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Effect of dietary palmitic acid supplementation and milking frequency: 1. Milk production and composition in early-lactation dairy cows

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ABSTRACT

Dietary palmitic acid (PA) supplementation can be used to increase milk fat yield by dairy cows. Additionally, increasing milking frequency (MF) promotes milk production. Both practices are of interest to enhance milk performance, but their effects on milk properties are a concern from a milk processing perspective. Moreover, little is known regarding the possible interaction between those 2 practices on milk performance. This study aimed to evaluate whether milk responses of early-lactation dairy cows could benefit from the combined effects of PA supplementation and increased MF. Additionally, we determined the effects of the 2 practices on milk free fatty acids (FFA) content, milk protein fractions such as CN and whey proteins, and plasmin activity in milk. Eight early-lactation multiparous Holstein cows were randomly allocated to treatment sequences in a replicated 4 × 4 Latin square design with a 2 × 2 factorial arrangement of treatments. Levels of treatments were dietary PA (0% or 2% on a DM basis) and 2 or 3 milkings per day at equal intervals of time. Periods lasted 21 d with 16 d for treatment adaptation before data and sample collection. Except for lactose concentration, no significant interaction between MF and PA supplementation was observed. Dry matter intake was not affected by treatment. Increased MF and PA supplementation both increased milk, fat, and protein yields, as well as milk efficiency. Palmitic acid supplementation increased the yield of 16 C fatty acids (FA) in milk at the expense of shorter de novo FA. Calculated desaturase indexes increased with PA, indicating an increased Δ^9-desaturase activity or expression. In contrast, most desaturase indexes decreased with MF. Dry matter and NDF digestibilities were similar between treatments. However, PA decreased total and 16 C FA digestibilities. Transfer of 16 C FA into milk was increased with PA supplementation. Neither MF nor PA had an effect on FFA concentration in fresh milk. Milk concentrations of total N, true protein, CN, or whey proteins were similar between treatments. However, even if milk CP yield was increased by both PA and thrice-daily milking, CN yield was not affected by PA and only tended to be increased by thrice-daily milking. The effects of PA supplementation and MF on milk performance were additive. Under the conditions of this trial, increased MF and PA supplementation did not impair milk composition parameters of importance for milk processing.
Key words: palmitic acid, milking frequency, dairy cows, milk fatty acids

INTRODUCTION

On-farm practices have an impact on milk quality and composition, which greatly influence milk processing. The increasing use of automatic milking systems and associated increase in milking frequency (**MF**) have raised concerns regarding their effects on milk properties (Hogenboom et al., 2019). Another recent source of concern is the effects of dietary palmitic acid (**PA**; 16:0) supplementation in dairy cows on thermal properties of milk fat, properties that could affect the characteristics of final dairy products (Harvatine, 2021).

Many studies have looked at the effects of PA supplementation or MF on milk performance, but few of them looked at their effects on milk technological properties. Feeding PA can increase milk and fat yields, as well as milk efficiency (dos Santos Neto et al., 2021). Dietary PA supplementation also modifies milk fatty acids (**FA**) composition, increasing the proportion of 16:0 at the expense of other short- or long-chain FA (Mosley et al., 2007). Dietary PA supplementation could also increase the susceptibility of milk to lipolysis, resulting in a higher free FA (**FFA**) concentration (Wiking et al., 2003). Fat supplements can also modify milk N partitioning (DePeters and Cant, 1992), potentially reducing

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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-25. Nonstandard abbreviations are available in the Notes.

the CN N/total milk N ratio, which greatly influences the processing of milk into cheese.

On the other hand, increasing MF from twice to thrice daily has been reported to enhance milk yield by 10% to 15% (Klei et al., 1997; Smith et al., 2002). The effects of increased MF on milk composition are less clear. A decrease or no changes in concentrations of milk components have been reported (Klei et al., 1997; Sapru et al., 1997; Smith et al., 2002). The effects of MF on SCC or plasmin activity in milk influence proteolysis (Klei et al., 1997; Sapru et al., 1997). Milking frequency is expected to have few effects on milk FA composition (Delamaire and Guinard-Flament, 2006). It is generally accepted that milk FFA concentration increases with MF (Wiking et al., 2006), but the mechanism behind this effect is not well understood. Moreover, it is sometimes difficult to dissociate the effects of the increase in MF itself from the effects of other automatic milking system factors (e.g., pumping, pipeline, cooling, bacterial lipolysis; Wiking et al., 2006).

There is a need to study the effect of on-farm practices such as PA supplementation and MF while focusing on milk traits that affect milk processing. Moreover, to our knowledge, the potential interaction between PA supplementation and MF has never been investigated before. Hence, the aim of this study was to evaluate the effects of dietary PA, thrice-daily MF, and their interaction on milk production and composition. We hypothesized that PA and MF would interact to increase dairy performance, and that both PA and MF would influence milk properties.

Table 1. Ingredients, chemical composition, and fatty acid (FA) composition of experimental diets

Item	Treatment[1]	
	No PA	PA
Ingredient, % DM		
Corn silage	30.9	30.8
Grass silage	32.6	32.6
Ground corn	19.7	17.5
Soybean meal	13.7	13.2
Corn gluten meal	1.0	1.7
Mineral and vitamin mix[2]	2.1	2.1
PA supplement[3]	—	2.1
Chemical composition		
DM, % as fed	42.9	43.2
OM, % DM	93.6	93.5
NDF, % DM	35.2	34.9
ADF, % DM	22.4	22.4
CP, % DM	14.6	14.5
Starch, % DM	23.6	23.1
NEL, Mcal/kg DM[4]	1.71	1.78
FA, mg/g DM		
14:0	0.08	0.41
16:0	4.08	21.74
*c*9 16:1	0.05	0.09
18:0	0.52	0.99
*c*9 18:1	3.73	4.67
*c*9*c*12 18:2	9.97	10.13
*c*9*c*12*c*15 18:3	3.67	3.59
Others	0.47	0.61
Total	22.58	42.23

[1]No PA = no palmitic acid supplementation; PA = palmitic acid supplementation (Jefo Dairy Fat 99%, 88% free 16:0).

