or 15% of DM.

Both C22:2 (n-6 *cis*) and C20:5 (n-3 *cis*) in sphingomyelin decreased linearly with the inclusion of CSS supplement. The SFA proportion in sphingomyelin showed a cubic effect with CSS supplementation ($P < 0.01$).

Mammary Transcription of Sphingolipogenic Genes

The *SPTLC1* mRNA decreased linearly ($P = 0.03$) with CSS supplementation (Table 6). Similarly,

Table 4. Proportion of fatty acids (FA) in phospholipids (g of FA/100 g of FA of phospholipids) of milk from cows fed diets with increasing levels of crushed sunflower seeds (CSS)

Fatty acid	Treatment ¹				SEM	P-value for polynomial contrast		
	Control	5% CSS	10% CSS	15% CSS		Linear	Quadratic	Cubic
C12:0	0.38	0.30	0.066	ND ²	0.05	<0.01	0.92	0.25
C13:0	0.10	0.17	0.12	0.11	0.03	0.94	0.55	0.46
C14:0	4.38	4.00	0.81	0.46	0.45	<0.01	0.93	0.03
C15:0	1.02	0.88	0.35	0.25	0.08	<0.01	0.85	0.06
C16:0	24.7	21.4	14.4	13.0	1.1	<0.01	0.23	0.01
C16:1 (n-9 <i>cis</i>)	1.12	0.83	0.39	0.33	0.08	<0.01	0.09	0.10
C17:0	0.75	0.66	0.62	0.60	0.02	<0.01	0.12	0.71
C17:1 (n-9 <i>cis</i>)	1.77	0.31	0.89	0.45	0.26	0.19	0.41	0.21
C18:0	18.3	21.5	25.9	25.1	0.8	<0.01	0.03	0.11
C18:1 (<i>trans</i> -11)	0.53	1.03	1.69	1.95	0.04	<0.01	0.48	0.70
C18:1 (n-9 <i>cis</i>)	30.1	33.7	38.5	38.4	1.0	<0.01	0.40	0.66
C18:1 (n-9 <i>trans</i>)	0.32	0.45	0.51	0.69	0.04	<0.01	0.74	0.51
C18:1 (n-7 <i>cis</i>)	0.70	0.62	0.65	0.60	0.14	0.59	0.87	0.49
C18:2 (n-6 <i>cis</i>)	7.93	9.15	8.05	8.86	0.24	0.42	0.73	0.04
C18:3 (n-3 <i>cis</i>)	0.93	0.74	0.51	0.50	0.04	<0.01	0.03	0.13
C20:0 and C18:3 (n-6 <i>cis</i>) ³	0.22	0.26	0.27	0.28	0.01	0.22	0.23	0.58
C20:1 (n-9 <i>cis</i>)	0.65	1.06	1.57	2.38	0.18	<0.01	0.38	0.80
C20:3 (n-6 <i>cis</i>)	1.90	0.73	1.44	1.04	0.18	0.32	0.38	0.14
C20:4 (n-3 <i>cis</i>)	0.22	0.27	0.76	0.59	0.04	0.10	0.38	0.15
C20:4 (n-6 <i>cis</i>)	0.89	0.79	0.59	0.76	0.07	0.30	0.26	0.36
C20:5 (n-3 <i>cis</i>)	0.39	0.29	0.18	0.29	0.03	<0.01	<0.01	<0.01
C22:0	ND	ND	0.18	0.20	0.05	0.07	0.93	0.41
C22:5 (n-3 <i>cis</i>)	0.49	0.52	0.51	0.51	0.03	0.85	0.84	0.77
C22:5 (n-6 <i>cis</i>)	0.29	<0.01	0.88	1.97	0.25	<0.01	0.06	0.55
C23:0	1.00	<0.01	0.17	<0.01	0.17	0.05	0.21	0.25
C24:0	0.61	0.12	0.41	0.32	0.09	0.54	0.38	0.21
MUFA	35.4	38.6	44.2	45.0	1.1	<0.01	0.64	0.33
n-3	1.85	1.56	1.72	1.33	0.08	0.20	0.24	0.46
n-6	11.0	10.7	10.9	12.7	0.4	0.14	0.16	0.76
PUFA	12.8	12.5	12.7	13.9	1.0	0.40	0.27	0.93
SFA	51.3	49.1	44.8	40.1	1.1	<0.01	0.72	0.28
UFA	48.7	50.9	57.1	59.8	1.3	<0.01	0.72	0.28

¹Cows were fed a diet without lipid supplement (control) or diets supplemented with CSS at 5, 10, or 15% of DM.

