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ABSTRACT

Spontaneous lipolysis results in the breakdown of milk fat by the lipoprotein lipase (EC: 3.1.1.34), an enzyme present in milk. Free fatty acids (**FFAs**) and by-products released in milk during lipolysis can alter both the organoleptic value of milk (off-flavors release) and technological properties of dairy products (decrease in creaming capabilities). Current climate change is having significant impacts on the feeding of grazing animals, with negative consequences on the availability and quality of grass. We and others have demonstrated that dietary restriction increases milk lipolysis in the cow species. However, no data about the impact of feed restriction on milk lipolysis is available in the ewe species. Thus, this paper aims to investigate the effect of feed restriction on milk characteristics with regard to lipolysis values in dairy ewes. Two groups of 24 multiparous Lacaune ewes in mid-lactation received a "non-restricted" control diet (100% of *ad libitum* DM intake) or a "restricted" (**RESTR**) diet (65% of *ad libitum* DM intake) according to a 2 × 2 crossover design. Milk gross composition together with lipolysis analyses were performed. Blood samples were also screened for metabolites or hormone concentrations. The RESTR treatment induced a decrease in milk production (− 21% compared with control treatment) and a modification of the metabolism of dairy ewes characterized by an increase in plasma non-esterified fatty acids (**NEFAs**), which represents the balance between adipose tissue mobilization and the use of NEFA by other tissues (+153%), cholesterol (+17%) and β-hydroxybutyrate (+4 %) levels. As a result, a decrease in BW of dairy ewes was observed (−7%). Feed restriction also resulted in a decrease in milk lipolysis estimated by the milk FFA measured by the copper-soap method (−63 and −62%, respectively, for morning and evening milking) or by the reference Bureau of Dairy Industry method (−51 and −57%, respectively, for morning and evening milking). The decrease in milk spontaneous lipolysis under feed restriction was not associated with a decrease in lipoprotein lipase activity in ewes. These results will be completed with proteomic and lipidomic studies in milk samples to better understand mechanisms initiated in the ewe species specifically with regard to lipolysis in milk.

Implications

Spontaneous lipolysis, i.e. the breakdown of milk fat by the enzyme lipoprotein lipase present in milk, can have an impact on the organoleptic and technological properties of milk and dairy products. We demonstrate here for the first time that lipolysis in milk is lower when ewes are underfed. This observation sharply contrasts to the well-known increase of lipolysis in milk produced by underfed cows. Our study reveals a dramatic species-specific feature with regard to the regulation of lipolysis−related mechanisms in milk. The implications of our findings are discussed with regard to a similar study we performed on the cow.

Introduction

Sheep milk accounts for less than 2% of worldwide dairy production but it may be critical to the economic system of countries from the Mediterranean area displaying mainly a pastoral production system. The ongoing climate change is exerting a substantial influence on the grazing animals' diet, with consequences on both the quantity and quality of milk produced. Therefore, it is impera-

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tive to conduct research aimed at evaluating the impact of limited feed intake on the milk quality of dairy species.

Lipolysis, that is milk fat breakdown by the lipoprotein lipase (**LPL** enzyme; EC: 3.1.1.34), can contribute to change radically the quality of final dairy products and their ability to further processing. Indeed, the release of free fatty acids (**FFAs**) from the milk fat globule (**MFG**) and their subsequent oxidation can alter the organoleptic qualities of milk by the release of non-desired off-flavors. In addition, the release of mono and di-glycerides as the first steps of milk fat breakdown can cause serious problems during milking, such as foaming and plugging in the milking machine, or even in postmilking processes (Deeth, 2006). To maintain consistency of dairy product quality, lipolysis should be measured and monitored continuously, especially in the ewe species whose milk fat, the LPL substrate, has almost twice the fat content of cow's milk (Williams et al., 2009).

Spontaneous lipolysis results from a complex interplay between farming practices, animal physiology and animal genetics. We have previously demonstrated that cows fed a low-energy diet produce milk with higher levels of spontaneous lipolysis (Vanbergue et al., 2018; Hurtaud et al., 2023). In these conditions, feed intake does not meet energy requirements for body maintenance and milk production, which results in negative energy balance and high adipose tissue mobilization as evidenced by a sharp increase in plasma non-esterified fatty acids (**NEFAs**) (Hurtaud et al., 2023).

Although the effect of feed restriction is well characterized in the cow and to a lesser extent in goats (Chilliard et al., 2014), to the best of our knowledge, nothing is known about the impact of a restricted diet with regard to milk lipolysis in the ewe species. The objective of the present study was therefore to investigate the effect of feed restriction on global milk performances and overall metabolism in dairy ewes, with special regard to lipolysis. Moreover, because in the cow species lipolysis values are dependent upon the milking time (morning vs evening), we wondered whether this was also true in the sheep species. Results are discussed in the light of the specific composition of ewe milk and related dairy products.

