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The effect of dietary forage to concentrate ratio and forage type on milk phospholipids and fatty acid composition of polar lipids

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ABSTRACT

The effects of grass silage and red clover silage on milk fatty acid (FA) composition are extensively studied, but little is known of their impacts on minor lipid constituents of milk fat globule membrane. We investigated the effects of forage:concentrate (FC) ratio in grass silage-based diets and forage type (grass silage vs. red clover silage) on selected molecular species of milk phospholipids (PL) and the FA composition of PL. Ten multiparous Nordic Red cows were offered following dietary treatments: grass silage-based diets containing 70:30 (HG) or 30:70 (LG) FC ratio or a red clover silage-based diet (RC) comprising 50:50 FC ratio on a dry matter basis. The most abundant molecular species within the phosphatidylcholines was 16:0–18:1 phosphatidylcholine that was increased by 18% in HG compared with LG milk. Dietary treatments did not affect the relative proportion of 18:1–18:1+18:0–18:2 phosphatidylethanolamine that was the most prevalent species (ca. 44–45%) in that class. We identified the d18:1–22:0 sphingomyelin as the most abundant sphingomyelin species that tended to increase in HG milk compared with LG. The FC ratio did not affect the relative proportions of saturated FA nor monounsaturated FA in PL, but the proportion of *cis*-9 18:1 was elevated in HG vs. LG milk, whereas the proportion of 18:2n-6 was 50% higher in LG vs. HG milk. The RC diet increased monounsaturated FA and 18:3n-3 levels in PL compared with grass silage-based diets and decreased the relative proportion of saturated FA. However, the RC diet did not affect the relative proportion of polyunsaturated FA in PL, although red clover silage typically increases the proportion of polyunsaturated FA in milk fat. This study provides valuable knowledge of the minor lipid components in milk on species level in relation to common feeding strategies in high-forage systems.
Keywords: grass silage, red clover silage, phospholipid, polar lipid, fatty acid composition

INTRODUCTION

Milk polar lipids (**PL**) are amphiphilic molecules found in the surrounding trilayer membrane of milk fat globules (**MFGM**) and in smaller milk exosomes that are nanovesicles having an important role in transporting other signaling molecules between cells (Ortega-Anaya and Jiménez-Flores, 2019). Increasing evidence has demonstrated that the PL fraction of milk has beneficial effects on neuronal development of infants (Timby et al., 2014), regulation of immune responses, protection against bacteria and the modulation of gut microbiome (Bhinder et al., 2017; Ortega-Anaya and Jiménez-Flores, 2019; Anto et al., 2020). Bovine milk consists of relatively low concentrations of PL compared with the abundant triglycerides (**TG**), typical concentration varying from 9 to 40 mg/100 g of raw milk (Ortega-Anaya and Jiménez-Flores, 2019) and 0.25–0.98 g/100 g of milk fat (Rombaut et al., 2005, 2006; Ali et al., 2017). To maximize the value of these minor lipid constituents of bovine milk, more knowledge is needed especially on how dairy cow diets affect these constituents as a mean of naturally modifying the milk fat globule.

The predominant PL classes in milk are phosphatidylethanolamine (**PE**) (26.8–46.4% of total polar lipids), phosphatidylcholine (**PC**) (19.2–37.3%), sphingomyelin (**SM**) (18–28.7%), phosphatidylserine (2.8–16.1%) and phosphatidylinositol (0.6–13.6%) as reviewed by Ortega-Anaya and Jiménez-Flores (2019). The complexity of PL fraction arises from the wide variety of structural and positional isomers within a PL class (Fong et al., 2007; Donato et al., 2011; Ali et al., 2017). Currently more than 500 molecular spe-

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cies and 15 classes of PL have been characterized in bovine milk (Liu et al., 2020). While extensive research on the identification of molecular species have been conducted, studies investigating how dairy cow diets affect PL on molecular species level are scarce (Craige Trenerry et al., 2013; Fougère et al., 2021). Although with feeding strategies it is possible to naturally modify the fatty acid (**FA**) composition to more beneficial direction for human health, the possible impact of dairy cow diets on MFGM structure and functionality should not be overlooked. Furthermore, as discussed in Liu et al. (2018), understanding of biological functions on molecular species level is still severely lacking. As an example, some lysophosphatidylcholine species could be used as potential biomarkers indicating cow's health during heat stress (Liu et al., 2017).

The effects of red clover silage on cow's FA metabolism are extensively studied, but research on minor lipid constituents in MFGM are lacking. It is well elucidated that feeding red clover silage increases the proportion of PUFA in milk fat because of reduced biohydrogenation of PUFA, especially 18:3n-3 and 18:2n-6, in rumen (Vanhatalo et al., 2007; Halmemies-Beauchet-Filleau et al., 2013, 2014; Jaakamo et al., 2019). According to Heid and Keenan (2005) and Lashkari et al. (2020b) the FA composition of PL most likely reflects the general changes induced by diet as the MFGM is derived from the membranes of the mammary epithelial cells. In fact, dietary supplementation of oil seeds rich in UFA to either corn silage or grass clover silage-based diets have been shown to increase the incorporation of UFA into polar lipid fraction (Lopez et al., 2008; Lashkari et al., 2020b). Therefore, it seems plausible that feeding red clover silage increases not only the overall PUFA level in milk fat, but also the incorporation of PUFA to polar lipid fraction. Furthermore, high concentrate content in grass silage-based diet showed higher PUFA level in milk fat due to higher PUFA intake from feed when compared with a diet with low concentrate content (Jaakamo et al., 2019). It remains a question whether the effects of these forage types are seen in FA composition of PL.