[2]Containing 18.0% Ca; 5.0% P; 6.0% Mg; 9.5% Na; 45 mg/kg I; 3,500 mg/kg Fe; 600 mg/kgCu; 2,000 mg/kgMn; 3,000 mg/kg Zn; 20 mg/kg Co; maximum 480 mg/kg F; minimum 300,000 IU/kg vitamin A; 100,000 IU/kg vitamin D_3; and 1,500 IU/kg vitamin E.

[3]Containing 1.23% 14:0, 88.36% 16:0, 0.11% 16:1, 3.33% 18:0, 5.63% *c*9 18:1, 0.81% *c*9*c*12 18:2, 0.01% *c*9*c*12*c*15 18:3, and 0.51% other FA.

[4]Estimated based on NASEM (2021).

MATERIALS AND METHODS

A companion paper (Landry et al., 2025) focuses on milk processing parameters and butter manufacture and properties.

Animals and Treatments

All procedures in this study were approved by the institutional animal care committee (2021-859) based on the current guidelines of the Canadian Council on Animal Care (2009).

Eight multiparous Holstein cows averaging 45 ± 14 DIM and 681 ± 48 kg BW at the beginning of the trial were used in a replicated 4 × 4 Latin square design with a 2 × 2 factorial arrangement of treatments. Cows were assigned to square according to DIM, and then randomly allocated to treatment sequences balanced for carryover effects in subsequent periods. Cows received a diet (Table 1) with or without a PA supplement (88% of free 16:0, Jefo Dairy Fat 99%, Jefo Nutrition, Saint-Hyacinthe, QC, Canada) at 2% of the diet on a DM basis as a replacement for ground corn. They were also milked twice or thrice daily at regular intervals (12 or 8 h between milkings). Each experimental period lasted 21 d, with 16 d for adaptation to treatments, and the last 5 d for data and sample collection. Cows were housed in a tiestall barn and had free access to water throughout the experiment. A post hoc power analysis was performed using G*Power 3.1 (Faul et al., 2007) with a sample size of 8, an α-value of 0.05, and a power of 0.80. This power analysis determined that our experimental design provided sufficient power to detect an absolute difference of 0.25 percentage units in milk fat content.

Based on feed ingredient composition, diets were formulated to meet or exceed the National Academies of Sciences, Engineering, and Medicine requirements (NASEM, 2021; Table 1). Diets were fed as TMR at 0800 h daily, and the amount offered was adjusted based on the previous day's intake to allow for 10% refusals. Silages were sampled twice a week and dried for 3 d at 55°C to

determine DM concentration, and to adjust the composition of diets on an as-fed basis.

Experimental Measurements and Sampling

Body weight was measured at 1400 h on d 17, 18, and 19 of each period. Refusals were weighed daily just before feeding. Samples of TMR and refusals were collected from d 18 to 21 of each period, composited by dietary treatment within period for TMR, or composited by cow within period for refusals, and dried at 55°C for 3 d to determine DM. Dry samples were ground to pass a 1-mm screen using a Wiley mill (standard model 3; Arthur M. Thomas Co., Philadelphia, PA), and kept frozen at −20°C for further analyses. Fecal samples were collected via rectal stimulation or grab-sampling from each cow every 6 h on a 48 h period on d 18 and 19 to obtain 8 equal aliquots per cow. Feces aliquots were frozen at −20°C and later lyophilized (VirTual 50L, VirTis SP Scientific, Stone Ridge, NY). Feces samples were then composited to obtain one sample per cow per period and ground to 1-mm as previously described.

Cows milked twice daily were milked at 0530 and 1730 h, whereas cows milked thrice daily were milked at 0530, 1330, and 2130 h. Milk production was weighed in a bucket, and milk samples were collected at each milking from d 17 to 19. Milk was manually and gently stirred before sampling. A first sample was pooled by cow and by day according to each milking production, preserved in bronopol, and stored at 4°C until analyzed for milk composition by infrared spectroscopy. A second subsample without preservative was stored at −20°C for later FA and protein fractions determination. An additional milk sample was collected from each cow at every morning milking at 0530 h from d 18 to 20, and immediately cooled at 4°C for same-day determination of FFA concentration in fresh milk. On d 17, at 0530 h, 2 additional milk samples were manually taken from each individual cow directly from teats after their disinfection with isopropanol 70%. One of these samples was cooled and immediately centrifuged for 30 min at 17,800 × *g*, 8°C, then defatted and finally frozen at −20°C for later determination of plasmin activity. The second sample was stored at 4°C for 48 h before centrifugation and defatting for the same purpose.

Chemical Analyses

Feed and Digestibility Analyses. Total mixed rations and feces samples were analyzed for residual moisture (934.01; AOAC International, 2023), ashes (942.05; AOAC International, 2023), ADF (Ankom Technology, Fairport, NY; method 5: ADF in feeds-filter bag technique for A200; solutions as in method 973.18; AOAC International, 2023), NDF (Ankom Technology method 6: NDF in feeds-filter bag technique for A200; solutions as in Van Soest et al. (1991) including heat-stable α-amylase), and CP (N × 6.25; Kjeldahl method 954.01; AOAC International, 2023). The NDF and ADF reported were not corrected for ash. Total mixed rations were analyzed for starch content according to Hall (2009). Feed and feces FA were directly transesterified to methyl esters, then extracted and analyzed as described by Jenkins (2010) with modifications (Villeneuve et al., 2013). Fatty acid composition was determined using a gas chromatograph (GC7820; Agilent Technologies Canada Inc., Mississauga, ON, Canada), equipped with a polar capillary column (HP-Innowax 30-m length, 0.320 mm i.d., 0.25 µm film thickness; Agilent Technologies Canada Inc.), and a flame ionization detector. Column temperature at sample injection was maintained at 185°C for 0.5 min, then ramped up at 3°C/min to 220°C. Inlet and detector temperatures were 240°C and 250°C, respectively, and the split ratio was 50:1. The flow rate for hydrogen carrier gas was 1 mL/min.

