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Milking frequency and dairy cow susceptibility to lipolysis interact to alter milk lipolysis and composition

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ABSTRACT

Lipolysis is an ongoing issue for the French dairy industry that must be minimized. Milk lipolysis is defined as the hydrolysis of triglycerides, the major component of milk fat, resulting in the release of short-chain fatty acids responsible for rancid flavor and partial glycerides that impair functional properties such as foaming and creaming abilities. Milk lipolysis is a complex phenomenon that depends on both animal parameters and farming factors. Milk spontaneous lipolysis is higher in milk from automatic milking systems, which could be due to the number and intervals of milking, as lipolysis is lower in the case of a single daily milking. In addition, considerable interindividual variability in milk lipolysis has been observed, with some dairy cows being highly susceptible or nonsusceptible to lipolysis. The objective of this study was therefore to evaluate the impact on spontaneous milk lipolysis of different milking frequencies (i.e., 1 [morning or evening], 2, or 3 milkings per day) with evenly spaced milking intervals while accounting for individual susceptibility to lipolysis. To achieve this goal, 32 primiparous and multiparous dairy cows in mid-lactation were conducted using a continuous design with milking frequency as the main factor for a period of 3 wk. Four treatments were applied on 4 groups of cows: 1 milking per day at 6:00 a.m. (1M*6am*), 1 milking per day at 6:00 p.m. (1M*6pm*), 2 milkings per day at 6:00 a.m. and 6:00 p.m. (2M), and 3 milkings per day at 6:00 a.m., 2:00 p.m., and 10:00 p.m. (3M). In each group, there were 4 susceptible (SUS) dairy cows (lipolysis of SUS >0.70 mEq/100 g fat) and 4 nonsusceptible (NONSUS) dairy cows (lipolysis of NONSUS <0.70 mEq/100 g fat). As expected, 2M and 3M milkings increased milk yield by up to 30% compared with once-a-day milking. We confirmed that milk spontaneous lipolysis was influenced by increased milking frequency: compared with 2M, we observed more lipolysis with 3M and less with 1M. Regardless of the lipolysis susceptibility, the 1M*6am* and 1M*6pm* treatments caused a similar reduction in lipolysis. On the other hand, lipolysis was significantly higher in SUS cows with 2M and 3M treatments. In conclusion, although increased milking frequency results in greater milk yield, our results indicate that it can adversely impact milk quality with regard to free fatty acid concentrations. Conversely, although once-a-day morning or evening milking lead to decreased milk yield, they significantly reduced milk lipolysis regardless of a cow's susceptibility to lipolysis.
Key words: free fatty acid, automatic milking system, once-a-day milking

INTRODUCTION

Milk lipolysis is defined as the hydrolysis of triglycerides, the primary component of milk fat, by the lipoprotein lipase (**LPL**) enzyme (Deeth, 2006). This leads to the release of monoglycerides, diglycerides, and free fatty acids (**FFA**), including the short-chain fatty acids (**FA**) responsible for rancid flavor. In the Pays de Loire region and in Brittany, the main milk-producing regions in France, lipolysis is one of the criteria for milk payment. When it exceeds the threshold of 0.89 mEq/100 g of fat, a penalty of €3 per 1,000 L is incurred by the farmer. In addition, milk exceeding this threshold may become unsuitable for drinking and for processing certain products, particularly cream and butter. Lipolysis therefore represents an ongoing issue for the French dairy industry and must be minimized. Lipolysis can occur in milk in 3 principal ways: spontaneous lipolysis, which is the focus of this article; induced lipolysis, which is linked to milking systems and milk transport and arises due to thermal shocks and physical agitation on milk fat globules (**MFG**); and microbial lipolysis due to microbial lipase, which

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develops in milk after several days of cold storage. Spontaneous lipolysis in cows occurs following cooling of the milk, in the absence of mechanical shocks beyond those that occur during milking.

Spontaneous lipolysis depends on both the animal as well as factors related to farming conditions. In addition, considerable interindividual variability has been observed, with some cows being more susceptible to milk lipolysis than others (Vanbergue et al., 2017).

Milk susceptibility to lipolysis may be explained by the presence of components in skim milk that activate or inhibit the LPL enzyme (Cartier and Chilliard, 1990; Delosière et al., 2023), as well as by the MFG membrane (**MFGM**) characteristics, which determine its resistance and role as a barrier to lipase access to the triacylglycerol core. Potential explanations for the fragility of the MFGM include milk fat surface area, which is associated with milk susceptibility to lipolysis (Vanbergue et al., 2017), wherein small globules display a larger membrane surface area for lipase enzyme action than large ones (Murphy et al., 1979), as well as MFGM composition (Lu et al., 2016; Bernard et al., 2025), which affects its fluidity and LPL access to the triacylglycerols. Conversely, larger MFG may be more fragile (Wiking et al., 2019) and thus more accessible to the action of LPL. Furthermore, damage to MFGM during mechanical milking and cooling could be more pronounced in milks predisposed to spontaneous lipolysis (Cartier and Chilliard, 1990).

Lipolysis may also vary depending on milking practices, notably the use of an automatic milking system (**AMS**), which leads to increased lipolysis rates compared with a conventional milking parlor (De Marchi et al., 2017). This may be related to an increased milking frequency when using AMS. In dairy farms, once-a-day milking is also practiced, particularly in grassland systems to lighten the workload.

The objective of this study was therefore to evaluate the impact on spontaneous milk lipolysis of various milking frequencies: 1 milking per day at 6.00 a.m. (**1M*6am***), 1 milking per day at 6.00 p.m. (**1M*6pm***), 2 milkings per day at 6.00 a.m. and 6.00 p.m. (**2M**), and 3 equally spaced milkings per day at 6.00 a.m., 2.00 p.m. and 10.00 p.m. (**3M**). The individual susceptibility to lipolysis was also taken into account for each treatment (milking frequency). Our hypothesis was that milk lipolysis would be affected by milking frequency, with a decrease for once-a-day milking and an increase for 3 milkings per day, and that susceptible (**SUS**) dairy cows would be more affected by these milking frequencies than nonsusceptible (**NONSUS**) dairy cows. We further sought to evaluate the effect of milking time (morning or evening) for once-a-day milkings on milk composition and lipolysis.