Previously, we examined the effect of forage: concentrate (**FC**) ratio in grass silage-based diets and forage type (grass silage vs. red clover silage) on the size of milk fat globule and on FA metabolism from feed to milk (Jaakamo et al., 2019). The aim of the present study was to investigate the effect of dietary FC ratio in grass silage-based diets and forage type on selected polar lipid molecular species and FA composition of PL. The diets examined in this study represented common feeding strategies used in organic milk production and high-forage systems. We hypothesized that the changes in milk fat caused by different dairy cow diets would be reflected in the PL composition and FA profile of MFGM. More specifically, we hypothesized that the decreased biohydrogenation of long-chain PUFA in cows fed red clover silage would increase the proportion of long-chain PUFA in polar lipid fraction.

MATERIALS AND METHODS

Experimental Design, Diets and Sampling

The design of this feeding experiment is explained in detail in Jaakamo et al. (2019). All experimental procedures were approved by the National Animal Ethics Committee (ESAVI/5344/04.10.07/2014, Finland) in accordance with the guidelines established by the European Council (86/609/EEC, European Council 1986). Briefly, 10 multiparous Nordic Red cows were allocated at random to experimental diets in 3 × 3 Latin square design replicated 3 times. An extra replicate was randomly assigned to each diet once in each 35-d experimental period leading to total of 10 replicates for each diet. The experiment was conducted in 2 blocks (blocks A and B) of 4 and 6 cows to have the cows in the same lactation stage. The experimental diets were as follows: 1) high-forage, low-concentrate diet (**HG**) consisting of 70% grass silage-based forage and 30% cereal-based concentrate on DM basis, 2) low-forage, high-concentrate diet (**LG**) that contained 30% grass silage-based forage and 70% cereal-based concentrate and 3) diet consisting of 50% red clover silage and 50% cereal-based concentrate (**RC**). Originally the RC treatment was intended to consist of 70:30 FC ratio, but concentrate was added up to 50:50 FC ratio to compensate the low concentration of digestible OM of red clover silage. Silages consisted of mixed timothy (*Phleum pratense*), meadow fescue (*Festuca pratensis*) and red clover (*Trifolium pratense*) swards treated with 5 L/t of formic acid-based additive (760 g of formic acid and 55 g of ammonium formate per each liter, AIV 2 Plus, Kemira Ltd.). The concentrate consisted of barley, molassed sugar beet pulp, rapeseed meal and vitamin and mineral premix (Mahti-Mira, Vilomox Finland). The detailed composition of each experimental diet was reported in Jaakamo et al. (2019). The diets were offered as TMR ad libitum 4 times a day and cows were milked twice a day. Milk samples of 4 consecutive milkings were collected on d 30 to 32 and combined according to the milk yield. Samples were stored in −20°C before analysis.

Chemicals and Reagents

The following phospholipid reference standards and an internal standard (**ISTD**) were purchased

from Avanti Polar Lipids Inc.: 18:1–18:1 (*cis*-9) PE, 16:0–18:1 PE, 16:0–16:0 PC, 16:0–18:1 PC, 18:0–18:0 PC, 18:1–18:1 (*cis*-9) PC, d18:1–16:0 SM, d18:1–24:0 SM and d18:1–16:0-d31 SM. For the quantification of SM species, d18:1–16:0-d31 SM was used as ISTD. All chemicals used were of analytical grade or HPLC grade. Ultrapure water was used.

Standard solutions and calibration

Individual stock solutions (5 mg or 10 mg per milliliter 8:2 chloroform:methanol) of phospholipid reference standards were prepared, combined and diluted to form one single standard mix of 500 μg/mL concentration of each phospholipid analyte. Internal standard stock solution of 200 μg/mL was prepared in 8:2 chloroform:methanol. Calibration levels (0.5; 1; 5; 10; 25; 50; 75; and 100 μg/mL) were prepared in 1:1 chloroform:methanol. Before milk fat extraction 50 μL ISTD solution (200 μg/mL) was added to milk samples for phospholipid molecular species analysis by LC-MS/MS.

The concentrations of selected phospholipid species were determined with corresponding calibration standards which are listed in detail in Supplemental Table S1 (https://doi.org/10.17632/yy6hv32pbz.1; Jaakamo et al., 2023). Internal standard SM d18:1–16:0-d31 was used for the calculations of SM species to mitigate the possible loss of analytes during sample preparation.

Fat Extraction

Milk fat was extracted according to reference procedures (IDF 1986, International Dairy Federation, Brussels, Belgium) and Shingfield et al. (2003) with slight modifications: 400 μL ammonia was added to 2 mL of milk sample following 2 mL of ethanol. Fat was extracted with 5:4 diethyl ether:hexane and the upper layer was collected. One milliliter of ethanol was added to the residual sample and the extraction with diethyl ether:hexane was repeated. The extracts were combined and evaporated to dryness with constant flow of nitrogen in 50°C. The amount of total extracted fat was measured.