Indigestible NDF (**iNDF**) was determined in triplicate according to the Norfor feed evaluation system (Åkerlind et al., 2011) to estimate apparent total-tract digestibility. Briefly, 0.35 g of dried ground feed and fecal samples were weighed into filter bags (F57; 25-µm porosity; Ankom Technology) to obtain a 10- to 20-mg sample/cm^2. Bags were presoaked in tap water, and then incubated for 288 h into the ruminal ventral sac of a fistulated cow receiving a diet similar to the experimental control diet. After retrieval from the rumen, filter bags were rinsed in cold water and washed without spinning in a washing machine. The NDF analysis was performed on the incubated samples to determine iNDF.

Nutrients apparent digestibilities were calculated as follows:

$$100 - \{100 \times [(\text{diet iNDF, \%DM/feces iNDF, \%DM}) \times (\text{nutrients in feces, \%DM/nutrients in diet, \%DM})]\}.$$

Apparent digestibility of each nutrient allowed calculation of the amount of nutrient digested, which was used to calculate the true transfer of FA. Apparent and true recoveries of dietary 16 C and 18 C FA into milk were calculated as [g FA in milk/g FA ingested × 100] and [g FA in milk/g FA absorbed × 100], respectively (Adeniji et al., 2025).

Milk Analyses. Milk fat, protein, lactose, SCC, and MUN concentrations were determined on samples with bronopol by infrared spectroscopy using a Foss MilkoScan FT 6000 (Foss, Hillerød, Denmark), combined with a Fossomatic FC (Foss), by a commercial laboratory (Lactanet, Sainte-Anne-de-Bellevue, QC, Canada).

For milk FA determination, samples without preservative were thawed and composited proportionally to milk yield to obtain one sample per cow per period. Lipid was then extracted and methylated according to Rico et al. (2021), and milk FA profiles were determined according to the procedure described by Boivin et al. (2013).

Free FA concentration was determined on individual milkings by colorimetry according to the copper-soap method (IDF, 1991). Briefly, 0.5 mL of milk was added to a tube containing 5 mL solvent (chloroform/heptane/methanol; 49/49/2; vol/vol/vol), 0.1 mL EDTA 8% (wt/vol), 0.1 mL HCl 0.7 *N*, and 2.25 mL copper reagent (10 mL $Cu(NO_3)_2 \cdot 3H_2O$ 1 *M*, 5 mL triethanolamine, and 85 mL NaCl 270 g/L; pH 8.3). Tubes were agitated for 30 min, then centrifuged at 20°C and 350 × *g* for 15 min. Then, 1 mL of the supernatant was transferred to another tube containing 1 mL of sodium diethyldithiocarbamate (0.1% wt/vol in n-butanol). Resulting tubes were vortexed for 10 s, and the absorbance of each sample was read with a spectrophotometer at 440 nm. Solutions of PA were used to produce a standard curve.

To determine protein fractions, samples without preservative were thawed and composited proportionally to milk yield to obtain 1 sample per cow per period. True protein, CN, whey proteins, and NPN concentrations in milk were determined based on the indirect approach (ISO 8968-4:2016; ISO, 2016; and ISO 17997-1:2004; ISO, 2004). Nitrogen concentration in all protein fractions was determined by Kjeldahl titration with HCl 0.01 *M*. For total milk nitrogen (**TN**), 0.5 mL of raw milk was used. For NPN isolation, 40 mL of trichloroacetic acid 15% (wt/vol) was added to 10 mL of milk at 38°C and lightly stirred. After 5 min, the solution was filtered through Whatman no. 40 filter paper to collect the NPN filtrate, which was frozen to −20°C before N determination by Kjeldahl titration. For non-CN N (**NCN-N**) isolation, the ISO 17997–1:2004 method (ISO, 2004) was used with some modifications. Twenty-five milliliters of demineralized water at room temperature was added to 6 mL of milk prewarmed at 38°C. Acetic acid 10% (vol/vol) was then progressively added until the pH of the solution reached 4.6. Samples were then stirred and left to stand for 5 min, allowing the precipitate to settle. The solution was then filtered through a Whatman no. 40 filter paper to collect the NCN-N filtrate. Filtrate was frozen to −20°C before N determination by Kjeldahl titration. Nitrogen fractions were used to calculate true protein [(TN − NPN) × 6.38], CN [(TN − NCN-N) × 6.38], and whey proteins [(NCN-N − NPN) × 6.38].

Milk plasmin and plasminogen activity were determined according to de Vries et al. (2016). Briefly, 0.5 mL of defatted milk was incubated with 7.5 mL of plasmin buffer (53 m*M* Trizma buffer, 117 m*M* NaCl, and 20 m*M* ε-amino-n-caproic acid; pH 7.4) for 1 h. Then, milk serum was separated by ultracentrifugation (Optima L-100 XP, Beckman Coulter, Brea, CA) with a SW40Ti rotor at 100,000 × *g* for 1 h at 4°C. For the determination of plasmin activity, 150 µL of the milk serum and 40 µL of the substrate pyro-GLU-Phe-Lys-p-nitroanilide hydroxychloride chromogenic (2.5 mg/mL) were added in a 96-well plate (BioLite, Thermo Fisher Scientific, Waltham, MA). For plasminogen activity, urokinase (16,667 IU/mL) was also added to the 150 µL of milk serum and 40 µL of substrate to activate plasminogen into plasmin, which allows calculation of plasminogen activity by the difference between total (plasmin + plasminogen activity) and plasmin activity. The change in absorbance at 405 nm and 37°C was measured every 3 min for 120 min with a multimode microplate reader (Varioskan, Thermo Fisher Scientific). The linear part of the absorbance curve was used to calculate enzymatic activity (from 30 to 120 min; Korycha-Dahl et al., 1983) in 150 µL then reported by milliliter of milk serum. Every change in absorbance of 0.001 per min equals 1 U of enzyme activity.