Material and methods

Animals and experimental design

The experiment was conducted at the INRAE *La Fage* Experimental Farm (*Causse du Larzac*, F-12250 Saint-Jean Saint-Paul, France; https://doi.org/10.15454/1.548325523466425E12). The study used 48 multiparous Lacaune ewes in mid-lactation according to a crossover design experiment with the main factor "level of feeding". At the beginning of the experimental period, on average, days in milk were 102 ± 2.0 d (mean ± SD), ewes produced 2.6 ± 0.5 L (mean ± SD) of milk/d characterized by 6.58 ± 0.92% (mean ± SD) fat content and 5.22 ± 0.38% (SD) protein content. Their BW was 76.0 ± 10.1 kg (mean ± SD), and the lactation rank was 4 ± 1 (mean ± SD). Two dietary treatments were used differing by the level of feeding. Ewes were allocated to two groups of 24 animals according to the following criteria and in this order: number of lambs (1 or 2, or 3), lactation stage, lactation rank, milk yield, milk fat and protein contents, somatic cell count (**SCC**), and BW. Two levels of feeding were applied: "non-restricted" (**NON RESTR**) with ewes fed at 100% of *ad libitum* DM intake and "restricted" (**RESTR**) with ewes fed at 65% of *ad libitum* DM intake. In March 2021, the trial was conducted during 2 weeks split into two 1-week periods. During period 1, each group of ewes received a grass–silage-based diet at 100% of *ad libitum* DM intake, or a grass–silage-based diet at 65% of *ad libitum* DM intake. During period 2, the level of feeding was reversed.

Treatments and feeding

Ingredients, chemical composition and nutritional value of the diets are given in Supplementary Tables S1 and S2. All the ewes received a diet consisting of 76% forage (37% grass silage and 39% alfalfa hay) and 24% concentrate (20% barley and 4% Fortolis), and 14 g of minerals. All ewes were fed with this diet *ad libitum* for 4 weeks prior to a 1-week pre-experimental period. During the *ad libitum* period, the food distribution was adjusted for each ewe to an allowance rate of 115% of the previous day's voluntary intake. The individual DM intakes were measured in order to calculate the individual 100% of *ad libitum* DM intake (the calculation of the *ad libitum* DM intake was performed during 4 weeks prior to the1-week pre-experiment). Ewes allocated to the RESTR treatment were given a 3-day transition period to switch to the restricted diet for 4 days. For the second experimental period, the level of feeding was reversed for 4 days following a 3-day transition period. Ewes allocated to the NON RESTR treatment were fed at 100% of their *ad libitum* DM intake (Fig. 1). Diets were formulated to meet energy and protein requirements when distributed at 100% of *ad libitum* DM intake (Institut National de la Recherche Agronomique, 2018).

Measures, sample collection and laboratory analysis

Feeds and refusals

The ewes were housed in sheepfolds, in a pen of 48 on straw-bedding and had permanent access to fresh water. Ewes had access

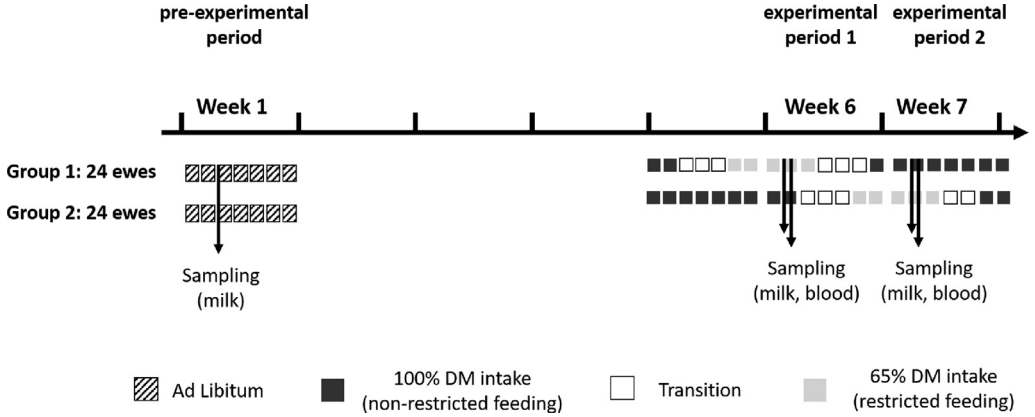

Fig. 1. Description of the experimental protocol for dairy ewes (n = 48).

to an individual feeding postcontrolled by an electronic device that allows each animal to get into its right place using individual electronic identification. Milking took place in the morning at 0800 h., during which time the feed was distributed and the ewes had access to individual troughs when they returned from milking between 0830 and 0900 h. Evening milking took place at 1700 h., with access to feed at around 1730 h. Refusals were collected and weighed daily to evaluate each ewe's DM intake. Throughout the experiment, the methods to calculate DM intake and diet chemical and nutritional composition (DM, mineral matter, CP, NDF, ADF, starch, organic matter digestibility, phosphorus, calcium, fat) of grass silage, alfalfa hay, and energy concentrate were the same as in Vanbergue et al. (2018).