Lipid Fractioning

The fat extracts were dissolved in 1 mL of 2:1 chloroform:methanol and fractionated with solid phase extraction (**SPE**) cartridges (Supelclean LC-Si, bed weight 1 g, 6 mL volume, Supelco) according to a procedure by Avalli and Contarini (2005). Fat extracts were introduced to SPE cartridges after a conditioning step with hexane. The cartridge was washed first with 8:2 and then 1:1 hexane:diethyl ether mixtures to remove non-polar lipids. The polar lipids were eluted first with 4 mL of methanol, second with 2 mL of methanol and finally with 2 mL of 3:5:2 chloroform:methanol:water. The eluted fraction was evaporated to dryness with constant flow of nitrogen in 50°C. The extracts were dissolved in 1 mL of 1:1 methanol:chloroform for analysis of phospholipid molecular species in LC-MS/MS.

Fatty Acid Methyl Esters

For the determination of the FA composition of PL, milk samples were re-extracted without internal standard and re-fractionated with SPE. After the SPE the lipid extracts were dissolved in hexane:methyl acetate and methylated to FAME via transesterification with methanolic sodium methoxide (Shingfield et al., 2003). The extracts were dissolved in 0.5 mL of hexane before analysis by GC coupled with flame ionization detector.

Instrumentation

The analyses of phospholipid molecular species were performed with Acquity UPLC I-Class solvent delivery system coupled with Xevo TQ mass spectrometer (Waters). Phospholipid species were separated with 100 × 2.1 mm, 1.6 μm CORTECS UPLC HILIC column fitted with a HILIC 1.6 μm VanGuard pre-column (Waters) held in 35°C. Mobile phase consisted of A) 10 mM ammonium acetate and 0.1% formic acid in water and B) 0.1% formic acid in acetonitrile. Phospholipids were separated by a gradient program; 10% A 0 to 5 min, 20% A 10 to 15 min and 10% A at 16 min. Flow rate was 0.4 mL/min and injection volume 1 μL. The ESI source temperature was set to 150°C, capillary and cone voltages to 2 kV and 35 V, desolvation temperature to 600°C and desolvation and cone gas flows to 990 and 30 L/hr. The MS/MS was operated in positive ESI mode. Collision energies were set to 25, 30 or 35 eV depending on the mass pair. The molecular species were measured as multiple reaction monitoring with transitions listed in Supplemental Table S1 (https://doi.org/10.17632/yy6hv32pbz.1; Jaakamo et al., 2023).

The composition of FAME of polar lipids were analyzed with a GC coupled with flame ionization detector (6890N, Agilent Technologies). The FAME were separated with a temperature gradient program on an CP-Sil 88 column (100 m × 0.25 mm id., 0.2 μm film thickness; Agilent Technologies) while maintaining the injector and detector in a constant temperature of 255°C. The FA content of PL was determined as g/100 g total FA in polar lipid SPE extract using theoretical relative response factors (Halmemies-Beauchet-Filleau et al., 2011).

Statistical analysis

Statistical analyses of data were performed by ANOVA by SAS (SAS Institute Inc.) as reported in Jaakamo et al. (2019). The PROC MIXED model was used to test the effect of dietary FC ratio and forage type on selected phospholipids and FA composition of total PL including fixed effects of period, diet and diet by period interaction and random effects of cow, block, and block by period interaction. To test the effect of FC ratio and forage type, orthogonal contrasts were used as follows: A) HG vs. LG (FC ratio in grass silage-based diets), and B) RC vs. grass silage diets. Least squares means are reported, with significant effect at $P \leq 0.05$ and a trend toward significance $0.05 < P < 0.10$.

RESULTS

Phospholipid molecular species

Within the class of phosphatidylcholines 16:0–18:1 PC was the most abundant single molecular species (Table 1). The relative proportions of other measured PC species were roughly around 10% each. Compared with HG, feeding LG increased ($P < 0.01$) the relative proportions of 16:0–14:0 PC by 14% and 16:0–16:0 PC by 17%, whereas 16:0–18:1 PC was increased ($P = 0.041$) by 18% in HG milk compared with LG milk. Forage type had an impact on 18:0–18:0 PC, 18:0–18:1 PC and 18:1–18:1 PC by increasing those species ($P < 0.05$) in RC milk.

The 18:1–18:1+18:0–18:2 PE accounted for 43.9–45.3% of all measured PE species, but it was not affected by dietary treatments. The relative proportions of 16:0–18:2 PE and 18:1–18:2+18:0–18:3 PE were increased ($P < 0.05$) in LG milk compared with HG, whereas the effect was vice versa in 16:0–18:1 PE and 18:0–18:1 PE. Forage type had no effect on the relative proportions of PE molecular species.

Within SM class, d18:1–22:0 SM was the most abundant species accounting for 26–27% of all SM species. The dietary treatments had only minor effects on SM species as the relative proportion of d18:1–22:0 SM tended to increase ($P = 0.074$) in HG versus LG milk and d18:1–23:0 SM was 12% higher ($P < 0.01$) in RC milk compared with grass silage diets.