Statistical Analysis

Data were analyzed with the MIXED procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC) as a replicated 4 × 4 Latin square design according to the following model:

$$Y_{ijklm} = \mu + D_i + M_j + (D \times M)_{ij} + P_k + S_l + C{:}S_{l:m} + \varepsilon_{ijklm},$$

where Y_{ijklm} is the individual observation, µ is the overall mean, D_i is the fixed effect of dietary treatment (i = 1 to 2), M_j is the fixed effect of MF (j = 1 to 2), $(D \times M)_{ij}$ is the interaction between dietary treatment and MF, P_k is the fixed effect of period (k = 1 to 4), S_l is the fixed effect of square (l = 1, 2), C_m the random effect of cow (m = 1 to 4) nested within square, and ε_{ijklm} is the residual error term. Differences between treatments were declared at $P \leq 0.05$, and tendencies from $P > 0.05$ to $P \leq 0.10$.

RESULTS AND DISCUSSION

Animal Performance

No interaction was found between PA supplementation and MF on cow performance except for lactose concentration (Table 2). Body weight was lower for cows milked 3 times daily, but not affected by PA supplementation. Dry matter intake was not different between treatments (Table 2). It could be hypothesized that the supplemental daily milking was challenging to cows (increase in daily milk yield with no increase in DMI), leading to a decreased BW because DMI remained the same. However, it is also plausible that the decrease in BW observed is a

Table 2. Effect of palmitic acid (PA) supplementation and twice or thrice-daily milkings on BW, DMI, and milk yield, composition and efficiency

	Treatment[1]							
	No PA		PA			*P*-value[2]		
Item	2×	3×	2×	3×	SEM	FAT	MF	FAT × MF
BW, kg	696	684	699	684	14.3	0.65	<0.01	0.79
DMI, kg/d	28.4	28.2	27.8	28.6	0.82	0.82	0.51	0.32
Milk yield, kg/d								
Actual	44.7	47.7	46.4	49.6	1.21	<0.01	<0.01	0.85
4% Fat corrected[3]	43.5	45.3	46.5	49.4	1.29	<0.01	<0.01	0.30
Energy corrected[4]	43.5	45.4	46.1	48.9	1.13	<0.01	<0.01	0.37
Milk fat								
Content, %	3.86	3.68	4.05	4.01	0.219	<0.01	0.05	0.19
Yield, kg/d	1.71	1.75	1.86	1.97	0.082	<0.01	0.01	0.23
Milk crude protein								
Content, %	3.23	3.16	3.22	3.22	0.070	0.34	0.24	0.16
Yield, kg/d	1.43	1.50	1.49	1.59	0.036	<0.01	<0.01	0.41
Milk lactose								
Content, %	4.68	4.72	4.67	4.64	0.034	0.01	0.79	0.01
Yield, kg/d	2.09	2.25	2.17	2.30	0.055	0.03	<0.01	0.63
MUN, mg/dL	10.4	11.2	11.0	11.6	0.88	0.15	0.09	0.73
SCC, ’000/mL	15	14	15	15	3.4	0.70	0.93	0.83
Milk efficiency, ECM/DMI	1.54	1.61	1.66	1.72	0.025	<0.01	0.01	0.70

[1]No PA = no PA supplementation; PA = PA supplementation (2% on a DM basis; 88% 16:0); 2× = 2 milkings/d (12-h intervals); 3× = 3 milkings/d (8-h intervals).

[2]FAT = effect of PA supplementation; MF = effect of milking frequency.

[3]4% FCM = 0.4 × (milk yield in kg/d) + 15.0 × (fat yield in kg/d) per Gaines (1928).

[4]ECM = 0.01 × (milk yield in kg/d) + 12.2 × (fat yield in kg/d) + 7.7 × (protein yield in kg/d) + 5.3 × (lactose yield in kg/d) per Sjaunja et al. (1990).

consequence of the additional milking that occurred 30 min before weighing time, when we collected an average of 16.3 kg per cow, reducing milk present in the udder for cows milked thrice daily. Both PA supplementation and 3× MF increased milk yield, ECM, and 4% FCM (Table 2). Milk fat, protein, and lactose yields were increased by PA supplementation and 3 × MF. Consequently, milk efficiency was improved by both PA supplementation and increased MF. Milk fat concentration was increased by PA but decreased with 3× MF. There was no effect of treatments on milk protein concentration and SCC. Thrice-daily milking tended to increase MUN ($P = 0.09$; Table 2). To our knowledge, this is the first experiment testing the interaction between PA and MF. Based on the current results, we conclude that feeding a PA supplement does not modulate the response to an increased MF, their effects being additive.

In agreement with the results of this trial, PA supplementation usually increases milk fat yield and concentration (de Souza and Lock, 2018; Shepardson and Harvatine, 2021) without affecting (Piantoni et al., 2013) or with slightly decreasing milk protein concentration (Lock et al., 2013). In the current study, milk protein concentration was not affected by PA, whereas protein yield was increased. Milk protein concentration is sometimes lowered due to a dilution effect, when the increase in protein secretion does not match the increase of milk yield. However, DePeters and Cant (1992) suggested that milk yield response is not the only factor influencing protein content when fat is supplemented in the diets. They highlighted that the individual N fractions in milk should be evaluated when fat-supplemented diets are fed. The effect of PA on milk yield is inconsistent across studies. Dietary PA supplementation can either increase (Piantoni et al., 2013; de Souza and Lock, 2018) or have no effect (Lock et al., 2013; Shepardson and Harvatine, 2021) on milk yield. This inconsistency could be related to the reported effects of PA on DMI, which is also inconsistent across studies. When feeding PA, an increase (de Souza and Lock, 2018), a decrease (Lock et al., 2013; Shepardson and Harvatine, 2021), or no changes (Piantoni et al., 2013; de Souza et al., 2017) in DMI have been reported. The increase in milk efficiency with dietary PA supplementation appears to be more consistent. Authors reporting a decrease in DMI saw no effect on milk yield (Lock et al., 2013; Shepardson and Harvatine, 2021). In the current study, DMI was not different between treatments, but PA increased milk yield and consequently ECM/DMI efficiency. The early stage of lactation (45 ± 14 DIM) of cows in our study could have allowed them to benefit more from the supplementation and reach a higher level of production because DMI remained the same across treatments. Such a response differs from other trials with cows in a later stage of lactation that lowered their intake while maintaining their milk production (Lock et al., 2013; Shepardson and Harvatine,

Table 3. Effect of palmitic acid (PA) supplementation and twice or thrice-daily milkings on main fatty acids (FA) concentration in milk and Δ^9-desaturase indexes