²ND = not detected.

³Coeluting peaks were present between C20:0 and C18:3 (n-6 *cis*).

Table 5. Proportion of fatty acid (FA) in sphingomyelin (g of FA/100 g of FA of sphingomyelin) of milk from cows fed diets with increasing levels of crushed sunflower seeds (CSS)

Fatty acid	Treatment ¹				SEM	P-value for polynomial contrast		
	Control	5% CSS	10% CSS	15% CSS		Linear	Quadratic	Cubic
C14:0	2.08	1.67	2.23	2.71	0.26	0.02	0.86	0.31
C15:0	0.62	0.51	0.55	0.61	0.04	0.92	0.11	0.46
C16:0	37.5	31.7	32.5	33.8	2.6	0.28	0.11	0.53
C16:1 (n-9 <i>cis</i>)	0.90	0.91	0.94	0.89	0.06	0.95	0.62	0.68
C18:0	5.49	4.59	4.42	4.33	0.42	0.07	0.37	0.74
C18:1 (n-9 <i>cis</i>)	1.23	1.18	0.99	0.99	0.10	0.04	0.80	0.40
C18:2 (n-6 <i>cis</i>)	0.28	0.32	0.27	0.24	0.02	0.16	0.21	0.30
C20:0	1.41	1.43	1.50	1.47	0.06	0.26	0.62	0.41
C20:3 (n-3 <i>cis</i>)	0.33	0.30	0.26	0.24	0.02	0.01	0.89	0.81
C20:5 (n-3 <i>cis</i>)	0.99	0.98	0.88	0.83	0.05	0.01	0.79	0.47
C21:0	0.68	0.64	0.65	0.66	0.06	0.67	0.64	0.80
C22:1 (n-9 <i>cis</i>)	0.48	0.36	0.30	0.28	0.05	0.01	0.40	0.80
C22:0	15.2	19.9	23.2	24.5	0.8	<0.01	0.03	0.84
C22:2 (n-6 <i>cis</i>)	1.81	1.47	1.10	0.68	0.68	<0.01	0.65	0.91
C23:0	16.8	16.7	14.7	13.1	0.9	<0.01	0.50	0.59
C24:0	12.7	14.6	13.7	12.8	0.7	0.76	0.07	0.37
C24:1 (n-9 <i>cis</i>)	2.26	2.34	1.60	1.52	0.16	<0.01	0.62	0.04
C26:0	0.26	0.29	0.25	0.21	0.03	0.26	0.24	0.46
>C20	55.0	57.7	56.9	54.9	3.0	0.32	0.12	0.54
MUFA	4.88	4.80	3.83	3.69	0.15	<0.01	0.77	0.01
n-3	1.33	1.28	1.15	1.08	0.06	<0.01	0.87	0.53
n-6	2.09	1.80	1.38	0.92	0.09	<0.01	0.40	0.83
PUFA	3.43	3.08	2.53	2.00	0.10	<0.01	0.37	0.78
SFA	91.6	92.1	93.6	94.2	0.2	<0.01	0.38	<0.01
UFA	8.31	7.89	6.37	5.70	0.15	<0.01	0.38	<0.01

¹Cows were fed a diet without lipid supplement (control) or diets supplemented with CSS at 5, 10, or 15% of DM.

SPTLC2 mRNA showed a linear decrease ($P = 0.02$) with CSS supplementation.

DISCUSSION

Sphingolipids have a unique structure and FA composition that favor strong interaction with cholesterol in cell membranes. Sphingolipids are synthesized via a unique pathway, and FA incorporated into sphingolipids share no pathway intermediates with triglycerides or phospholipids. In contrast, triglycerides and phospholipids are derived from diglycerides, and their FA composition is similar (Bitman and Wood, 1990). The

effect of lipid supplementation on the FA composition of phospholipids and sphingomyelin reflects the different synthesis pathways of lipid fractions.