Fatty acid composition of polar lipids

Forage:concentrate ratio. The proportions of SFA, MUFA or the sum of UFA in polar lipid fraction did not differ between HG and LG treatments (Table 2). Total amount of PUFA was 15% higher in LG compared with HG with a tendency toward significance ($P = 0.055$). High grass treatment contained 9% more 16:0 ($P < 0.01$) which was the most abundant single FA accounting for 31 (LG) to 34% (HG) of total FA in PL fraction. However, HG lowered ($P < 0.001$) the proportions of saturated short and medium-chain FA 8:0, 10:0, 12:0 and 14:0 compared with LG. Feeding HG increased ($P < 0.05$) the proportions of 18:0, *cis*-9 18:1, sum of *cis* 18:1 and total sum of 18:1 in milk polar lipids, but did not affect the sum of *trans* 18:1 compared with LG. Feeding LG increased ($P < 0.01$) the sum of n-6 PUFA, total *cis* 18:2 and overall the sum of non-conjugated 18:2 that were mainly affected by the 1.5-fold increase in the proportion of *cis*-9,*cis*-12 18:2, which was the main 18:2 isomer (Supplemental Table S2; https://doi.org/10.17632/yy6hv32pbz.1; Jaakamo et al., 2023). On the contrary, HG milk contained 25% more n-3 PUFA ($P < 0.01$) than LG mostly affected by *cis*-9, *cis*-12, *cis*-15 18:3. The proportion of 22:0 in HG milk was 33% higher ($P < 0.01$) than in LG. In both treatments, 15:0 was the most abundant single odd-chain FA (Supplemental Table S3; https://doi.org/10.17632/yy6hv32pbz.1; Jaakamo et al., 2023) that was also increased ($P < 0.001$) in LG compared with HG.

Forage type. Feeding RC decreased ($P = 0.014$) SFA by 5% and increased ($P < 0.01$) MUFA by 11% and *trans* FA by 24% compared with grass silage-based diets (Table 2). The proportions of 12:0, 14:0 and 16:0 were lower ($P < 0.001$, $P = 0.016$ and $P < 0.01$) in RC compared with grass silage-based diets, but 18:0 was not affected by forage type. The sum of 18:1 was 12% higher ($P < 0.01$) in RC milk and accounted for more than 23% of all FA in polar lipid fraction. In addition, sums of both *trans* and *cis* 18:1 isomers were increased ($P < 0.001$ and $P = 0.045$) in RC relative to grass silage-based diets and the relative proportion of *cis*-9 18:1 was increased by 10% in RC milk with a trend toward significance ($P = 0.074$). The RC treatment did not affect total PUFA compared with grass silage-based diets although the sum of n-3 PUFA was 40% higher ($P < 0.001$) in RC than in grass silage-based diets. Feeding RC increased the proportion of 18:3n-3 by 1.5-fold compared with grass silage-based diets. Forage type did not affect 18:2n-6 nor total non-conjugated 18:2. Proportions of both 15:0 and 23:0 were increased ($P < 0.01$) in the PL fraction of RC milk compared with grass silage-based diets (Supplemental Table S3; https://doi.org/10.17632/yy6hv32pbz.1; Jaakamo et al., 2023).

DISCUSSION

We examined the effect of dietary FC ratio in grass silage-based diets, and an additional diet based on red clover to test the effect of forage type (grass silage vs. red clover silage), on selected molecular species of milk

phospholipids. There is plenty of literature on the effects of red clover silage or grass silage on total milk FA composition (Dewhurst et al., 2003; Halmemies-Beauchet-Filleau et al., 2014; Jaakamo et al., 2019), but studies testing the impact of these forage types on lipid composition of MFGM are scarce despite the importance of these forages in organic production and in conventional milking systems. Furthermore, as MFGM components have shown beneficial effects on human health (Timby et al., 2014; Bhinder et al., 2017; Anto et al., 2020), it is reasonable to study the MFGM composition also as a function of cow diet. Bovine milk is a major lipid source in Western diet, and with dairy cow diets it is possible to naturally modify the FA composition to healthier direction without changing consumer eating habits. However, modifying the FA composition by modulating dairy cow diet may have implications on MFGM structure and functionality. We focused on the most abundant PL molecular species in milk and FA composition of PL with forage types common in high-forage systems. Although the RC diet consisted of 50:50 FC ratio instead of the intended 70:30 FC ratio due to low concentration of digestible OM in red clover silage, the effect of forage type was examined with comparison between RC and grass silage-based diets (the average of 70:30 and 30:70 FC ratio).

Irrespective of the diet, we identified the 18:1–18:1+18:0–18:2 PE and 16:0–18:1 PC as the major PE and PC species consistent with Craige Trenerry et al. (2013), Liu et al. (2020) and Wei et al. (2022). In the present study, d18:1–22:0 SM was the prevalent (ca. 25–27%) SM species followed by d18:1–16:0 SM and d18:1–23:0 SM that are in line with the findings of Liu et al. (2015). Other studies have reported d18:1–16:0 SM as the dominant SM species followed by d18:1–22:0 SM and d18:1–23:0 SM (Craige Trenerry et al., 2013; Liu et al., 2020; Wei et al., 2022). This discrepancy can be explained by the fact that the relative proportions of these SM species (d18:1–16:0, d18:1–22:0 and d18:1–23:0) are much more evenly distributed compared with the relative proportions of PC or PE species, for example.