	Treatment[1]							
	No PA		PA			*P*-value[2]		
Item	2×	3×	2×	3×	SEM	FAT	MF	FAT × MF
FA group, g/100 g FA								
4:0	2.66	2.71	2.44	2.65	0.100	0.11	0.13	0.33
De novo (6 C to 14 C)	21.45	21.60	18.01	18.19	0.552	<0.01	0.56	0.96
Mixed (16:0 + *c*9 16:1)	32.22	32.02	38.02	37.53	0.709	<0.01	0.52	0.78
Preformed ≥18 C[3]	27.66	27.54	26.25	26.38	0.697	0.07	0.99	0.86
*c*9 18:1[4]	13.08	12.95	13.17	13.06	0.501	0.80	0.75	0.98
Saturated[5]	62.75	63.06	63.44	63.72	0.800	0.19	0.56	0.98
Unsaturated[6]	19.60	19.38	19.67	19.46	0.673	0.88	0.65	0.29
Saturated:unsaturated	3.24	3.28	3.24	3.33	0.158	0.81	0.51	0.84
^{9}-desaturase specific ratio[7]								
*c*9 14:1	0.084	0.080	0.092	0.088	0.0079	<0.01	0.05	0.91
*c*9 16:1	0.040	0.039	0.043	0.042	0.001	0.03	0.29	0.88
*c*9 18:1	0.644	0.640	0.678	0.663	0.016	<0.01	0.08	0.31
*c*9*t*11 18:2	0.355	0.342	0.380	0.360	0.010	<0.01	<0.01	0.50

[1]No PA = no PA supplementation; PA = PA supplementation (2% on a DM basis; 88% 16:0); 2× = 2 milkings/d (12-h intervals); 3× = 3 milkings/d (8-h intervals).

[2]FAT = effect of PA supplementation; MF = effect of milking frequency.

[3]FA of 18 C and more including *c*9 18:1 and excluding *iso* 18:0.

[4]Coelution with minor concentration of *c*10 18:1.

[5]∑Straight-chain SFA.

[6]*cis* UFA.

[7]Specific ratios calculated as the Δ^9-desaturase product divided by the sum of the Δ^9-desaturase product and substrate: *c*9 14:1 = *c*9 14:1/(*c*9 14:1 + 14:0); *c*9 16:1 = *c*9 16:1/(*c*9 16:1 + 16:0); *c*9 18:1 = *c*9 18:1/(*c*9 18:1 + 18:0); *c*9*t*11 18:2 = *c*9*t*11 18:2/(*c*9*t*11 18:2 + *t*11 18:1).

2021). A meta-analysis by dos Santos Neto et al. (2021) revealed that PA-enriched supplements increased milk yield by 1.60 kg/d, milk fat yield by 0.10 kg/d, and milk protein yield by 0.04 kg/d, compared with, respectively, 1.77 kg/d, 0.19 kg/d and 0.07 kg/d in the current study.

Milk production was 7% or 3.1 kg/d greater for 3× compared with 2× MF, and the increase in milk fat and protein yields were 73 and 86 g/d, respectively. It is well established that milk yield increases with MF (Erdman and Varner, 1995; Klei et al., 1997). This increase was reported to be from 10% to 15% (Klei et al., 1997; Smith et al., 2002). Erdman and Varner (1995) had previously suggested that going from 2 to 3 milkings per day would cause a fixed increase in milk production of 3.5 kg/d and an increase of 92 g of fat and 84 g of protein per day. In the current study, cows from the control group (no PA and 2× MF) produced an average of 44.7 kg/d of milk, which is much greater than the average milk production of cows in the studies cited previously (Erdman and Varner, 1995: 19.5 kg/d; Klei et al., 1997 and Smith et al., 2002: 29 kg/d). With greater milk performance, the potential for an increase in milk yield with MF likely decreases, which could explain why the 7% increase in milk yield in our study is slightly inferior to what is expected based on previous literature. The effects of MF on milk composition are not clear. Studies have reported a decrease (Klei et al., 1997; Smith et al., 2002) or no changes (DePeters et al., 1985; Wiking et al., 2006) in concentrations of milk components when increasing milkings from twice to thrice daily. Our study generally agrees with these findings: milk fat concentration was reduced by 3× MF and milk protein concentration was similar between treatments. The effect on fat concentration was most likely due to a dilution effect because the increase was slightly greater for milk yield (+7%) than for fat yield (+4%).

Milk FA Composition

Increasing MF had no significant effect on milk FA composition (Table 3). This is in agreement with the results of Delamaire and Guinard-Flament (2006), who also reported that milk FA composition was not different despite the milking interval varying from 8 to 24 h. In contrast, Wiking et al. (2006) reported a decrease in PUFA in milk from half-udders milked 4 times daily compared with their counterparts milked twice daily. The authors suggested that the increase in fat yield with MF could be due mainly to a greater de novo synthesis, and a consequent decrease in the proportion of PUFA in milk. In our trial, no effect of MF on milk preformed FA concentration was observed (Table 3). However, in

Table 4. Effect of palmitic acid (PA) supplementation and twice or thrice-daily milkings on milk fatty acid (FA) yields

	Treatment[1]							
	No PA		PA			*P*-value[2]		
Item	2×	3×	2×	3×	SEM	FAT	MF	FAT × MF
Milk FA yield, g/d								
4:0	46	48	46	53	3.3	0.17	0.02	0.18
De novo (6 C to 14 C)	367	378	336	358	19.0	0.01	0.05	0.50
Mixed (16:0 + *c*9 16:1)	553	564	709	741	37.0	<0.01	0.18	0.51
Preformed ≥18 C[3]	472	480	488	518	20.4	0.03	0.13	0.37
*c*9 18:1[4]	222	225	245	256	10.2	<0.01	0.33	0.51
Saturated[5]	1,076	1,108	1,184	1,258	63.3	<0.01	0.03	0.37

[1]No PA = no PA supplementation; PA = PA supplementation (2% on a DM basis; 88% 16:0); 2× = 2 milkings/d (12-h intervals); 3× = 3 milkings/d (8-h intervals).