Phospholipid and Sphingomyelin Yield and Concentration

Concentration of phospholipids determined in the present study (i.e., 0.93 to 1.47) was higher than the results found in cows fed linseed diets (Lopez et al., 2008). However, sphingomyelin concentration was lower than sphingomyelin concentration reported by Lopez et al. (2008). These discrepancies may be due to dif-

Table 6. Mammary transcription genes of sphingomyelin biosynthesis in cows fed diets with increasing levels of crushed sunflower seed (CSS)

Gene ¹	Treatment ²				P-value for polynomial contrast		
	Control	5% CSS	10% CSS	15% CSS	Linear	Quadratic	Cubic
<i>LASS2</i>	1.00 (0.80–1.25) ³	0.89 (0.72–1.20)	0.80 (0.67–1.05)	0.81 (0.57–0.95)	0.08	0.85	0.95
<i>SGMS2</i>	1.00 (0.76–1.32)	0.85 (0.76–1.42)	0.98 (0.86–1.51)	1.16 (0.69–1.30)	0.94	0.58	0.59
<i>SPTLC1</i>	1.00 (0.71–1.41)	0.89 (0.59–1.27)	0.77 (0.58–1.14)	0.70 (1.37–0.80)	0.03	0.45	0.72
<i>SPTLC2</i>	1.00 (0.77–1.30)	1.08 (0.79–1.41)	0.71 (0.60–1.02)	0.62 (0.48–0.87)	0.02	0.27	0.33
<i>UGCG</i>	1.00 (0.73–1.37)	1.48 (0.87–1.80)	1.48 (1.06–1.96)	1.41 (0.96–1.89)	0.37	0.39	0.71

¹*LASS2* = longevity assurance gene 1 homolog; *SPTLC1* = serine-palmitoyl transferase, long chain subunit 1; *SPTLC2* = serine-palmitoyl transferase, long chain subunit 2; *SGMS2* = sphingomyelin synthase 2; *UGCG* = UDP-glucose ceramide glucosyltransferase.

²Cows were fed a diet without lipid supplement (control) or diets supplemented with CSS at 5, 10, or 15% of DM.

³95% CI (lower limit–upper limit).

ferences in the stage of lactation, feeding of the cow, milk, and the methods of preparation and analysis of the samples. The results showed that concentration of milk phospholipids was decreased linearly by CSS supplementation. This is probably due to a lower surface area of smaller milk fat globules (Lopez et al., 2008). In the present study, the proportion of sphingomyelin in phospholipids increased and was accompanied by decreased concentration of phospholipids in milk fat, most likely to maintain rigidity of MFGM (Lopez et al., 2008, 2011), which has been discussed below.

FA Composition of Phospholipids

Our results show that the FA composition of milk phospholipids is responsive to dietary changes. In our experiment, CSS supplementation linearly decreased the phospholipid proportion of C16:0 by up to 12 percentage points, depending on the level of supplementation. The major FA substituting C16:0 in milk phospholipids are C18:0 and C18:1 (n-9, *cis*). In the present experiment, C18:0 in milk phospholipids increased by approximately 7 percentage points, which is in agreement with the proportion of C18:0 in milk phospholipids of cows fed linseeds (Lopez et al., 2008). However, in our experiment, C18:0 constituted between 18.3 and 25% of the total FA present in milk phospholipids, whereas Lopez et al. (2008) reported a C18:0 proportion between 10.1 and 17.3%. Also, the level of C18:1 (n-9, *cis*) in our experiment (range: 30 to 38%) is higher than that reported by Lopez et al. (2008). However, the variation in phospholipid proportion of FA is large, and the variation observed in the present experiment is in agreement with previous reports (Fong et al., 2007; Lopez et al., 2014).

Although in the present study, diet concentration of C18:2 (n-6 *cis*) increased with increasing CSS supplementation, a cubic effect of CSS supplement on C18:2 (n-6 *cis*) proportion of phospholipids was observed. These findings might reflect the limited influence of CSS levels on the FA composition of milk phospholipids. In addition, changes in allocation of phospholipid FA by mammary gland could be a reason for the cubic effect of treatments on C18:2 (n-6 *cis*) proportion of phospholipids. Supplementation of CSS linearly increased the proportion of UFA in milk phospholipids, suggesting that changes in the supply of UFA may have contributed to the alterations in the UFA concentration of milk phospholipids. This is in agreement with Lopez et al. (2008), who reported a more than 10 percentage point decrease of SFA in milk phospholipids following dietary supplementation of UFA. However, the proportion of SFA in milk phospholipids is associated with large variation. Lopez et al. (2008) and Menard et al.

(2010) detected more than 60% SFA in milk phospholipids from cows receiving a basal diet, whereas Fong et al. (2007) and Fauquant et al. (2005) report a proportion of SFA in milk phospholipids below 50%. The discrepancies found in the literature may be explained by differences in the basal diet or, more likely, differences in the methods used to extract phospholipids from milk.