Overall, the dietary treatments had very minor effects on the relative proportions of measured molecular species. In our study, the high concentrate content in LG decreased 16:0–18:1 PC, 18:1–18:2 PC, 16:0–18:1 PE and 18:0–18:1 PE, whereas Craige Trenerry et al. (2013) reported that increasing supplementation of 75:25 grain:forage from 8 to 16 kg DM total supplement/

Table 1. Effects of forage:concentrate ratio and forage type on relative proportions (%) of selected phospholipid species within polar lipid class in milk of lactating cows[1]

	Treatment[2]				Contrast (*P*-value)[3]	
% of polar lipid class	HG	LG	RC	SEM	FC ratio	Forage type
Phosphatidylcholine (PC)						
16:0–14:0	11.7	13.3	12.5	0.37	<0.01	0.98
16:0–16:0	10.3	12.1	11.0	0.39	<0.001	0.57
16:0–18:0	8.34	8.59	8.83	0.27	0.24	0.059
16:0–18:1	21.3	18.1	18.6	1.11	0.041	0.39
16:0–18:2	8.61	8.50	8.15	0.52	0.79	0.30
18:0–18:0	8.83	9.06	9.43	0.31	0.31	0.023
18:0–18:1	7.43	7.62	7.92	0.27	0.33	0.029
18:0–18:2	8.15	8.46	8.51	0.29	0.21	0.34
18:1–18:1	8.34	8.05	8.55	0.18	0.13	0.039
18:1–18:2	7.07	6.24	6.49	0.24	0.024	0.59
Phosphatidylethanolamine (PE)						
16:0–18:1	16.7	14.3	15.4	0.63	<0.01	0.88
16:0–18:2	10.3	13.5	10.9	1.36	<0.001	0.11
18:1–18:1+18:0–18:2	45.3	43.9	44.7	1.22	0.39	0.94
18:1–18:2+18:0–18:3	13.8	17.0	16.5	0.76	<0.01	0.19
18:0–18:1	14.0	11.3	13.2	0.59	<0.01	0.44
Sphingomyelin (SM)						
d18:1–14:0	3.94	3.53	3.38	0.26	0.28	0.26
d18:1–16:0	21.6	21.5	19.7	1.62	0.97	0.11
d18:1–20:0	11.5	11.8	11.5	0.39	0.52	0.73
d18:1–22:0	27.3	25.6	26.7	0.66	0.074	0.76
d18:1–23:0	20.2	21.3	23.2	1.02	0.24	<0.01
d18:1–24:0	15.6	15.7	16.0	0.87	0.89	0.58

[1]Values are LSM and pooled SEM, n = 10 for each treatment. Values represent the mean over d 30 to 32.

[2]Refers to grass silage diets with forage:concentrate (FC) ratio 70:30 (HG) and 30:70 (LG), and red clover silage-based diet (RC) with FC ratio 50:50.

[3]FC ratio = forage:concentrate ratio effect (30:70 vs. 70:30 in grass silage diets); Forage type = forage effect (red clover silage vs. grass silage).

Table 2. Effects of forage:concentrate ratio in grass silage diets and forage type (red clover silage vs. grass silage) on fatty acid composition of total polar lipids in milk of lactating cows[1]