MATERIALS AND METHODS

Animals and Experimental Design

The experiment was conducted at the experimental farm IE PL, INRAE, Dairy Nutrition and Physiology (IE PL, Le Rheu, France; https://doi.org/10.15454/yk9q-pf68; animal housing agreement number C-35-275-23) in accordance with French legislation on animal experimentation and with approval by the French National Committee for Consideration of Ethics in Animal Experimentation (authorization: APAFIS #34355-2021121510194440 v1, delivered on January 20, 2022). For the experiment, we used 32 Holstein cows (8 primiparous; 130 ± 42 DIM; 666 ± 65.1 kg of BW before batching; average ± SD) producing on average 36.0 ± 6.3 kg of milk per day with 39.5 ± 4.4 g/kg fat, 31.6 ± 2.3 g/kg protein, and a spontaneous lipolysis estimated to 0.82 ± 0.91 mEq/100 g of fat for morning milk (6:30 a.m.) and 1.51 ± 1.50 mEq/100 g of fat for evening milk (4:30 p.m.). The cows were chosen according to their susceptibility to lipolysis, measured by both the Fourier transformed infrared (**FTIR**) mid-infrared spectrometry (MyLab Dairy Laboratory, Châteaugiron, France) and copper soap methods (Hurtaud et al., 2023). Cows were allocated to 4 groups of 8 animals each, according to the following criteria and in this order: milk yield, spontaneous lipolysis (2 groups, less than or greater than 0.70 mEq/100 g fat), lactation stage, parity (primiparous vs. multiparous), milk fat and protein contents, SCC, and BW. Each group included 4 susceptible dairy cows (lipolysis of SUS >0.70 mEq/100 g fat) and 4 nonsusceptible dairy cows (lipolysis of NONSUS <0.70 mEq/100 g fat). The lipolysis values of NONSUS cows were 0.24 ± 0.05, 0.29 ± 0.16, 0.22 ± 0.06, 0.35 ± 0.25 mEq/100 g fat for the 1M*6am*, 1M*6pm*, 2M, and 3M groups, respectively. The lipolysis values of SUS cows were 0.75 ± 0.19, 1.42 ± 1.27, 0.81 ± 0.28, 0.73 ± 0.20 MEq/100 g fat for the 1M*6am*, 1M*6pm*, 2M, and 3M groups, respectively. All cows were kept indoors, with a mean area of 8.75 m^2 per cow.

Starting in February 2022, the experiment was conducted using a continuous design with milking frequency as the main factor for a period of 3 wk. Four treatments were applied: one milking per day at 6:00 a.m. (1M*6am*), one milking per day at 6:00 p.m. (1M*6pm*), 2 milkings per day at 6:00 a.m. and 6:00 p.m. (2M), and 3 milkings per day at 6:00 a.m., 2:00 p.m. and 10:00 p.m. (3M).

Feeding

Throughout the experiment, cows had free access to water and were fed via individual electronic gating twice daily at 8:00 a.m. and 6:00 p.m. All cows were offered the same diet consisting of 65% maize silage (offered

ad libitum), 12.5% energy concentrate (20% wheat, 20% maize, 20% barley, 20% beet pulp, 15% wheat bran, 3% cane molasses, 1% vegetable oil, and 1% salt), 12.5% soybean meal, 10% dehydrated alfalfa, and 300 g of minerals. Diets were formulated to meet energy and protein requirements when distributed at 100% of ad libitum DMI (INRA, 2018).

All feed refusals were collected and weighed daily to determine individual DMI. To calculate DMI, refusals were assumed to have the same composition as the offered diet.

Sample Collection and Laboratory Analyses

Milk Yield and Traits. Cows were milked in the milking parlor at different times according to their milking frequency treatment. Milk yield was recorded individually at each milking. Milk fat, protein and lactose contents, and SCC were determined from all milkings on Monday, Tuesday, and Wednesday during the pre-experimental period (1 wk just before the beginning of the experiment) and during each week of the experimental period. These analyses were performed by FTIR mid-infrared spectrometry for fat, protein, and lactose contents and by flow cytometry for SCC at the MyLab Dairy Laboratory (Châteaugiron, France).

An additional individual milk sample was collected from milk cans from individual milkings on Wednesday, and then stored in containers of different volumes at 4°C or at −20°C according to the analyses. Spontaneous lipolysis, FA profile, MFG diameter, and milk protein and mineral composition were determined from these milk samples.

Spontaneous Lipolysis of Milk. Two samples of the same milk were immediately heated after milking in a water bath at 100°C for 3 min for the first sample, and after 24 h of storage at 4°C for the second sample to stop the activity of LPL. The FFA were measured by the copper soap method as described in Hurtaud et al. (2023). Spontaneous lipolysis of milk was quantified using the FFA content of milk after 24 h of storage at 4°C, from which the initial FFA content was subtracted.

Milk Fat Globule and Casein Sizes. After adding bronopol (Merck, Darmstadt, Germany), vials of milk were kept at room temperature for a maximum of 16 h to assess MFG diameter distribution using laser-light scattering (Mastersizer 3000, Malvern, United Kingdom). Using the Malvern software (V5.30), we calculated mean diameters as $d_{4,3} = \Sigma\left(N_i \times d_i^4\right) / \Sigma\left(N_i \times d_i^3\right)$ and $d_{3,2} = \Sigma\left(N_i \times d_i^3\right) / \Sigma\left(N_i \times d_i^2\right)$, and MFG area as $s = 6/(\rho \times d_{3,2})$, with N_i = the number of MFG in diameter class d_i and ρ = the density of the particle considered (0.92 for fat). Another sample of milk without bronopol was skimmed by 2 successive centrifugations (3,157 × *g*, 4°C, 10 min). The mean diameter $d_{4,3}$ of casein micelles was measured on the skim milk using the Mastersizer 3000.

Milk Fatty Acid Composition and LPL Activity. Milk FA methyl esters of the freeze-dried milk samples were then prepared and analyzed after injection into a gas chromatograph (Agilent 7890A GC System, Massy, France) equipped with a flame ionization detector and a CP-Sil 88 capillary column (100 m × 0.25 mm, 0.2 µm thickness; Agilent Technologies Inc., Santa Clara, CA), as previously described (Fougère et al., 2018). Milk LPL (EC 3.1.1.34) activity was measured from morning milk stored at −20°C, as described by Bernard et al. (2005).