[2]FAT = effect of PA supplementation; MF = effect of milking frequency.

[3]FA of 18 C and more including *c*9 18:1 and excluding *iso* 18:0.

[4]Coelution with minor concentration of *c*10 18:1.

[5]∑Straight-chain SFA.

support of the hypothesis of Wiking et al. (2006), thrice-daily MF increased de novo FA yield by 5%, but mixed and preformed FA yields were not significantly affected (Table 4). Detailed FA yields are presented in Supplemental Table S1 (see Notes).

Feeding a PA supplement increased the proportion of 16:0 (+17%) and *cis*-9 16:1 (+25%; Supplemental Table S2, see Notes) at the expense of de novo FA (−16%; 6–4 C), whereas the proportion of preformed FA only tended to diminish ($P = 0.07$; Table 3). The increase in the proportion of 16:0 was expected and is in agreement with previous studies (Chamberlain et al., 2016; Shepardson and Harvatine, 2021). Dietary PA decreased the yield of de novo FA (−7%) but increased the yield of mixed (+30%) and, to a lesser extent, preformed (+6%) FA (Table 4). Previous studies also reported that the increase in 16 C FA yield observed with dietary PA supplementation was at the expense of de novo FA (Piantoni et al., 2013), whereas others reported no effect on de novo FA yield (de Souza and Lock, 2018). Secretion of 4:0 and desaturation of 18:0 into *cis*-9 18:1 by the mammary tissue have been suggested as mechanisms to maintain milk fluidity when secretion of saturated FA such as 16:0 increases (Barbano and Sherbon, 1980). Dietary PA did not affect the concentration or secretion of 4:0 nor *cis*-9 18:1 concentration in milk. However, secretion of *cis*-9 18:1 in milk was increased when cows received PA.

All desaturase indexes increased with PA supplementation (Table 3). Used as a proxy (Baumgard et al., 2002), these desaturation indexes indicate an increase in the Δ^9-desaturase enzyme activity or expression with PA. Interestingly, 3× MF decreased *cis*-9 14:1/(*cis*-9 14:1 + 14:0) and *cis*-9 *trans*-11 18:2/(*cis*-9 *trans*-11 18:2 + *trans*-11 18:1) and tended to decrease *cis*-9 18:1/(*cis*-9 18:1 + 18:0) indexes. Only the de novo FA yield was increased by MF. If the increase in milk fat yield with MF is mainly associated with de novo synthesis, the need for Δ^9-desaturation could be diminished because of the lower melting point of those shorter-chain FA compared with 16:0. Changes in milk FA composition are expected to affect milk processing parameters such as fat thermal properties (Landry et al., 2025).

Nutrient Total-Tract Apparent Digestibility and FA Transfer in Milk

Milking frequency had no effect on nutrient digestibility (data not presented). Dietary PA had no effect on DM, OM, and NDF intakes or digestibilities (Table 5). The intake and the calculated absorption of total FA, total 16 C FA, and total 18 C FA increased with PA. However, PA decreased the digestibility of total FA and total 16 C FA, and tended to increase total 18 C digestibility ($P = 0.06$). The meta-analysis by dos Santos Neto et al. (2021) revealed that PA-enriched supplements increased DM and NDF digestibility without affecting total FA digestibility in dairy cows. Average total FA digestibility for diets supplemented with PA was also higher in this meta-analysis (73.9%) than in the current study (63.8%). The authors suggested that the positive effect of PA supplementation on DM digestibility could be linked with the uptake and membrane incorporation of this FA by fibrolytic bacteria (dos Santos Neto et al., 2021). Many experimental factors can affect digestibility. For instance, DMI was greater in our study (28.3 kg/d) compared with the average of the trials included in the meta-analysis (24.9 kg/d). Moreover, in many studies reporting an increase in DM and NDF digestibilities, the PA supplement replaced soyhulls in the diet (Piantoni et al., 2013; Rico et al., 2017; de Souza and Lock, 2018), which was not

Table 5. Effect of palmitic acid (PA) supplementation on nutrient and fatty acids (FA) intakes, total-tract apparent digestibilities, and transfer of total 16 C and 18 C FA into milk fat

Item	Treatment[1,2]		SEM	*P*-value
	No PA	PA		
DM				
Intake, kg/d	28.3	28.2	0.75	0.82
Digestibility,[3] %	64.5	64.4	0.62	0.82
Digested,[4] kg/d	18.3	18.1	0.53	0.69
OM				
Intake, kg/d	26.5	26.3	0.70	0.77
Digestibility, %	66.2	66.2	0.61	0.92
Digested, kg/d	18.7	18.6	0.56	0.59
NDF				
Intake, kg/d	10.0	9.8	0.26	0.35
Digestibility, %	48.9	47.8	1.31	0.48
Digested, kg/d	4.9	4.7	0.17	0.21
Total FA				
Intake, g/d	638	1,190	28.3	<0.01
Digestibility, %	71.5	63.8	2.05	<0.01
Absorbed, g/d	459	761	33.6	<0.01
∑ 16 C FA				
Intake, g/d	117	615	15.1	<0.01
Digestibility, %	67.7	52.3	1.98	<0.01
Absorbed, g/d	79	325	18.9	<0.01
Secreted in milk, g/d	577	743	37.6	<0.01
Apparent transfer,[5] %	491	121	14.0	<0.01
True transfer,[6] %	729	237	23.2	<0.01
∑ 18 C FA				
Intake, g/d	512	552	15.2	<0.01
Digestibility, %	75.1	78.5	1.91	0.06
Absorbed, g/d	386	434	19.3	<0.01
Secreted in milk, g/d	462	489	19.6	0.03
Apparent transfer, %	91	89	3.2	0.55
True transfer, %	122	113	5.1	0.09

[1]No PA = no PA supplementation; PA = PA supplementation (2% on a DM basis; 88% 16:0).

[2]Milking frequency had no effect on nutrient digestibilities, and there was no PA × MF interaction.

[3]Nutrients apparent digestibilities were calculated as follows: 100 − {100 × [(diet iNDF, %DM/feces iNDF, %DM) × (nutrients in feces, %DM/nutrients in diet, %DM)]}.