Milk yield and traits

Ewes were milked every day at 0800 and 1730 h at the milking parlor, and milk yield was recorded individually at each milking. Milk fat, protein contents and SCC were determined from two consecutive milkings every week. These analyses were performed by mid-IR spectrometry (MilkoScan™ FT + spectrometer, Foss, Hillerød, Denmark) for fat, and protein contents and by flow cytometry for SCC, all at the LIAL dairy laboratory (Aurillac, France).

Milk for lipolysis, fatty acid profile, MFG size and milk protein and mineral composition determinations was collected from the same milking. Milk samples were individually collected on the total milking recovered from morning and evening milkings at the end of the pre-experimental period and at the end of each experimental period (one day) and then stored at −20 °C for analysis of FA composition. Milk LPL (EC 3.1.1.34) activity was measured on morning milking as described in Bernard et al. (2005).

Milk lipolysis

Two vials with colorless bronopol (Merck, Darmstadt, Germany) per ewe were collected for the measure of FFA contents, an indicator of milk lipolysis, and kept at 4 °C. FFA analyses were performed on both samples by the copper-soap method (Shipe et al., 1980) and by ISO/TS 22113 standard (Bureau of Dairy Industry (**BDI**) method; Actalia, Poligny, France).

Milk fat globule and casein size

A milk vial per ewe was collected and kept at room temperature with bronopol (Merck, Darmstadt, Germany) for further evaluation of MFG size distribution by laser light scattering (Mastersizer 3000, Malvern, UK) (Hurtaud et al., 2023). The milk was skimmed by two successive centrifugations which removed the fat. The mean diameter $d_{4,3} = \Sigma (N_i x d_i^4) / \Sigma (N_i x d_i^3)$ (with N_i the number of particles in diameter class d_i) of the casein micelles was measured with the Mastersizer 3000.

Milk fatty acid composition

Two milk vials per ewe were collected and stored at −20 °C to perform the milk FA analysis. Samples of morning and evening milk samples were freeze-dried and pooled according to the calculated mean milk fat yield at each milking (60/40) to create a representative 100-mg sample. The fatty acid composition was measured as previously described (Fougere et al., 2018).

Milk nitrogen and minerals

Milk samples (250 mL) were collected per ewe and stored −20 °C for total nitrogen, non-protein nitrogen, non-casein nitrogen, casein and urea analysis determined according to the Kjeldahl methods described by (Alais, 1984). Total and soluble calcium were analyzed analysis by inductively coupled plasma optical emission spectroscopy (ICP-OES 5110 Agilent Technology, Les Ulis, France) on milk and milk ultrafiltrate, respectively, as previously described (Hurtaud et al., 2023). Total and soluble phosphorus contents were determined using a KONE PRO multiparameter analyzer (ThermoFisher Scientific, Illkirch, France) by the Allen method for phosphorus (Pien, 1969).

Plasma metabolites and hormones

Jugular blood samples were collected using 5 mL heparinized tubes (VT-050SHL, Venoject, Terumo Europe, Leuven, Belgium) at the end of each experimental period. Blood sampling took place in the morning after milking and before feeding. After centrifugation of blood and storage of plasma at −20 °C until analysis (Hurtaud et al., 2023), plasma glucose, urea, acetate, NEFA, triglycerides, cholesterol, lactose and β-hydroxybutyrate contents were assayed using colorimetric enzymatic reactions on 2 replicates as reported in Delamaire and Guinard-Flament (2006). Plasma insulin (lowest limit of quantification = 2.34 μUI/mL, CV intraassay: 4.5% (39 μUI/mL), 27% (4.7 μUI/mL), CV interassay: 11% (39 μUI/mL), 26% (4.7 μUI/mL)) and IGF-1 (lowest limit of quantification = 0.6 ng/mL, CV intraassay: 9% (206 ng/mL), 10% (63 ng/mL), CV interassay: 6.3% (206 ng/mL), 5% (63 ng/mL)) concentrations were determined by radioimmunoassays as in Hurtaud et al. (2023). Plasma concentrations of prolactin (limit of detection: 0.89 ng/mL, CV intraassay: 4.6%, CV interassay CV: 2.8%) were assessed using the method described by Herve et al. (2019).