Milk N and Mineral Composition. Total nitrogen, NPN, non-CN, and CN were determined according to the Kjeldahl method described by Alais (1984). Urea was analyzed on milk ultrafiltrate (Vivaspin Turbo 15 Centrifugal Concentrator Polyethersulfone, Sartorius, Göttingen, Germany) in 2 replicates with colorimetric enzymatic reactions assessed using a multiparameter analyzer (KONE Instruments 200 Corporation, Espoo, Finland). Total and soluble Ca, total Na, total K, and total Mg were respectively analyzed via inductively coupled plasma optical emission spectroscopy (ICP-OES 5110, Agilent Technology, Les Ulis, France) of milk and milk ultrafiltrate (Ca only), as previously described by Hurtaud et al. (2023). Total and soluble P and Cl contents were determined using a KONE PRO multiparameter analyzer (ThermoFisher Scientific, Illkirch, France) according to the Allen method for P (Pien, 1969) and as described by Henry et al. (1974) for Cl.

Plasma Metabolites and Hormones. Blood was sampled from the tail using 5-mL heparinized and EDTA tubes (VT-050SHL, Venoject, Terumo Europe, Leuven, Belgium) after the morning milking during the last day of each experimental period. Blood was centrifuged at 2,264 × *g* for 15 min at 4°C, and plasma was removed and stored at −20°C until analysis. Plasma metabolites were analyzed in 2 replicates by colorimetric enzymatic reactions using a multiparameter analyzer (Kone Instrument Corp., Espoo, Finland). Plasma glucose, urea, and nonesterified FA (**NEFA**) contents were measured as reported by Corset et al. (2024); plasma acetate, triglyceride, lactose, and BHB contents were measured as reported by Anger et al. (2024); and plasma lactose was analyzed as reported in Guinard-Flament et al. (2011). Plasma insulin concentration was determined by RIA using the Wizard2 gamma counter 2470 (Perkin-Elmer) with commercial kits (Insulin RIA kit, PI-12K, Millipore, Billerica, MA). Plasma prolactin concentrations were measured using an indirect competitive ELISA with a rabbit-anti-prolactin antibody (Kollmann et al., 2008).

Statistical Analyses

Unless otherwise noted, all statistical analyses were performed using the SAS software (SAS 9.2, SAS Institute Inc., Cary, NC). The results were averaged so as to have only one result per treatment and per cow. For 2M and 3M, daily values were obtained by calculating the average of each milking values weighted by milk yield or by fat content (for lipolysis). Milking frequency (1M*6am*, 1M*6pm*, 2M, and 3M), susceptibility to lipolysis (SUS and NONSUS), and the interaction between milking frequency and cow susceptibility were evaluated using analyses of covariance for DMI, BW, milk traits, and plasma parameters. The statistical model was a mixed model (MIXED procedure of SAS) that included milking frequency, cow susceptibility, and the interaction of milking frequency and cow susceptibility as fixed effects, and a covariate that corresponded to the value of variable Y_{ij} during the pre-experiment period ($CovY_{ij}$):

$$Y_{ij} = \mu + Susceptibility_i + MilkingFrequency_j + Susceptibility_i \times MilkingFrequency_j + CovY_{ij} + \varepsilon_{ij},$$

where μ is the mean, Y_{ij} is the variable dependent on the fixed effects, and ε_{ij} is the residual error associated with each ij observation. For all analyses, the threshold for statistical significance was set at $P < 0.05$, and that for a trend was set at $0.05 < P < 0.10$.

We complemented these univariate analyses of milk content and composition parameters by considering a multivariate analysis called redundancy analysis (**RDA**; Oksanen et al., 2024). Briefly, RDA enables a global visualization of trends between a set of explanatory variables and a set of milk parameter variables by first fitting individual multiple regression to each milk parameter and subsequently performing dimensionality reduction on fitted values using a principal components analysis. Here we performed an RDA in the R programming language (v4.4.0; R Core Team, 2021) with the vegan package (2.6-6.1) using milking treatment and lipolysis susceptibility as fixed effects and considering a nonredundant subset of milk content and composition parameters as response variables (milk yield, milk ejection rate, milk protein content, SCS, milk lactose content, milk fat content, lipolysis, MFG $d_{4,3}$, milk casein $d_{4,3}$, milk casein content, milk soluble protein, milk urea content, milk total P content, milk total Ca content, milk K content, milk citrate content, milk Na content, and milk Na^+/K^+ ratio). An *F*-statistic with permutation test was used to evaluate the overall significance of milking treatment and lipolysis susceptibility. Samples and milk parameters were simultaneously visualized on an ordination plot.

RESULTS

Milking Frequency Affects Milk Yield, Composition, and Lipolysis

Compared with 2M, milking frequency had a significant ($P = 0.005$) effect on DMI, with a significantly lower DMI with 1M*6am* (−1.7 kg DM/d) and a slightly higher DMI with 3M (+0.6 kg DM/d) without any effect on BW. Milk yield and milk lactose content and yield were lower for 1M*6am* and 1M*6pm* (compared with 2M, −10.1 kg, $P < 0.001$; −1.65 g/kg, $P = 0.022$; −537 g/d, $P < 0.001$, respectively; Table 1). On the contrary, milk fat and protein contents were higher for 1M*6am* and 1M*6pm* (+6.77 g/kg, $P = 0.007$; +1.96 g/kg, $P = 0.001$, respectively), but milk fat and protein yields were lower (−191 g/d, $P = 0.001$; −260 g/d, $P < 0.001$). We found no significant difference between 2M and 3M on milk yield, fat and protein contents, and yields despite a numerical gap of 0.9 kg for milk yield, 2.0 g/kg for fat content, 0.6 g/kg for protein content, 141 g/d for fat yield, and 57 g/d for protein yield. Milking frequency had no effect on SCS.

Free FA after 24 h of storage at 4°C and lipolysis were higher for 2M and 3M compared with 1M*6am* and 1M*6pm* (+0.53 mEq/100 g fat, $P < 0.001$; +0.50 mEq/100 g fat, $P < 0.001$, respectively) and also higher for 3M compared with 2M (+0.25 mEq/100 g fat, $P < 0.001$; +0.27 mEq/100 g fat, $P < 0.001$, respectively; Table 2). Lipoprotein lipase activity was higher for 2M and 3M compared with 1M*6am* and 1M*6pm* (+524 nmol/min per mL, $P < 0.001$) and was also higher for 3M compared with 2M (+142 nmol/min per mL, $P < 0.001$). Milk fat globule diameters measured as $d_{4,3}$ or $d_{3,2}$ were larger with 3M compared with other milking frequencies (+0.40 µm, $P = 0.014$, +0.27 µm, $P = 0.008$, respectively), and MFG area (s) was lower (−0.14 m^2, $P = 0.005$).