	Treatment[2]				Contrast (*P*-value)[3]	
Fatty acid, g/100 g of total polar lipids	HG	LG	RC	SEM	FC ratio	Forage type
4:0	1.84	1.51	1.71	0.094	0.021	0.78
6:0	1.80	1.87	1.80	0.14	0.45	0.66
8:0	1.12	1.36	1.16	0.096	<0.001	0.14
10:0	2.54	3.76	2.70	0.35	<0.001	<0.01
cis-9 10:1	0.26	0.34	0.29	0.012	<0.001	0.21
12:0	3.06	4.83	3.23	0.45	<0.001	<0.001
14:0	11.3	12.7	11.3	0.64	<0.001	0.016
16:0	33.5	30.7	29.4	1.04	<0.01	<0.01
Σ *cis* 16:1	2.13	2.35	2.22	0.10	<0.001	0.63
Σ *trans* 16:1	0.16	0.16	0.19	0.010	0.85	0.015
Σ 16:1	2.29	2.51	2.41	0.11	<0.01	0.80
18:0	9.26	7.72	9.04	0.42	<0.01	0.18
cis-9 18:1	18.1	15.8	18.6	1.82	0.036	0.074
Σ *cis* 18:1	18.7	16.4	19.4	1.86	0.032	0.045
trans-11 18:1	0.94	0.63	0.91	0.041	<0.001	0.02
Σ *trans* 18:1	3.06	3.25	3.91	0.11	0.20	<0.001
Σ 18:1	21.8	19.7	23.3	1.9	0.045	<0.01
cis-9,*cis*-12 18:2 (18:2n-6)[4]	2.72	4.10	3.19	0.25	<0.001	0.33
Σ *cis* 18:2	2.74	4.12	3.21	0.25	<0.001	0.34
Σ *trans* 18:2	1.38	1.03	1.46	0.069	<0.01	<0.01
Σ 18:2[5]	3.53	4.74	4.03	0.27	<0.01	0.73
cis-9,*trans*-11 18:2	0.42	0.31	0.49	0.030	0.01	<0.01
Σ CLA	0.55	0.39	0.61	0.040	<0.01	<0.01
cis-9,*cis*-12,*cis*-15 18:3 (18:3n-3)	0.81	0.62	1.08	0.045	<0.01	<0.001
18:3n-6	0.030	0.040	0.035	0.0059	<0.01	0.96
cis-9,*trans*-11,*cis*-15 18:3	0.068	0.046	0.096	0.017	0.017	<0.001
trans-9,*trans*-12,*cis*-15 18:3[6]	0.0096	0.011	0.014	0.0014	0.58	0.032
cis-9,*trans*-11,*trans*-15 18:3	0.029	0.0087	0.012	0.0032	<0.001	0.099
18:4n-3	0.015	0.0070	0.021	0.0021	<0.01	<0.001
20:0	0.14	0.12	0.22	0.0072	0.063	<0.001
Σ *cis* 20:1	0.057	0.083	0.15	0.0070	<0.01	<0.001
Σ 20:1	0.19	0.20	0.36	0.026	0.44	<0.001
cis-11,cis-14 20:2 (20:2n-6)	0.044	0.050	0.057	0.0048	0.17	0.011
cis-11,*cis*-14,*cis*-17 20:3 (20:3n-3)	0.011	0.0070	0.014	0.0020	0.15	0.047
cis-8,*cis*-11,*cis*-14 20:3 (20:3n-6)	0.21	0.27	0.21	0.083	0.021	0.16
cis-8,*cis*-11,*cis*-14,*cis*-17 20:4 (20:4n-3)	0.082	0.038	0.081	0.016	<0.001	0.02
cis-5,*cis*-8,*cis*-11,*cis*-14 20:4 (20:4n-6)	0.24	0.27	0.20	0.080	0.34	0.10
22:0	0.10	0.075	0.11	0.011	<0.01	0.011
Σ total 22:1	0.066	0.068	0.086	0.0099	0.83	0.056
cis-7,*cis*-10,*cis*-13,*cis*-16 22:4 (22:4n-6)	0.023	0.037	0.019	0.0046	0.011	0.012
cis-7,*cis*-10,*cis*-13,*cis*-16,*cis*-19 22:5 (22:5n-3)	0.14	0.16	0.14	0.036	0.026	0.73
cis-4,*cis*-7,*cis*-10,*cis*-13,*cis*-16,*cis*-19 22:6 (22:6n-3)	0.0070	0.0069	0.0053	0.0016	0.97	0.41
c15–24:1	0.0066	0.0077	0.0058	0.0018	0.61	0.49
24:0[7]	0.19	0.13	0.20	0.038	<0.01	0.016
26:0	0.064	0.054	0.050	0.0067	0.062	0.043
28:0	0.0070	0.0054	0.0035	0.0021	0.41	0.13
Σ *trans* fatty acids	4.88	4.72	5.93	0.14	0.42	<0.001
Σ SFA	68.1	68.8	65.2	2.36	0.61	0.014
Σ MUFA	26.2	24.6	28.1	1.82	0.13	<0.01
Σ PUFA	5.79	6.64	6.61	0.56	0.055	0.29
Σ UFA[8]	32.0	31.2	34.8	2.36	0.60	0.014
Σ n-3 PUFA[9]	1.11	0.89	1.40	0.087	<0.01	<0.001
Σ n-6 PUFA[10]	3.25	4.75	3.70	0.42	<0.001	0.26

[1]Values are LSM and pooled SEM, n = 10 for each treatment. Values represent the mean over d 30 to 32. Full fatty acid profile is shown in Supplemental Tables S2 and S3 (https://doi.org/10.17632/yy6hv32pbz.1; Jaakamo et al., 2023).

[2]Refers to grass silage diets with forage:concentrate (FC) ratio 70:30 (HG) and 30:70 (LG), and red clover silage-based diet (RC) with FC ratio 50:50.

[3]FC ratio = forage:concentrate ratio effect (30:70 vs. 70:30 in grass silage diets); Forage type = forage effect (red clover silage vs. grass silage).

[4]Co-elutes with *cis*-9,*cis*-15 18:2 and *cis*-9 19:1.

[5]Sum of 18:2 excluding conjugated 18:2 isomers.

[6]Co-elutes with *cis*-9,*cis*-12,*trans*-15 18:3.

[7]Co-elutes with *cis*-5,*cis*-8,*cis*-11,*cis*-14,*cis*-17 20:5 (20:5n-3).

[8]Sum of MUFA and PUFA.

[9]Sum of 22:6n-3, 22:5n-3, 20:4n-3, 20:3n-3, 18:3n-3, 18:4n-3 and *trans*-12,*cis*-15 18:2.

[10]Sum of 22:4n-6, 20:4n-6, 20:3n-6, 20:2n-6, 18:3n-6 and *cis*-9,*cis*-12 18:2.