FA Composition of Sphingomyelin

The main FA of milk sphingomyelin were C16:0, C22:0, C23:0, and C24:0 in agreement with Sanchez-Juanes et al. (2009). The major effect of CSS supplementation on milk sphingomyelin composition was a quadratic effect in C22:0, which increased by almost 10 percentage points. In contrast, C23:0 proportion linearly decreased by 5 percentage points in cows receiving 15% CSS supplementation. The substitution of C23:0 with C22:0 in milk sphingomyelin probably reflects an increased availability of C22:0 in the mammary gland. Supplementation of CSS linearly decreased milk sphingomyelin proportion of C22:2 (n-6 *cis*), C20:5 (n-3 *cis*), and C22:1 (n-9 *cis*), which resulted in a cubic response in proportion of UFA in milk sphingomyelin. This is in contrast to the effect of CSS supplementation on phospholipid composition, where CSS supplementation linearly increased the proportion of UFA. Therefore, the different FA composition in phospholipids and sphingomyelin may reflect the different synthesis pathway. In addition, CSS supplementation did not affect milk sphingomyelin proportion of C18:0, C16:0, or C18:2 (n-6, *cis*) in the present experiment. These findings reflect that the regulation of milk sphingomyelin secretion is not solely dependent on the dietary FA composition, but may involve the coordinated and highly regulated FA catabolism and synthesis in body tissues, in particular mammary glands (Christie, 1981; Bauchart, 1993). The cubic effect of CSS on sphingomyelin SFA could be due to the key role of saturated long-chain FA in maintaining rigidity of the MFGM. High proportion of SFA and high proportion of long-chain FA (>C20) of sphingomyelin are 2 properties that contribute to less fluidity and tend to support a structural role of sphingomyelin in maintaining rigidity of the MFGM (Bitman and Wood, 1990; Et-Thakafy et al., 2018). Our data show that the increase in SFA proportion in sphingomyelin was accompanied by a decrease in SFA proportion of phospholipids. Variation in the FA content of phospholipids from MFGM may be an adaptation to membrane fluidity requirements. Therefore, MFGM have more very-long-chain FA (more rigidity), compensated by more UFA (less rigidity). In addition, deposition, de novo synthesis, elongation, de-

saturation, and mobilization of these FA in adipocytes (Halmemies-Beauchet-Filleau et al., 2013) may be a reason for the lack of CSS supplementation on sphingomyelin proportion of C16:0, C18:0, and C18:2 (n-6 *cis*). The measured levels of the individual FA in the milk sphingomyelin are within the variations reported in the literature (Bitman and Wood, 1990; Noh and Koo, 2004; Fong et al., 2007). The changes in FA composition caused by dietary supplementation of CSS do not induce conformational changes in the sphingomyelin, as C22:0 and C23:0 are both long-chain SFA with similar physiological capacities. Thus, the potential to interact with cholesterol and form lipid rafts is most likely not affected.

Mammary Transcription of Lipogenic Genes

Mammary transcription of sphingolipogenic genes is activated at the onset of lactation (Bionaz and Looor, 2008), supporting that the synthesis of sphingomyelin is important for milk production. The *SPTLC1* and *SPTLC2* are considered rate-limiting factors in sphingomyelin synthesis, and decreased transcription indicates a decreased mammary de novo synthesis of sphingomyelin (Rabionet et al., 2014). This could be explained by a decreased demand for mammary sphingomyelin synthesis caused by a decreased demand for milk fat globule formation. Lopez et al. (2008) showed that milk fat globule size was decreased in cows receiving lipid supplements, which decrease the demand for membrane formation and sphingomyelin synthesis.

Although not significant, the more than 40% increase observed in *UGCG* transcription after CSS supplementation may suggest that the mammary epithelial cells are directing newly synthesized ceramides to glucosphingolipid synthesis instead of sphingomyelin, which is the major sphingomyelin in bovine milk. The reason for this change may be due to a limited availability of choline for mammary sphingomyelin synthesis, and limited availability of choline may affect sphingomyelin synthesis (Lyman et al., 1975).

CONCLUSIONS

In summary, dietary supplementation of CSS increased the proportion of UFA in milk phospholipids. The SFA proportion of sphingomyelin increased with CSS supplementation. However, the UFA composition of milk sphingomyelin is regulated in a way that only allows minor structural changes to the sphingomyelin. Furthermore, mammary transcription of the gene coding for the rate-limiting enzyme in sphingomyelin de novo synthesis was decreased by CSS supplementation.

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