Soluble Ca and P were higher for 2M and 3M compared with 1M*6am* and 1M*6pm* (+49 mg/kg, $P = 0.001$; +21 mg/kg, $P = 0.019$, respectively). The content of Na was lower, the content of K was higher, and the Na^+/K^+ ratio was lower for 2M and 3M compared with 1M*6am* and 1M*6pm* (−76 mg/kg, $P = 0.003$; +142 mg/kg, $P < 0.001$; −0.036, $P = 0.002$, respectively). Milk citrate was higher for 2M and 3M compared with 1M*6am* and 1M*6pm* (+0.39 g/L, $P < 0.001$; Table 3).

Milking frequencies had no significant effect on milk FA except a higher *cis*-9-C14:1/C14:0 ratio for 1M*6pm* compared with 1M*6am*, 2M, and 3M (0.015, $P = 0.049$) and a tendency for higher $\sum$ <C16:0 (1.24 percentage units, $P = 0.077$) FA percentage compared with 2M and 3M (Table 4 and Supplemental Table S1, see Notes). Regarding plasma parameters, lactose and urea tended to be higher with 1M6*am* compared with 2M and 3M (+14.7 mg/L, $P = 0.083$; +60.7 mg/L, $P = 0.060$, respectively).

Table 1. Dry matter intake, BW, milk production and composition of cows submitted to different milking frequencies and having different susceptibilities to lipolysis

		Milking frequency				Susceptibility			Effect (*P*-value)		
Item	ATOL[1] ID	1M*6am*	1M*6pm*	2M	3M	NONSUS	SUS	RMSE	Susceptibility	Milking frequency	S × MF[2]
DMI, kg/d	ATOL_0005395	24.9^{c}	25.7^{bc}	26.6^{ac}	27.1^{a}	26.6^{A}	25.5^{B}	1.17	0.019	0.005	0.498
BW, kg	ATOL_0000351	676	—[3]	686	678	691^{A}	669^{B}	13.3	0.002	0.299	0.771
Milk yield, kg/d	ATOL_0001518	26.4^{b}	25.9^{b}	36.2^{a}	37.1^{a}	31.7	31.1	3.82	0.676	<0.001	0.523
Milk fat content, g/kg	ATOL_0001520	46.2^{a}	43.1^{ac}	37.9^{b}	39.9^{bc}	40.1^{B}	43.4^{A}	4.48	0.044	0.007	0.736
Milk fat yield, g/d	ATOL_0000549	1,202^{b}	1,105^{b}	1,346^{a}	1,487^{a}	1,244	1,326	162.8	0.169	0.001	0.934
Milk protein content, g/kg	ATOL_0001521	33.0^{a}	33.4^{a}	31.2^{b}	31.8^{b}	32.1	32.7	0.98	0.101	0.001	0.784
Milk protein yield, g/d	ATOL_0000550	865^{b}	860^{b}	1,122^{a}	1,179^{a}	1,006	1,007	117.8	0.988	<0.001	0.534
Milk lactose content, g/kg	ATOL_0000619	47.8^{b}	48.0^{b}	49.6^{a}	49.0^{ab}	47.8^{B}	49.4^{A}	1.17	0.002	0.022	0.684
Milk lactose yield, g/d	ATOL_0000618	1,269^{b}	1,245^{b}	1,794^{a}	1,816^{a}	1,528	1,535	202.2	0.920	<0.001	0.495
SCS	ATOL_0000991	1.89	1.85	1.47	1.42	1.63	1.68	0.517	0.786	0.166	0.446

$^{a–c}$Means for milking frequency effect in the same row with no common superscript differ ($P < 0.05$).

^{A,B}Means for susceptibility effect in the same row with no common superscript differ ($P < 0.05$).

[1]Traits in reference to Animal Trait Ontology for Livestock (ATOL; https://www.umrh.inrae.fr/ontologies/visualisation/public/).

[2]S × MF = interaction between susceptibility and milking frequency.

[3]For the 1M*6pm* treatment, there is no BW value because the cows were only weighed after morning milking.

Table 2. Milk fat content, free fatty acids, lipolysis, and milk fat globule parameters of cows submitted to different milking frequencies and having different susceptibilities to lipolysis

		Milking frequency				Susceptibility			Effect		
Item	ATOL[1] ID	1M*6am*	1M*6pm*	2M	3M	NONSUS	SUS	RMSE	Susceptibility	Milking frequency	S × MF[2]
Milk fat content T0, g/kg	ATOL_0001520	46.7^{a}	43.8^{ac}	38.4^{b}	40.2^{bc}	40.9	43.7	4.03	0.060	0.002	0.800
FFA T0, mEq/100 g fat	ATOL_0001753	0.23^{a}	0.26^{ab}	0.29^{b}	0.29^{b}	0.29^{A}	0.25^{B}	0.028	0.002	0.001	0.220
Milk fat content T24, g/kg	ATOL_0001520	46.6^{a}	43.6^{ac}	38.5^{b}	40.1^{bc}	40.8	43.6	4.03	0.065	0.003	0.775
FFA T24, mEq/100 g fat	ATOL_0001753	0.38^{c}	0.32^{c}	0.75^{b}	1.00^{a}	0.51^{B}	0.71^{A}	0.200	0.045	<0.001	0.004
Lipolysis, mEq/100 g fat	—	0.14^{c}	0.05^{c}	0.46^{b}	0.73^{a}	0.23^{B}	0.45^{A}	0.189	0.020	<0.001	0.004
Lipoprotein lipase activity, nmol/min per mL	ATOL_0000188	190^{c}	181^{c}	639^{b}	781^{a}	447	449	12.6	0.628	<0.001	0.131
Milk fat globules[3]											
$d_{4,3}$, µm	ATOL_0000729	4.23^{b}	4.09^{b}	4.10^{b}	4.54^{a}	4.24	4.24	0.273	0.944	0.014	0.742
$d_{3,2}$, µm	ATOL_0000729	3.63^{b}	3.50^{b}	3.56^{b}	3.83^{a}	3.63	3.63	0.177	0.930	0.008	0.702
s, m^2	ATOL_0000730	1.81^{a}	1.88^{a}	1.84^{a}	1.70^{b}	1.81	1.81	0.087	0.885	0.005	0.787

$^{a–c}$Means for milking frequency effect in the same row with no common superscript differ ($P < 0.05$).