day in grazing cows decreased the relative proportions of 18:0–18:1 PE and 18:0–18:1 PC. They also reported a similar pattern of decreasing long-chain d18:1–22:0 SM and d18:1–23:0 SM when increasing supplementation from 8 to 16 kg DM/day. Similarly, we observed a trend toward decrease in d18:1–22:0 SM when high concentrate content was fed to the cows. Forage type showed even less changes in the relative proportions of PL species. Feeding RC did not cause any differences in the PE species when compared with grass silage diets, whereas only minor increases in 4 PC species and 1 SM species in RC milk were observed. These observations suggest that the FC ratio would have a greater impact on PL species than the forage type, but in general, the relative proportions of individual molecular species within a PL class are only slightly affected by dairy cow diets. Same type of observations can be made when PL are studied on the class level. For example, in the studies of Lopez et al. (2008) and Lopez et al. (2014) the relative proportions of PE, PC and SM classes were not affected by linseed supplementation to corn silage-based diet or when the cows were fed either fresh pasture or corn silage-based diets. Furthermore, Mesilati-Stahy et al. (2015) studied the relative proportions of PL classes in milk from cows fed high-concentrate low-forage or low-concentrate high-forage diets based on corn silage, oat and clover hay and observed that the experimental diets only tended to affect the proportions of PE and SM in the milk fat globule size fraction with largest globules (mean diameter 3.3 μm). Even though we focused on the molecular species level and not the class level, it seems that dairy cow diets do not induce remarkable differences on the relative proportions at species level nor at class level. However, it must be said that although the relative proportions of PL classes remain unchanged, the concentrations of PL are affected by dietary treatments. On a molecular species level, a diet with high oil and starch content led to an average of 78% increase in 10 of 21 PC species and affected 6 PI species (Fougère et al., 2021). Furthermore, higher PL concentrations in milk have been obtained with diets containing lipid supplements (Lopez et al., 2008), with a diet based on corn silage, oat and clover hay with 35:65 instead of 65:35 FC ratio (Argov-Argaman et al., 2014) or when cows were fed fresh pasture instead of corn silage-based diet (Lopez et al., 2014).

For the identification of molecular species we used a triple quadrupole MS instrument with an ESI source and HILIC stationary phase for the separation of the PL. One limitation in our research was related to the quantification of PL molecular species and to the use of ISTD. The instrument response in MS is affected by the acyl chain length, the degree of acyl chain unsaturation and overall lipid concentration that should be considered to obtain accurate concentrations (Koivusalo et al., 2001; Wang et al., 2017). To overcome the loss of analytes in sample preparation and also the behavior in the MS, there should be an ISTD for every compound or at least for each PL class. In this study, we made a compromise by using the d18:1–16:1-d31 SM as an internal standard for SM compounds but other PL were measured with external calibrations. Furthermore, based on previous research (Craige Trenerry et al., 2013; Ali et al., 2017), we selected the most abundant PL molecular species as reference standards to quantify other molecular species within a PL class. As the aim of this study was to test the effect of dairy cow diets, the relative proportions of individual PL species are comparable although we did not have comprehensive set of reference standards within a PL class and used only one ISTD. In addition, the number of measured molecular species was greatly limited, because we used partly overlapping multiple reaction monitoring time windows of each molecular species within a class that reduced the instrument's ability to measure sufficient number of data points per chromatographic peak. Because of this limitation, we carefully selected the most abundant molecular species of each class based on previous literature (Craige Trenerry et al., 2013; Liu et al., 2020; Wei et al., 2022) and compared the effects of dairy cow diets on these PL species of milk.

In addition, we investigated the effect of dairy cow diets on FA composition of PL. As it is well elucidated, glycerophospholipids, PE and PC being the most abundant in MFGM, contain relatively high amounts of 18:1, 18:2 and 18:3, whereas sphingomyelin is rich in long-chain saturated FA 16:0, 22:0, 23:0 and 24:0 (Bitman and Wood, 1990; Fong et al., 2007). Consistent with previous studies (Lopez et al., 2008, 2014; Sánchez-Juanes et al., 2009), 16:0 and 18:1 were the major FA detected in polar lipid fraction irrespective of dietary treatment. In general, the effects of dietary treatments on the FA profile of PL were similar to the effects on FA profiles of total milk fat previously reported in Jaakamo et al. (2019). This similarity in milk and PL FA profiles has been observed also in the study of Lashkari et al. (2020a) with dairy cow diets based on grass-clover silage supplemented with crushed high oleic sunflower seeds alone or with rumen-protected choline. In present study, the FC ratio did not affect SFA content of PL as similar to milk fat in our previous study (Jaakamo et al., 2019), but it was decreased by 5% in RC milk compared with grass silage-based diets. Irrespective of the diet, the SFA content was more than 65% of all FA in PL fraction (Table 2) being in agreement with Lopez et al. (2008) that reported more than 60% SFA in PL and 10–20% relative difference in PL versus total FA in corn silage-based diet. In contrast,

Lashkari et al. (2020a) reported 46.8% SFA content in PL and 73.9% SFA in total milk fat in their control diet based on grass-clover silage leading to a greater relative difference compared with our present and previous results (Jaakamo et al., 2019). These variations in SFA content of PL fraction can be explained by dairy cow diets or by different extraction methods as discussed in Lashkari et al. (2020b). Many previous studies (Lopez et al., 2008, 2014; Wei et al., 2022) have used similar silica-bonded SPE cartridges to separate the nonpolar and polar lipid fractions as we did, but other cartridge-materials such as aminopropyl (Lashkari et al., 2020a,b) have been used as well. The differences in cartridge materials might explain some discrepancies found in the literature, but it should be noted that incomplete recovery is a risk and should be compensated with the use of internal standards (Rombaut and Dewettinck, 2006).