Calculations and statistical analyses

The experimental design was a crossover design with two 1-week subsequent periods with the main factor being the feeding level. Effects of feeding level were evaluated on daily values for DM intakes, energy requirements and balance, BW, milk traits, and plasma parameters. For milk traits, daily values of milk composition were obtained by calculating the average of the morning and evening values weighted by milk yields of each milking. Effects of milking time (morning vs evening), type of feeding level and their interactions were evaluated on twice-daily values for lipolysis, MFG size, protein and mineral composition. The statistical model was a mixed model including "group of ewes" (as described above in the paragraph "Animals and experimental design"), "feeding level", "milking time", and the interaction between "milking time" and "feeding level" as fixed effects, ewes within a group as a random effect, and a covariable corresponding to the value of the variable Y_{ijkl} during the pre-experiment period ($CovY_{ijkl}$):

$$Y_{ijkl} = \mu + \text{Group}_i + \text{Milking Time}_j + \text{Period}_k + \text{Feeding level}_l + \text{Milking Time}_j \times \text{Feeding level}_l + CovY_{ijkl} + \varepsilon_{ijkl}$$

with the effects defined above.

The statistical significance threshold was set at $P < 0.05$. Trend was set at $P < 0.10$.

All statistical analyses on the dataset for the 48 ewes were performed using the MIXED procedure of SAS software (SAS 9.2 Institute Inc., Cary, NC). The statistical scripts and methods described here have all been validated and published (Hurtaud et al., 2023).

Results

Intake, energy balance and milk gross composition

By design, feed restriction induced a decrease in DM intake (−0.9 kg/d), net energy for lactation (−1.9 MJ/d), and BW (−5.5 kg) (Table 1). Detailed composition of experimental diets is given in Supplementary Tables S1 and S2. RESTR treatment caused a decrease in milk yield (−0.41 L/d) whereas milk fat and protein contents slightly increased (+5.3 and + 1.0 g/L, respectively;

Table 1
BW, DM intake, and energy balance based on the non-restricted (NON RESTR) and restricted (RESTR) feeding treatments for dairy ewes (n = 48).

Characteristic	ATOL[1] id	Feeding		SEM	*P*	
		NON RESTR	RESTR		Group	Feeding
BW, kg	ATOL_0000351	74.9	69.4	1.41	0.6312	<0.0001
DM intake, kg/d	ATOL_0005395	2.98	2.08	0.060	0.0656	<0.0001
Total net energy requirements for maintenance and lactation[2], MJ/d		16.46	14.58	0.309	0.0279	<0.0001
Net energy for lactation[2], MJ/d	ATOL_0002559	10.03	8.12	0.272	0.0142	<0.0001

Abbreviations: NON RESTR = non-restricted feeding treatment; RESTR = restricted feeding treatment.
[1] Traits in reference to ATOL: Animal Trait Ontology for Livestock, https://www.atol-ontology.com/en/erter-2/.
[2] according to INRA, 2018.

Table 2
Milk yield and composition based on non-restricted (NON RESTR) and restricted (RESTR) feeding treatments for dairy ewes (n = 48).

Characteristic	ATOL[1] id	Feeding		SEM	*P*	
		NON RESTR	RESTR		Group	Feeding
Milk yield, L/d	ATOL_0001518	1.92	1.51	0.056	0.1126	<0.0001
Milk fat content, g/L	ATOL_0001520	78.6	83.9	1.63	0.1987	<0.0001
Milk fat yield, g/d	ATOL_0000549	149.7	125.0	4.333	0.0194	<0.0001
Milk protein content, g/L	ATOL_0001521	58.8	59.8	0.78	0.0202	0.0254
Milk protein yield, g/d	ATOL_0000550	112.4	89.37	3.0284	0.0111	<0.0001
SCC, $\times 10^3$/mL	ATOL_0000991	259.1	574.08	237.31	0.3182	0.3324

Abbreviations: NON RESTR = non-restricted feeding treatment; RESTR = restricted feeding treatment; SCC = somatic cell count.
[1] Traits in reference to ATOL: Animal Trait Ontology for Livestock, https://www.atol-ontology.com/en/erter-2/.

Table 3
Milk yield and composition (fat, protein, CP, true protein, casein, soluble protein, casein micelle diameter and minerals) based on non-restricted (NON RESTR) and restricted (RESTR) feeding treatments for dairy ewes and milk on the sampling day (n = 48).