^{A,B}Means for susceptibility effect in the same row with no common superscript differ ($P < 0.05$).

[1]Traits in reference to Animal Trait Ontology for Livestock (ATOL; https://www.umrh.inrae.fr/ontologies/visualisation/public/).

[2]S × MF = interaction between susceptibility and milking frequency.

[3]$d_{4,3} = \Sigma\left(N_i \times d_i^4\right)/\Sigma\left(N_i \times d_i^3\right)$, with N_i = the number of MFG in diameter class d_i. $d_{3,2} = \Sigma\left(N_i \times d_i^3\right)/\Sigma\left(N_i \times d_i^2\right)$, with N_i = the number of MFG in diameter class d_i. s: milk fat globule area = $6/(\rho \times d_{3,2})$, with ρ = the density of the particle considered (0.92 for fat).

Table 3. Milk mineral composition of cows submitted to different milking frequencies and having different susceptibilities to lipolysis

Item	ATOL[1] ID	Milking frequency				Susceptibility			Effect		
		1M*6am*	1M*6pm*	2M	3M	NONSUS	SUS	RMSE	Susceptibility	Milking frequency	S × MF
Total Ca, mg/kg	ATOL_0000705	1,301	1,357	1,329	1,329	1,315	1,343	59.9	0.262	0.379	0.278
Soluble Ca, mg/kg	ATOL_0000706	331[b]	323[b]	376[a]	376[a]	362[A]	341[B]	28.3	0.042	0.001	0.504
Colloidal Ca, mg/kg	ATOL_0000708	966	1,030	960	955	956	1,000	61.2	0.080	0.121	0.133
Total P, mg/kg	ATOL_0000271	867	836	955	899	854	925	119.5	0.137	0.325	0.503
Soluble P, mg/kg	ATOL_0000272	343	331	353	362	347	347	18.9	0.915	0.019	0.230
Colloidal P, mg/kg	ATOL_0000273	544	513	578	534	522	563	124.9	0.379	0.777	0.853
Na, mg/kg	ATOL_0000624	388[a]	428[a]	327[b]	337[b]	381	359	47.6	0.232	0.003	0.550
K, mg/kg	ATOL_0000623	1,627[b]	1,605[b]	1,750[a]	1,767[a]	1,687	1,687	69.8	1.000	<0.001	0.038
Na/K ratio, mg/mg	—	0.231[b]	0.258[a]	0.202[c]	0.214[b]	0.227	0.226	0.0238	0.881	0.002	0.513
Citrate, g/L	ATOL_0001765	1.56[b]	1.47[b]	1.85[a]	1.97[a]	1.77[A]	1.66[B]	0.145	0.046	<0.001	0.801

[a–c]Means for milking frequency effect in the same row with no common superscript differ ($P < 0.05$).
[A,B]Means for susceptibility effect in the same row with no common superscript differ ($P < 0.05$).
[1]Traits in reference to Animal Trait Ontology for Livestock (ATOL; https://www.umrh.inrae.fr/ontologies/visualisation/public/).

Plasma glucose tended to be lower with 2M compared with 1M*6am* and 3M (−4.2 mg/L, $P = 0.066$; Table 5).

Susceptibility of Dairy Cows to Lipolysis

In the experimental period, SUS dairy cows had a lower DMI (−1.1 kg/d, $P = 0.019$) and also a lower BW (−22 kg, $P = 0.002$) with a similar rank of lactation (2.0 vs. 2.5). NONSUS and SUS dairy cows produced the same quantity of milk, but milk fat and lactose contents were higher in SUS dairy cows milk (+3.3 g/kg, $P = 0.044$ and +1.6 g/kg, $P = 0.002$, respectively; Table 1). Milk FFA measured just after milking were lower in SUS cows (−0.04 Meq/100 g fat, $P = 0.002$), but FFA measured after 24 h of milk storage at 4°C were higher in SUS cows (+0.20 Meq/100 g fat, $P = 0.020$; Table 2). For FFA measured after 24 h of storage at 4°C and lipolysis, we observed a significant interaction between lipolysis susceptibility and milking frequencies ($P = 0.004$). Whether the cows were NONSUS or SUS, the 1M*6am* and 1M*6pm* treatments caused a similar reduction in lipolysis. On the other hand, lipolysis was significantly ($P = 0.004$) higher in SUS cows with 2M and 3M treatments (Table 2). Among milk minerals, only soluble Ca was lower in SUS cows (−21 mg/kg, $P = 0.042$), and colloidal Ca tended to be higher (+44 mg/kg, $P = 0.080$; Table 3). The SUS cow milk contained a lower percentage of ∑ <C16:0 FA (−1.5 percentage units, $P = 0.014$; Table 4). Dairy cow susceptibility to lipolysis had no effect on plasma parameters (Table 5).

To provide a global overview on the impact of milking frequency and lipolysis susceptibility, we performed an RDA to obtain a simultaneous visualization of samples (i.e., dairy cows) and variables (i.e., nonredundant milk content and composition parameters), as shown in Figure 1. The RDA revealed a global statistically significant association between milk parameters and both milking frequency ($F = 5.0021$, $P < 0.001$) and lipolysis susceptibility ($F = 4.2448$, $P < 0.001$). Separation according to milking frequency was explained by the first RDA component, whereas that for lipolysis susceptibility was aligned with the second RDA component. The RDA visualization further highlighted correlations among parameters and their association with each of the first 2 RDA components, with, for example, higher values of lipolysis observed in the 3M milking group as compared with the 1M groups (i.e., arrow nearly parallel to the first RDA component and directed toward the 3M samples) and to a lesser extent in the SUS group compared with the NONSUS group (i.e., arrow directed toward the SUS samples along the second RDA axis). Taken together, this RDA visualization provides a useful overview of the shared trends in univariate results described in Tables 1, 2, 3, 4, and 5 and Supplemental Table S1 (see Notes).