Previously, we reported 16% increase in MUFA level in RC milk compared with grass silage-based diets (Jaakamo et al., 2019), mostly affected by the high proportion of *cis*-9 18:1. Although *cis*-9 18:1 was the most abundant MUFA in the MFGM in RC milk, the relative proportion of *cis*-9 18:1 increased only 10% in RC compared with grass silage-based diets with only tendency toward significance. Furthermore, we found that FC ratio in grass silage-based diets did not affect MUFA level although high FC ratio increased *cis*-9 18:1 that was similar to the results in total milk fat (Jaakamo et al., 2019). In other words, the increases in *cis*-9 18:1 induced by dietary treatments were more pronounced in total milk fat than in polar lipid fraction. This has been observed in other studies as well (Lopez et al., 2008; Lashkari et al., 2020a) and especially in dietary treatments with oil seeds rich in UFA. For example, a 10% addition of crushed high oleic sunflower seeds to grass-clover silage-based diet increased the *cis*-9 18:1 level more than 2-fold in total milk fat versus 28% in PL fraction (Lashkari et al., 2020a). When the *cis*-9 18:1 intake from diet is high, the escape of oleic acid from ruminal biohydrogenation is increased, although the main effect is the increased production of stearic acid in the rumen that is then partly converted to oleic acid by stearoyl-CoA desaturase in mammary gland tissue (Chilliard and Ferlay, 2004; Shingfield et al., 2010). Even though the *cis*-9 18:1 intake from red clover silage is not as high as from LG diet (Jaakamo et al., 2019) or in diets with oil supplements, the apparent biohydrogenation of *cis*-9 18:1 in the rumen is lowered (Halmemies-Beauchet-Filleau et al., 2013; Jaakamo et al., 2019) and leads to relatively higher increase in milk fat than in MFGM.

In red clover silage-based diets, higher ruminal escape of PUFA leads to increased PUFA levels in milk, especially in elevated concentrations of 18:2n-6 and 18:3n-3 (Vanhatalo et al., 2007; Jaakamo et al., 2019). In the present study, this was also observed in the elevated proportion of 18:3n-3 in PL fraction, suggesting that the decreased biohydrogenation leads not only to increased amounts of 18:3n-3 in total FA but also in the MFGM. In our previous research, we reported 22% increase in PUFA level in RC vs. grass silage-based diets and 20% increase in LG vs. HG (Jaakamo et al., 2019), but this was not the case in PL fraction as there was no statistical difference in the relative proportions of PUFA between RC and grass silage-based diets and only a tendency toward significance in LG vs. HG. This increase in PUFA level in LG is due to the increase in the relative proportion of 18:2n-6 that is also explaining the increase in n-6 PUFA level. In corn silage diet supplemented with linseed rich in PUFA, the relative proportion of PUFA was increased almost 2-fold in total milk fat whereas in MFGM this increase was 1.01-fold (Lopez et al., 2008). Furthermore, when high oleic acid supplementation was fed to the cows, PUFA level in MFGM showed no statistical difference between treatments, but decreased in total milk fat (Lashkari et al., 2020a). It is well established that the relative proportion of PUFA is higher in MFGM than in total milk fat, but according to our results and previous evidence (Lopez et al., 2008; Lashkari et al., 2020a) it should be noted that the impact of cow's diet on PUFA level is greater in total milk fat than MFGM. One explanation could be that as the structure of PL consists of a glycerol backbone with 2 esterified FA, the incorporation of PUFA in TG is more favored. Furthermore, given that the PL fraction comprises less than 1% of milk fat and that the PL composition is affecting the stability of intracellular lipid droplet (Argov-Argaman, 2019), the changes induced by diet are more evident in TG fraction to maintain rigidity of the MFGM. Long-chain, preformed FA absorbed as very low density lipoproteins (originating from feed and activity of ruminal microbiota) or as nonesterified FA (from adipose tissues) have been suggested to act as limiting factor for the synthesis of PL in mammary epithelial cells (Argov-Argaman, 2019). In the mammary epithelial cell, the long-chain fatty acids can be used either for PL or TG synthesis in the endoplasmic reticulum, in the cytoplasm or directly in the intracellular lipid droplet surface. However, the exact mechanisms governing the FA distribution between TG and PL remain to be elucidated.

Our results show that dietary treatments had only minor effects on the relative proportions of PL molecular species. We measured the most abundant molecular species based on previous studies, but a more comprehensive identification of the molecular species would give more detailed information on the effects of

diets commonly used in high-forage systems. Although the molecular composition of PL species in bovine milk has been well characterized with lipidomics tools, the understanding of diet-dependent changes on species level lags far behind. Our findings show that the dairy cow diets affect not only the composition of total FA in milk, but also the membrane FA composition. Overall, the differences induced by diets were similar in MFGM as in milk fat. More research is needed on the biological functions of individual molecular species and mechanisms governing the PL synthesis in terms of FA distribution. A better understanding on the impact of dairy cow diets on these minor lipid constituents helps to take advantage of their nutritional and technological properties.

In conclusion, FC ratio in grass silage-based diets modified the relative proportions of PL molecular species by influencing the abundances of 4 PC species, 4 PE species and 1 SM species. Forage type had fewer effects as feeding red clover silage affected 4 PC species and 1 SM species, but no PE species. The changes induced by dairy cow diets on FA profile of PL reflected the general changes previously observed in milk fat. Low FC ratio led to higher relative proportion of n-6 PUFA due to the higher 18:2n-6 level in PL compared with high FC ratio. We hypothesized that the high ruminal escape of PUFA in cows fed red clover silage would lead to increased incorporation of PUFA into the PL fraction. However, feeding RC did not affect PUFA level in MFGM when compared with grass silage-based diets although 18:3n-3 was increased suggesting more strict regulation of FA composition in the MFGM.

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