[4]Apparent digestibility of each nutrient allowed to calculate amount of nutrient digested.

[5]Secreted in milk/ingested × 100.

[6]Secreted in milk/absorbed × 100.

the case in our experiment in which PA replaced ground corn. Other studies that did not use soyhulls as a replacement reported no effect of a PA-enriched supplement on DM and NDF digestibilities at concentration similar to those in the current study (Rico et al., 2014; Shepardson and Harvatine, 2021).

In the study by Shepardson and Harvatine (2021), the PA supplement increased total FA and total 18 C digestibilities, unlike in the current study, where only total 18 C digestibility tended to increase. The observed decrease in total FA digestibility could be the consequence of an important decrease in 16 C FA digestibility (from 67.7% to 52.3%), whereas the 16 C digestibility was not significantly decreased in the study by Shepardson and Harvatine (2021). Many other studies reported a decrease in total and 16 C FA digestibilities with dietary PA supplementation (Piantoni et al., 2013; Rico et al., 2017; de Souza and Lock, 2018). In agreement with the current observations, Piantoni et al. (2013) reported a decrease in total FA digestibility from 71.3% to 61.2%, also mainly due to a decrease in 16 C FA digestibility (from 67.5% to 50.2%) when feeding 2% (DM basis) of a highly enriched PA supplement (99% 16:0). The decrease in FA digestibility with PA was expected because fat digestibility is consistently decreased when intake increases (Palmquist, 1991).

Apparent and true transfers of 16 C FA, which is of mixed origin, into milk decreased with dietary PA (491% to 121% for apparent transfer and 729% to 237% for true transfer; Table 5). Even if total 18 C ingested increased with PA supplementation, apparent transfer of 18 C FA was not significantly different between dietary treatments, whereas true transfer tended to decrease with the PA-enriched diet (122% to 113%; $P = 0.09$). The additional milk FA yield as a percentage of additional FA consumed was 27.8% for total FA, 33.3% for 16 C FA, and 67.2% for 18 C FA. In the study of Lock et al. (2013), with a similar PA supplementation, these values were 20.1% for total FA and 29.7% for 16 C FA.

Milk Properties

FFA Concentration. Free FA concentration in fresh milk was similar across treatments (0.61 ± 0.105 mEq/100 g of milk fat; Table 6). This result was unexpected and in contrast with previous studies. It is generally accepted that milk FFA concentration increases with MF (Klei et al., 1997; Wiking et al., 2006). However, Wiking et al. (2006) also found that there was no difference in milk FFA concentration right after milking, from half-udders milked 2 or 4 times daily. It is only after 24 h of storage at 5°C that milk from half-udders milked 4 times daily had a greater FFA concentration. Their results support the hypothesis that one of the causes of elevated FFA in milk with increased MF is the weakened milk fat globule membrane (**MFGM**). When the MFGM is disturbed, milk lipoprotein lipase can access and hydrolyze triacylglycerols located in the core of the globule. Indeed, a fragilized fat globule is prone to lipolysis. The cause of this increased fragility of fat globules with MF is still unknown but could possibly be the increase in milk fat globule diameter as reported by Wiking et al. (2006). It could be hypothesized that when fat yield increases, MFGM becomes limiting, creating larger and weaker fat globules that are more susceptible to lipolysis (Wiking et al., 2003). In this study, we determined FFA concentration in very fresh milk (a few hours after milking). Hence, it is possible that lipolysis

Table 6. Effect of palmitic acid (PA) supplementation and twice or thrice-daily milkings on free fatty acid (FFA) content, protein fractions concentrations and yields, and plasmin activity in milk

	Treatment[1]							
	No PA		PA			*P*-value[2]		
Item	2×	3×	2×	3×	SEM	FAT	MF	FAT × MF
FFA, mEq/100 g of fat	0.63	0.68	0.51	0.62	0.105	0.39	0.45	0.73
Milk concentration, %								
Total N	0.522	0.507	0.518	0.513	0.0147	0.96	0.42	0.69
True protein	3.18	3.09	3.15	3.12	0.092	1.00	0.41	0.65
CN	2.71	2.63	2.68	2.67	0.095	0.99	0.52	0.65
Whey proteins	0.47	0.45	0.47	0.45	0.013	0.88	0.01	0.89
NPN	0.023	0.024	0.024	0.024	0.0009	0.22	0.65	0.15
CN N/Total N	0.814	0.813	0.811	0.814	0.0072	0.84	0.85	0.59
Yield, kg/d								
True protein	1.41	1.47	1.45	1.54	0.049	0.12	0.05	0.64
CN	1.21	1.25	1.24	1.32	0.048	0.17	0.08	0.64
Whey proteins	0.209	0.216	0.216	0.224	0.0083	0.02	0.02	0.88
NPN	0.043	0.045	0.045	0.047	0.0015	<0.01	<0.01	0.69
Plasmin activity, U/mL of milk serum								
Fresh milk	4.80	5.73	4.73	5.83	0.733	0.97	0.04	0.85
After 48 h at 4°C	5.10	5.20	3.85	3.68	0.770	0.03	0.95	0.82
Plasminogen activity, U/mL of milk serum								
Fresh milk	57.90	52.92	60.55	51.46	5.683	0.84	0.03	0.50
After 48 h at 4°C	51.49	50.22	59.73	48.29	6.013	0.42	0.11	0.20
Plasminogen:plasmin activity ratio								
Fresh milk	14.4	12.4	15.6	11.4	3.07	0.97	0.03	0.44
After 48 h at 4°C	19.4	13.4	18.2	16.3	5.07	0.81	0.29	0.58
Total plasmin + plasminogen activity, U/mL of milk serum								
Fresh milk	62.7	58.6	65.3	57.3	5.31	0.84	0.06	0.52
After 48 h at 4°C	56.6	55.4	63.6	52.0	5.62	0.63	0.10	0.17

[1]No PA = no PA supplementation; PA = PA supplementation (2% on a DM basis; 88% 16:0); 2× = 2 milkings/d (12-h intervals); 3× = 3 milkings/d (8-h intervals).