Table 4. Milk fatty acid composition of cows submitted to different milking frequencies and having different susceptibilities to lipolysis

		Milking frequency				Susceptibility			Effect		
Item	ATOL[1] ID	1M*6am*	1M*6pm*	2M	3M	NONSUS	SUS	RMSE	Susceptibility	Milking frequency	S × MF[2]
C16:0, %	ATOL_0000648	34.9	34.4	35.3	35.3	34.7	35.2	1.83	0.519	0.767	0.628
cis-9-C18:1, %	ATOL_0005766	14.7	15.2	14.1	15.0	14.6	14.9	1.65	0.656	0.577	0.085
cis-9-C14:1/C14:0	—	0.091[b]	0.106[a]	0.096[ab]	0.088[b]	0.095	0.095	0.0131	0.881	0.049	0.800
cis-9-C16:1/C16:0	—	0.041	0.048	0.042	0.045	0.044	0.044	0.0067	0.853	0.225	0.374
cis-9-C18:1/C18:0	—	1.79	2.06	1.98	1.83	1.85	1.98	0.249	0.161	0.129	0.530
cis-9, *trans*-11-CLA/*cis*-9-C18:1	—	0.48	0.53	0.49	0.46	0.48	0.50	0.073	0.551	0.297	0.936
∑ <C16:0,[3] %	—	30.9	31.7	31.3	29.6	31.6[A]	30.1[B]	1.53	0.014	0.077	0.078
∑ C16,[4] %	—	37.1	36.8	37.5	37.4	37.0	37.4	1.86	0.636	0.879	0.715
∑ >C18:0,[5] %	—	31.7	31.2	30.8	32.4	31.5	31.6	2.66	0.902	0.646	0.215

[a,b]Means for milking frequency effect in the same row with no common superscript differ ($P < 0.05$).

[A,B]Means for susceptibility effect in the same row with no common superscript differ ($P < 0.05$).

[1]Traits in reference to Animal Trait Ontology for Livestock (ATOL; https://www.umrh.inrae.fr/ontologies/visualisation/public/).

[2]S × MF = interaction between susceptibility and milking frequency.

[3]∑ <C16:0 = sum of FA from C4:0 to C15:0.

[4]∑ C16 = sum of C16:0, *iso*C16:0, *cis*-9-C16:1, *trans*-9-C16:1, and *cis*-7-C16:1.

[5]∑ >C18:0 = sum of FA from C18:0 to C20:4 n-6.

Table 5. Plasma composition of cows submitted to different milking frequencies and having different susceptibilities to lipolysis

		Milking frequency				Susceptibility			Effect		
Item	ATOL[1] ID	1M*6am*	1M*6pm*	2M	3M	NONSUS	SUS	RMSE	Susceptibility	Milking frequency	S × MF[2]
NEFA, µmol/L	VT:0001553	88.8	126.5	94.6	136.2	129.7	93.4	90.58	0.404	0.736	0.784
BHB, µmol/L	VT:0010996	475	599	586	550	551	554	137.5	0.945	0.378	0.898
Cholesterol, mg/L	VT:0000180	1,838	1,731	1,871	1,893	1,803	1,863	194.0	0.444	0.476	0.645
Lactose, mg/L	—	54.3	44.8	40.7	38.5	47.3	41.8	11.30	0.230	0.083	0.397
Glucose, mg/L	ATOL_0000097	79.7	78.0	75.5	79.8	78.1	78.4	2.80	0.803	0.066	0.085
Triglycerides, mg/L	VT:0002644	61.6	70.8	55.5	67.3	66.1	61.5	15.93	0.551	0.386	0.300
Urea, mg/L	VT:0005265	272	230	218	205	229	234	43.9	0.784	0.060	0.381
Acetate, mmol/L	—	0.88	0.94	0.93	0.99	0.95	0.92	0.310	0.833	0.938	0.815
Insulin, µIU/mL	—	16.4	22.1	14.4	17.5	18.8	16.4	7.53	0.407	0.246	0.815
Prolactin, ng/mL	—	35.3	35.8	40.9	33.8	35.7	42.5	11.9	0.604	0.104	0.165

[1]Traits in reference to ontologies: Animal Trait Ontology for Livestock (ATOL; https://www.umrh.inrae.fr/ontologies/visualisation/public/) and Vertebrate Trait Ontology (VT; https://bioportal.bioontology.org/ontologies/VT/?p=summary).

[2]S × MF = interaction between susceptibility and milking frequency.