Characteristic	ATOL[1] id	Milking	Feeding		SEM	Group	*P*		
			NON RESTR	RESTR			Feeding	Milking	F*M[2]
Milk yield, L	ATOL_0001518	Morning	1.18	0.93	0.117	0.121	<0.001	<0.001	0.007
		Evening	0.74	0.58					
Milk fat content, g/L	ATOL_0001520	Morning	73.8	78.2	5.23	0.226	<0.001	<0.001	0.151
		Evening	86.4	93.0					
Milk protein content, g/L	ATOL_0001521	Morning	58.9	60.7	1.99	0.023	0.009	<0.001	<0.001
		Evening	58.5	58.2					
SCC, $\times 10^3$/mL	ATOL_0000991	Morning	227	432	1693.2	0.292	0.126	0.269	0.483
		Evening	326	875					
CP content, g/kg	ATOL_0000617	Morning	60.1	61.7	1.91	0.016	0.012	0.006	0.003
		Evening	60.2	60.1					
Protein content, g/kg	ATOL_0001521	Morning	57.8	59.5	1.88	0.037	<0.001	0.002	0.003
		Evening	57.5	57.5					
Non-protein nitrogen, g/kg	ATOL_0000251	Morning	2.36	2.14	0.144	0.239	<0.001	<0.001	0.887
		Evening	2.48	2.26					
Urea, mg/L	ATOL_0000727	Morning	518	431	35.7	0.026	<0.001	<0.001	0.009
		Evening	566	506					
Soluble protein content, g/kg	ATOL_0001566	Morning	12.3	13.2	0.874	0.040	<0.001	0.010	0.471
		Evening	12.1	12.8					
Casein content, g/kg	ATOL_0000612	Morning	45.6	46.4	1.68	0.027	0.714	<0.001	0.003
		Evening	45.3	44.6					
Casein/protein ratio, %	/	Morning	78.6	77.8	1.20	0.381	<0.001	0.470	0.372
		Evening	78.9	77.8					
Casein micelle diameter, nm	ATOL_0000723	Morning	140	148	10.0	0.007	<0.001	<0.001	0.075
		Evening	145	159					
Total calcium content, mg/kg	ATOL_0000705	Morning	1909	1932	77.6	0.463	0.297	0.077	0.335
		Evening	1942	1943					
Soluble calcium content, mg/kg	ATOL_0000706	Morning	374	364	23.1	0.040	0.119	0.002	0.122
		Evening	380	380					
Colloidal calcium content, mg/kg	ATOL_0000708	Morning	1538	1571	74.7	0.659	0.122	0.681	0.139
		evening	1559	1560					
Total phosphorus content, mg/kg	ATOL_0000271	Morning	1197	1213	4.0	0.587	0.919	0.215	0.334
		Evening	1191	1172					
Soluble phosphorus content, mg/kg	ATOL_0000272	Morning	315	313	0.82	0.594	0.151	0.037	0.315
		Evening	311	301					
Colloidal phosphorus content, mg/kg	ATOL_0000273	Morning	880	898	4.0	0.305	0.853	0.496	0.446
		Evening	882	871					

Abbreviations: NON RESTR = non-restricted feeding treatment; RESTR = restricted feeding treatment; SCC = somatic cell count.
[1] Traits in reference to ATOL: Animal Trait Ontology for Livestock, https://www.atol-ontology.com/en/erter-2/.
[2] Feeding * Milking.

Table 4
Plasma metabolites and hormone concentrations based on non-restricted (NON RESTR) and restricted (RESTR) feeding treatments for dairy ewes (n = 48).

Characteristic	Ontology[1] id	Feeding		SEM	*P*	
		NON RESTR	RESTR		Group	Feeding
Acetate, mmol/L	/	0.899	0.702	0.0205	0.0012	<0.0001
Non-esterified fatty acids, µmol/L	VT:0001553	183	464	18.9	0.1951	<0.0001
β-hydroxybutyrate, µmol/L	VT:0010996	530	550	13.5	0.6984	0.0466
Glucose, mg/L	ATOL_0000097	605	590	7.1	0.6426	0.0527
Lactose, mg/L	/	36.6	30.6	1.33	0.2149	0.0006
Triglycerides, mg/L	VT:0002644	108	111	4.59	0.3181	0.6707
Cholesterol, mg/L	VT:0000180	767	900	28.90	0.943	<0.0001
Urea, mg/L	VT:0005265	596	503	12.384	0.9248	<0.0001
Insulin, µUI/mL	VT:0001560	14.33	13.06	0.969	0.0071	0.2866
IGF-1, ng/mL	ATOL_0000990	285	284	7.721	0.8125	0.9418
Prolactin, ng/mL	ATOL_0001699	127	164	8.873	0.7046	<0.0001

Abbreviations: NON RESTR = non-restricted feeding treatment; RESTR = restricted feeding treatment.
[1] Traits in reference to ontologies: ATOL (Animal Trait Ontology for Livestock, https://www.atol-ontology.com/en/erter-2/) and VT (Vertebrate Trait ontology, https://bioportal.bioontology.org/ontologies/VT/?p=summary).