[2]FAT = effect of PA supplementation; MF = effect of milking frequency.

was limited and, consequently, not different between MF. Moreover, because our milk was collected with minimal manipulation (was not pumped through a pipeline, and was manually and gently stirred before sampling), it is possible that lipolysis was minimized, explaining the discrepancies. Noteworthy was the great variability in milk FFA concentration between cows, but also between days of sampling for the same cow. It is also plausible that milking interval (i.e., time between milkings) rather than MF (number of milkings per day) per se affects milk FFA concentration. The shortest milking interval in the current study was 8 h. It is possible that shorter or uneven intervals could emphasize the release of FFA, but further research is needed to validate this hypothesis.

In contrast with our results, saturated fat supplementation has also been reported to increase milk FFA concentration (Astrup et al., 1980; Wiking et al., 2003). It is possible that fat globule size increases with milk fat yield, making the globules more susceptible to lipolysis. The correlation between fat yield and fat globule size was previously reported (Wiking et al., 2004). However, in the current study no effect of PA supplementation on FFA was observed. This discrepancy could be explained by the fact that FFA concentration was determined only a few hours after milking, limiting the time for lipolysis to occur.

Milk N Fractions and Plasmin Activity. In this study, no effects of treatments were observed on milk CP concentration as determined by infrared spectroscopy (Table 2). However, previous studies led to the hypothesis that fat supplementation can affect milk protein fractions (DePeters and Cant, 1992). In the current study, treatments had no effect on TN, true protein, CN, or NPN concentrations (Table 6). The ratio of CN-N to TN was also similar between treatments. Whey proteins concentration was slightly decreased with 3× MF (0.45% vs. 0.47%; $P = 0.01$). This decrease could be representative of a dilution effect in the additional milk volume produced with 3× MF. True protein and CN concentrations were also numerically lower with 3× MF. The calculated CP yield based on infrared results was increased by both PA and 3× MF (Table 2). However, increasing MF increased true protein, whey proteins, and NPN yields, but only tended to increase CN yield ($P = 0.08$; Table 6). Dietary PA only increased significantly whey proteins and NPN yields.

Milk protein concentration has been reported to decrease with fat supplementation while protein yield of-

ten remains unaffected (DePeters and Cant, 1992). Milk protein secretion does not always match the increase in milk production when fat is added to the diet, but this response is still not completely understood. In this study, milk CP concentration was unaffected by PA, and consequently protein yield increased. The ratio of CN to true protein was also unaffected by PA. DePeters and Cant (1992) suggested that CN were more depressed by dietary fat supplementation than other protein fractions. In the current study, PA increased total CP, whey proteins, and NPN yields, but not significantly CN and true protein yields. However, CN concentration in milk was not affected by PA.

Some authors reported an increase in the CN:total protein ratio with intensified MF (Klei et al., 1997; Sorensen et al., 2001). This observation could be explained by plasmin activity. Plasmin is the main protease in milk. Its principal substrates are β- and α-CN (Stelwagen et al., 1994). Originating from blood, this enzyme passes in milk in its active and inactive form (plasminogen). Plasminogen activators and their inhibitors regulate the conversion of plasminogen into plasmin (Stelwagen et al., 1994). In our study, plasmin activity in fresh milk increased with MF, but there was no effect of MF after 48 h of storage (Table 6). After 48 h of storage at 4°C, plasmin activity was lower in milk of PA-supplemented cows.

By activating plasminogen into plasmin, it is possible to measure plasminogen potential activity. In our study, plasminogen activity was lower in milk of cows milked thrice compared with twice daily. However, there was no effect of treatments on milk plasminogen activity after 48 h of storage at 4°C. The plasminogen-to-plasmin ratio in fresh milk was lower for 3× MF. This ratio is associated with a lower proteolytic potential in milk (Sorensen et al., 2001). After 48 h of storage at 4°C, the difference was no longer significant. The total plasmin and plasminogen activity tended to be lower for 3× MF ($P = 0.06$; Table 6). It was suggested that during a long milking interval, plasmin and plasminogen can migrate from blood to milk through the increased permeability of mammary tight junctions when milk accumulates in the udder (Stelwagen et al., 1994). Another hypothesis is that increased MF limits the time during which plasminogen can be activated into plasmin in the udder (Klei et al., 1997; Sorensen et al., 2001). Looking at plasminogen-to-plasmin ratio and total plasmin and plasminogen activity, our results tend to support this hypothesis. In previous research, increased plasmin activity when MF decreased was mostly observed between once and twice milkings daily or in late lactation (Stelwagen et al., 1994; Sorensen et al., 2001). In the study by Klei et al. (1997), plasmin activity was not affected by the increase of MF from 2 to 3 milkings per day. Moreover, many other factors, such as advanced DIM, greater parity, and elevated SCC, increase plasmin activity (Politis et al., 1989; Bastian et al., 1991). In our study, the ratio of CN to true protein was not affected by MF. It is possible that the duration of milking intervals did not allow us to see any differences, the longest interval between milkings being 12 h. Overall, there was no effect of treatments on milk CN concentration, but CN yield was not increased by dietary PA or thrice-daily milking. More research is needed to understand how fat supplementation and MF affect CN production in milk because of its importance in cheese manufacture.

CONCLUSIONS

In this experiment, both PA supplementation and increased MF enhanced milk performance but no significant interaction between the 2 practices was observed; their effects were additive. Milking frequency had no significant effect on milk FA composition, whereas PA supplementation increased 16 C FA secretion and Δ^9-desaturase activity. Fresh milk FFA concentration was not affected by PA supplementation or MF. Under the conditions of this trial, PA and MF had limited effects on overall protein fractions (CN, whey proteins, and so on) concentrations in milk. This study investigated, on a small scale, the effects of 2 on-farm practices. It remains to be determined to what extent the effects observed in the current study would reproduce in commercial settings. The effects of on-farm practices such as fat supplementation and MF need to be evaluated throughout the processing steps of milk up to the final dairy products.

NOTES

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Nonstandard abbreviations used: FA = fatty acid; FFA = free fatty acids; iNDF = indigestible NDF; MF = milking frequency; MFGM = milk fat globule membrane; NCN-N = non-CN nitrogen; PA = palmitic acid; TN = total milk nitrogen.

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