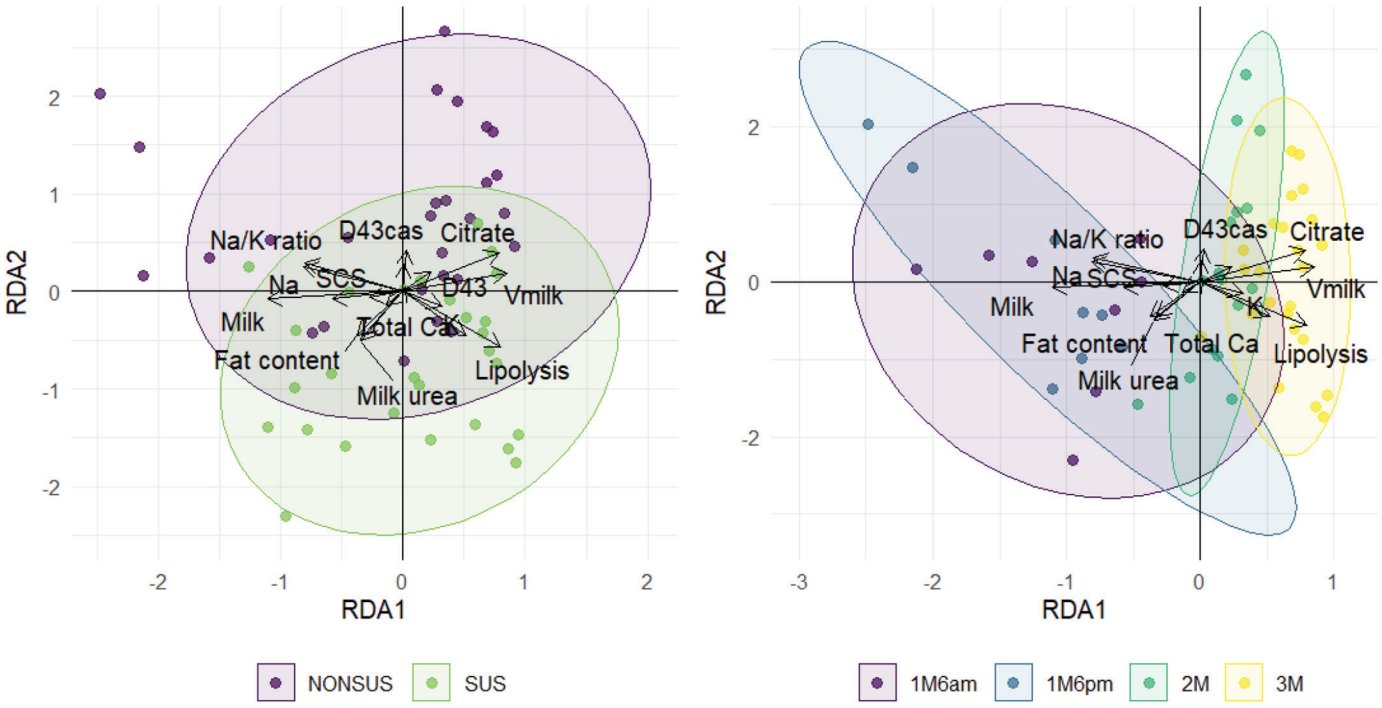

Figure 1. First 2 RDA components (RDA1, RDA2) of sample and variable biplots for nonredundant milk content and composition parameters (milk yield, milk secretion rate [Vmilk], protein content, SCS, lactose, fat content, lipolysis, MFG diameter [D43], mean diameter of casein micelles [D43cas], soluble protein, milk urea, total P, total CA, K, citrate, Na, Na/K ratio), with milking treatment (1M*6am*, 1M*6pm*, 2M, 3M) and lipolysis susceptibility (SUS, NONSUS) as fixed effects. Dairy cow samples are represented by points, arrows correspond to milk content and composition parameters, and ellipses correspond to normal data ellipses based on a multivariate *t*-distribution. In the left plot, samples are colored according to lipolysis susceptibility group (NONSUS = purple; SUS = green). In the right plot, samples are colored according to milking frequency group (1M*6am* = purple; 1M*6pm* = blue; 2M = green; 3M = yellow).

DISCUSSION

Milking Frequency

The results of milk yield and milk, protein, and lactose levels are those classically observed in the case of once-a-day milkings compared with twice-daily milkings: a 27.7% decrease in milk yield, in line with the −20% to 30% cited by Rémond and Pomiès (2005), increased fat and protein contents, and decreased lactose content (Stelwagen et al., 2013). Compared with twice-daily milking, the increase in fat content with 1M6*am* and 1M6*pm* can be explained by a concentration effect caused by reduced milk yield. Decreased milk lactose could be explained by decreased gene expression of key enzymes regulating lactose synthesis (Grala et al., 2011), lower mammary uptake of glucose (Guinard-Flament et al., 2007), or an increased leakiness of tight junctions between adjacent mammary epithelial cells, leading to elevated blood lactose concentrations (Stelwagen, 2001), for which a nonsignificant trend is observed, as well as an increased Na^+/K^+ ratio. Increased milk protein content in once-daily compared with twice-a-day milking may be due to an increase in casein content as in Claesson et al. (1959), Lacy-Hulbert et al. (1999), and Rémond et al. (2004).

Our study also examined the effect of 3 evenly spaced daily milkings, which has rarely been investigated thus far. These 3 daily milkings with 8-h intervals led to a lower increase in milk yield (0.8 kg on average) than previously reported (3.5 kg according to a review by Erdman and Varner, 1995) compared with classical twice-daily milkings. Several parameters could explain this result: one is the 8-h milking interval, which has not been widely reported in the literature (Erdman and Varner, 1995) because it is rarely encountered on farms that practice 3 milkings per day. However, a study (Hart et al., 2013) with 3 milkings per day at 8-h intervals reported a 2.9-kg increase in milk yield compared with 2 milkings per day. Another hypothesis could be linked to insufficient voluntary feed intake, as suggested by the trend toward a reduction in the percentage of refusals with the 3M versus the 2M treatment. The absence of a significant effect of 3M treatment on milk fat, protein, and lactose contents is consistent with the results of Hart et al. (2013).

Lipolysis is reduced with 1M*6am* and 1M*6pm* compared with 2M, in line with a previous study (Rémond and Pomiès, 2005). On dairy farms, once-a-day milking

is practiced, in particular in grassland systems. Although this practice results in a reduction of milk yield, it simplifies the workload while maintaining or even improving milk quality, particularly lipolysis. Indeed, once-a-day milking versus twice-daily milking has been shown to decrease spontaneous milk lipolysis (Vanbergue et al., 2016). This decrease in lipolysis is associated with and may be partly explained by a sharp reduction in the LPL activity (divided by 3.4 in 1M compared with 2M) as also observed by Rémond and Pomiès (2005) with 1 versus 2 milkings per day. Another explanation could be related to MFG size, with the hypothesis that smaller MFG are less sensitive to lipolysis, as suggested by some studies (Wiking et al., 2006; Vanbergue et al., 2018). However, MFG sizes with 1M*6am* and 1M*6pm* were not different from those obtained with 2M, suggesting that size or surface area of the MFG alone do not explain a propensity of milk to lipolysis. The DMI during once-a-day milking decreased slightly, but the energy and nitrogen intakes were largely sufficient to cover maintenance needs and milk yield without the cow having to mobilize its lipid reserves (no significant effect of milking frequency on plasma NEFA).

It is also essential to consider the impact of AMS on lipolysis, especially because it is an increasingly widespread practice. Indeed, many farmers install AMS to reduce their workload and stress. In 2022, 16.3% of French milking systems were AMS, and this percentage continues to rise. The number of milkings per day is higher with AMS and exceeds 2. With respect to the specific effect of 3 milkings per day, lipolysis is higher than for 1 and 2 milkings per day. Increased milking frequency has already been shown to increase FFA content in milk (Klei et al., 1997). One possible explanation is that the milk fat globule membrane composition is affected by the reduced time between milkings for the synthesis of its constituents (phospholipids and proteins). Indeed, in a recent study on dairy cows, we showed an association between milk lipolysis and a specific phospholipid and protein profile of the MFGM that could affect membrane fluidity and thus LPL anchoring (Bernard et al., 2025). The large increase in d_{90} with 3M indicated in Wiking et al. (2006) that larger MFG are synthesized, thus exacerbating their fragility (Wiking et al., 2019) and their accessibility to the action of LPL (Wiking et al., 2003) in favor of increased levels of FFA associated with a more frequent milking (Woodhouse and Kelton, 2023). Unlike Wiking et al. (2006), the synthesis of milk FA from C4:0 to C15:0 was lower with 3M. This result corroborates the previously mentioned hypothesis that with 3M, the quantities of food offered were probably not sufficient.