Table 2) as a probable consequence of the concentration effect due to the decline in milk production. Feed restriction did not affect significantly milk SCC (Table 2). No effect of feeding was observed on milk calcium and phosphorus contents, either in their soluble or colloidal forms (i.e. associated to casein micelles) (Table 3). The diameter of casein micelles in ewe milk increased upon feed restriction, especially in evening milking (+14 ηm, $P < 0.001$) suggesting that rearrangements occurred with regard to the colloidal fraction of milk (Table 3).

Regarding the milking effect, as compared with milk from morning milking, milk from the evening milking is lower in protein content (−0.4 and – 2.5 g/L, respectively, for NON RESTR and RESTR treatments; Table 3) due to a decrease in casein content (−0.3 and −1.8 g/kg, respectively, for NON RESTR and RESTR treatments) and soluble proteins (−0.2 and −0.4 g/kg, respectively, for NON RESTR and RESTR treatments), associated with higher casein micelle diameter (+5 and + 11 ηm, respectively, for NON RESTR and RESTR treatments; Table 3).

The RESTR treatment changed the metabolism of dairy ewes (Table 4), as indicated by the sharp increase in levels of cholesterol and plasma NEFA, which represents the balance between adipose tissue mobilization and the use of NEFA by other tissues (+133 mg/L and + 281 µmol/L, respectively; $P < 0.001$). Similarly, under feed restriction, milk FAs from de novo mammary synthesis (FA < C16) decreased, whereas long-chain FA taken up by the udder (FA > C16) increased, resulting mainly in a specific increase in milk cis9 C18:1 (+7.39% total FA; Table 5 and Supplementary Table S3). Under feed restriction, a sharp increase of prolactin levels in plasma was observed (+37 ng/mL, $P < 0.001$; Table 4).

Milk fat characteristics, lipolysis and milk lipoprotein lipase enzyme activity

The RESTR treatment caused a decrease in milk lipolysis estimated by the milk FFA measured by the copper-soap method (−0.25 and −0.47 mEq / 100 g of fat, $P < 0.001$, respectively, for morning and evening milking) or by the reference BDI method (−0.27 and −0.47 mEq / 100 g of fat, $P < 0.01$, respectively, for morning and evening milking) (Table 6). In a general way, data obtained by the copper soap method were well correlated with data gained with the reference BDI method, although they were underestimated ($R^2 = 0.94$; data not shown). On the whole, lipolysis was higher in evening milk regardless of the method used for analyses (copper soap or BDI; Table 6). The LPL enzyme activity in milk was not affected by feed restriction (Table 6). The decrease in milk lipolysis was associated with an increase in MFG diameter especially in evening milks (+0.21 µm) where lipolysis was higher, whatever the feeding level considered (Table 6).

The detailed composition of milk FA depending on the feed restriction level is given in Table 5. As we mentioned above, the milk FA rearrangements within the triglyceride core clearly reflected a decrease in lipogenesis-related mechanisms and an increase in adipose tissue mobilization upon feed restriction.

Discussion

Effect of feeding restriction on lipolysis

In the next decades, because of climate change, dairy ruminants will need to cope with prolonged droughts, reducing the amount of forage available. Consequently, studies need to be performed to assess the consequences of feed restriction on the quality of milk in dairy species. Numerous and recent studies have been published on the effects of breeding factors such as feed level on milk lipolysis in dairy cattle (Vanbergue et al., 2018; Hurtaud et al., 2023), and to a lesser extent, in dairy goats (Chilliard et al., 2003; Dønnem et al., 2011; Eknæs and Skeie, 2006). To our knowledge, no similar data has been reported for the ewe species. We therefore describe here for the first time the effect of feed restriction on the milk lipolysis in dairy ewes. Our results on dairy ewes show a response that contrasts sharply with that obtained in cows fed at 65% of *ad libitum* DM intake. Indeed, whereas a feed restriction in the cow species dramatically increases the lipolysis in milk (Hurtaud et al., 2023), fed−restricted ewes produce milk with lower values of lipolysis. This means that underfed dairy ewes produce milk with lower quantities of FFA, which is the opposite to what happens in cows displaying a negative energy balance. This result needs to be confirmed using breeds of ewe other than Lacaune (the one used in our study), because the breed may represent a key factor influencing the levels of FFA in the milk, whatever the species is (Vanbergue et al., 2017). Indeed, several studies have shown a link between cow's breed and lipolysis with lower susceptibility for more rustic, less productive breeds (Bachman et al., 1988; Ferlay et al., 2006; Vanbergue et al., 2017).