Overall, from studies on the effect of milking frequency or milking interval on lipolysis, a positive relationship between milk yield and lipolysis has been observed suggesting that low milk yield (and shorter intervals between milking) favors concentration of plasma components that may act as inhibitors of LPL enzyme, as previously demonstrated for the proteose peptone component 3 (Jellema, 1986) as well as MFGM composition (phospholipids and proteins) in favor of its strength.

With 2M treatment, lipolysis was lower in evening milks especially for SUS cows and was associated with higher milk yield. This result is in opposition with that of Ahrné and Björck, (1985) who found that milk obtained at the evening milking is more susceptible to spontaneous lipolysis than that obtained in the morning due to the shorter inter-milking interval before the evening milking, which results in lower milk yield (Suhren et al., 1981). Conversely, in our study, the 2 milkings were spaced with the same time interval (12 h). With the 3M treatment, lipolysis was higher at 2:00 p.m. This result is difficult to explain because it was not associated with a change in milk yield.

Once-a-Day Milking According to Time of Day and Lipolysis

Once-a-day milking is generally carried out in the morning so that the farmer is free of constraints for the rest of the day. However, dairy cows are sensitive to circadian rhythms, in particular the secretion of hormones such as melatonin, which occurs during the night (Teng et al., 2021) and metabolic activities that exhibit distinct periodic variations with the day-night cycle (Kumar Jha et al., 2015; Teng et al., 2021). In our study, neither lipolysis nor LPL activity were affected by the time at which the once-a-day milking was performed (6:00 a.m. vs. 6:00 p.m.; Table 2). It was recently shown that even though milk major composition (fat, protein, lactose, and total milk solids) was not different between day and night milk, small molecules, metabolites and lipids, hormones, and cytokines differed between day and night milk (Teng et al., 2021). These findings may explain differences observed between morning versus evening milkings in the classically used 2 milkings per day. Although crude milk composition and lipolysis were not affected by the milking time, understanding the variability of this factor would requires further studies to investigate other parameters involved in milk lipolysis (e.g., LPL, mRNA, and lipids components of the milk fat globule membrane).

Susceptibility of Dairy Cows to Lipolysis

According to Deeth and Fitz-Gerald (1975), the activator–inhibitor balance largely determines the susceptibility of milk to spontaneous lipolysis. However, other factors may be involved, such as a specific profile of the MFGM constituents that determine its strength. Ber-

nard et al. (2025) analyzed milks from dairy cows with high or low lipolysis coming from the experiment of Hurtaud et al. (2023). In milks with high lipolysis, they found 4 phosphatidylinositols, 8 phosphatidylcholines, 1 sphingomyelin, and 27 proteins; among them, the phosphatidylcholine/phosphatidylethanolamine ratio and ORM1 may contribute to the membrane remodeling of the MFGM. The abundance of CP, CHI3L1, NECTIN2, A2M were strongly positively correlated with high lipolysis. Conversely, 3 phosphatidylinositols, 1 phosphatidylcholine, and 2 phosphatidylethanolamines were assigned to the low lipolysis group. The milk with high lipolysis was associated with a specific MFGM phospholipids and proteins profile, suggesting an impact on membrane fluidity and lipid rafts composition intervening in LPL anchoring and activation, as well as on proinflammatory lipids and proteins.

Cows susceptible to lipolysis eat less, weigh less, and produce as much milk, which tends to have higher fat, lactose, and colloidal Ca contents. On the other hand, milk from susceptible cows contains less citrate and fewer short and medium-chain FA (C6:0, C8:0, C10:0, C14:0, and a trend for C12:0) that are de novo synthesized in the mammary gland. This result is opposite of what is classically observed in the literature, where a negative relationship between citrate content and FA synthesized by the udder has been reported (Garnsworthy et al., 2006; Akkerman et al., 2019). Taken together, these results suggest that cows susceptible to lipolysis may have specific features of their energy metabolism that are associated with greater efficiency, more reserve mobilization, or both (Guinguina et al., 2020). However, no difference was observed in plasma between susceptible and nonsusceptible cows for NEFA content, an indicator of adipose tissue mobilization, or for milk long-chain FA uptake by mammary tissue ($\sum$ >C16:0). The underlying physiological mechanisms of cow susceptibility to lipolysis require further investigation.

We highlighted a statistically significant interaction between the number of milkings per day and the susceptibility of cows to lipolysis, where greater susceptibility was associated with a stronger increase in lipolysis.

CONCLUSIONS

Increased milking frequency results in greater milk yield (e.g., increasing milking from once to 2 or 3 times per day can boost milk yield by up to 30%). However, more frequent milkings can have adverse effects on milk quality with respect to FFA concentrations. In this study, we demonstrated that the higher the milking frequency, the higher the LPL enzyme activity and lipolysis. This is especially true for cows with naturally higher levels of lipolysis in milk (SUS; lipolysis >0.7 mEq/100 g fat), although mechanisms underlying a cow's susceptibility to lipolysis need further investigation. These results are particularly relevant in light of the increased use of AMS where milking frequency is not fully controlled. On the other hand, once-a-day milking largely reduced milk lipolysis regardless of the susceptibility of dairy cows to lipolysis.

NOTES

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Nonstandard abbreviations used: 1M*6am* = 1 milking per day at 6:00 a.m.; 1M*6pm* = 1 milking per day at 6:00 p.m.; 2M = 2 milkings per day at 6:00 a.m. and 6:00 p.m.; 3M = 3 equally spaced milkings per day at 6:00 a.m., 2:00 p.m., and 10:00 p.m.; AMS = automatic milking system; ATOL = Animal Trait Ontology for Livestock; D43 = MFG diameter; D43cas = mean diameter of casein micelles; FA = fatty acid; FFA = free fatty acid; FTIR = Fourier transformed infrared; LPL = lipoprotein lipase; MFG = milk fat globule; MFGM = MFG membrane; NEFA = nonesterified FA; NONSUS = nonsusceptible; RDA = redundancy analysis; SUS = susceptible; Vmilk = milk secretion rate; VT = Vertebrate Trait Ontology.

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