Spontaneous lipolysis, that is the degradation of milk fat by the lipoprotein lipase enzyme, could impact both the organoleptic properties (taste defects, particularly rancid tastes caused by the oxidation of FFA in milk) and the technological properties of milk (creaming, foaming capabilities) (Deeth, 2006). Depending on the dairy product, too much (or too little) lipolysis may represent a default quality. Thus, lipolysis must be carefully monitored and controlled. This is particularly important in the ewe species where

Table 5
Milk fatty acid composition based on non-restricted (NON RESTR) and restricted (RESTR) feeding treatments for dairy ewes (n = 48).

Fatty acids (% total fatty acids)	ATOL[1] id	Feeding		SEM	*P*	
		NON RESTR	RESTR		Group	Feeding
C4:0	ATOL_0000638	2.75	2.60	0.044	0.0982	0.0004
C6:0	ATOL_0000640	2.67	2.29	0.028	0.7565	<0.0001
C8:0	ATOL_0000642	2.63	2.17	0.039	0.3076	<0.0001
C10:0	ATOL_0000644	8.75	6.64	0.150	0.1611	<0.0001
cis-9-C10:1	ATOL_0005629	0.31	0.26	0.008	0.9246	<0.0001
C12-0	ATOL_0000646	5.63	4.11	0.114	0.153	<0.0001
cis-9-C12:1	ATOL_0005628	0.23	0.16	0.007	0.3428	<0.0001
iso C14:0	ATOL_0000254	0.13	0.12	0.003	0.5035	0.0001
C14:0	ATOL_0000647	13.70	11.63	0.156	0.2305	<0.0001
iso C15:0	ATOL_0000256	0.28	0.28	0.005	0.1981	0.1405
anteiso C15:0	ATOL_0000257	0.42	0.38	0.008	0.2559	<0.0001
cis-9-C14:1	ATOL_0005627	0.29	0.24	0.008	0.6841	<0.0001
C15:0	ATOL_0000255	1.23	1.02	0.017	0.456	<0.0001
Σ <C16	/	39.45	32.17	0.432	0.1995	<0.0001
ΣC16	/	29.74	27.21	0.256	0.168	<0.0001
iso C16:0	ATOL_0000258	0.29	0.27	0.006	0.6939	0.0058
C16:0	ATOL_0000648	28.05	25.39	0.253	0.172	<0.0001
(iso C17:0) + trans-9-C16:1	ATOL_0000259	0.34	0.44	0.006	0.2787	<0.0001
anteiso C17:0	ATOL_0000260	0.28	0.36	0.006	0.9724	<0.0001
cis-9-C16:1	ATOL_0000702	0.92	0.89	0.019	0.473	0.0881
C17:0	ATOL_0000649	0.76	0.88	0.008	0.59	<0.0001
cis-9-C17:1	ATOL_0000660	0.22	0.32	0.007	0.3346	<0.0001
Σ>C18:0	/	30.43	40.01	0.456	0.662	<0.0001
ΣC18	/	27.47	36.67	0.448	0.496	<0.0001
C18:0 + cis-9-C18:1	/	21.83	30.01	0.429	0.2446	<0.0001
ΣC18:1	/	17.37	24.93	0.407	0.2468	<0.0001
ΣC18:2	/	1.94	2.42	0.034	0.0277	<0.0001
ΣC18:1 trans	/	2.09	2.26	0.031	0.0202	<0.0001
ΣC18:1 cis	/	15.28	22.67	0.401	0.1694	<0.0001
C18:0	ATOL_0000650	7.39	8.42	0.166	0.8418	<0.0001
trans-10-C18:1	ATOL_0000666	0.17	0.17	0.004	0.7521	0.5806
trans-11-C18:1	ATOL_0000661	0.99	1.14	0.019	0.0216	<0.0001
trans-12 + cis-6-C18:1	/	0.15	0.16	0.003	0.0677	0.0168
cis-9 C18:1	/	14.44	21.60	0.393	0.1532	<0.0001
cis-11-C18:1	ATOL_0000668	0.31	0.45	0.008	0.9457	<0.0001
cis-12-C18:1	ATOL_0000669	0.12	0.13	0.004	0.428	0.4357
cis-15-C18:1 + C19:0	ATOL_0000671	0.18	0.21	0.005	0.0392	0.0001
cis-9,trans-14-C18:2	ATOL_0005630	0.088	0.12	0.006	0.3426	0.0003
cis-9,cis-12-C18:2	ATOL_0000657	1.52	1.90	0.027	0.0633	<0.0001
C18:3 (n-3)	ATOL_0000699	0.77	0.91	0.015	0.025	<0.0001
cis-9,trans-11 CLA (+trans-7,cis-9 + trans-8,cis-10 CLA)	ATOL_0000657	0.60	0.75	0.016	0.0856	<0.0001
Σ≥C20:0	/	0.95	1.08	0.026	0.2322	<0.0001
cis-9 C14:1/C14:0	/	0.021	0.021	0.0006	0.8566	0.7578
cis-9 C16:1/C16:0	/	0.033	0.035	0.0007	0.8788	<0.0001
cis-9 C18:1/C18:0	/	1.97	2.62	0.059	0.3887	<0.0001
cis-9 trans-11 CLA/trans-11 C18:1	/	0.61	0.66	0.013	0.8541	<0.0001
ΣSFA	/	76.01	67.49	0.423	0.4556	<0.0001
ΣMUFA	/	19.84	27.48	0.410	0.2599	<0.0001
ΣPUFA	/	3.62	4.50	0.067	0.0310	<0.0001

Abbreviations: CLA = Conjugated Linoleic Acids; NON RESTR = non-restricted feeding treatment; RESTR = restricted feeding treatment; MUFAs = MonoUnsaturated Fatty Acids; PUFAs = PolyUnsaturated Fatty Acids; SFAs = Saturated Fatty Acids.
[1] Traits in reference to ATOL: Animal Trait Ontology for Livestock, https://www.atol-ontology.com/en/erter-2/.

milk fat is almost double the rate in cows and whose milk is increasingly marketed as UHT (Ultra-High Temperature) milk or yoghurt where the rancid flavor caused by lipolysis is not desired by the consumer.

Ewe milk is mostly processed into cheese worldwide. The organoleptic value of cheese is therefore more depending on the microbial lipolysis and proteolysis rather than the spontaneous lipolysis due to the native LPL enzyme. However, the FA profiles between milk and cheese are preserved and any change in the quality of the milk fat is directly reflected in the cheese (Coppa et al., 2011). We have demonstrated here that a restricted diet lowers the lipolysis levels in ewe milk. Feed restriction is expected to occur more frequently in the next years, because of an increased drought as a consequence of climate change, which can lead to a reduction in the quantity and/or quality of food available. This is of major importance in the Mediterranean region, where sheep's milk is particularly significant for the local economy. Thus, our research is of particular interest for sheep farmers and sheep's cheese producers, since a restricted diet in the ewe species may impact not only the quantity of the milk produced (over 20% less milk produced when ewes were fed at 65% of *ad libitum* DM intake) but also the quality of milk, especially with regard to milk fat (lipolysis rate reduced by 50% in milk from underfed ewes). This may impact the quality of sheep dairy products, depending on the type of cheese produced and consumer expectations, which may differ according to dietary habits from one country to another.

Another key point to notice is that the decreased lipolysis in milk produced from underfed ewes cannot be explained- solely or even partially- by a reduced activity of the LPL enzyme. We did not observe any modulation of lipoprotein lipase activity in

Table 6
Milk fat characteristics (spontaneous lipolysis, milk fat globule diameter and lipoprotein lipase activity only measured for morning milk) based on non-restricted (NON RESTR) and restricted (RESTR) feeding treatments for dairy ewes on the sampling day (n = 48).

Characteristic	ATOL[1] id	Milking	Feeding			*P*			
			NON RESTR	RESTR	SEM	Group	Feeding	Milking	F*M[2]
Lipolysis, mEq/100 g fat (copper soap method)	/	morning	0.40	0.15	0.36	0.144	<0.001	<0.001	0.036
		evening	0.76	0.29					
Lipolysis, mEq/100 g fat (BDI method)	/	morning	0.53	0.26	0.45	0.137	<0.001	0.002	0.146
		evening	0.83	0.36					
Lipoprotein lipase activity, ηmol/min per mL	ATOL_0000188	morning	452.6	451.6	1.805	0.013	0.512	nd[3]	nd[3]
		evening	nd[3]	nd[3]					
Milk fat content, g/L	ATOL_0001520	morning	73.8	78.2	5.23	0.226	<0.001	<0.001	0.151
		evening	86.4	93.0					
Milk fat globule diameter ($d_{4,3}$[4]), µm	ATOL_0000729	morning	5.30	5.35	0.226	0.818	<0.001	<0.001	0.021
		evening	5.06	5.27					
Milk fat globule diameter ($d_{3,2}$[5]), µm	ATOL_0000729	morning	4.40	4.47	0.242	0.208	0.006	<0.001	0.377
		evening	4.24	4.38					
Milk fat globule area (s[6]), m^2	ATOL_0000730	morning	1.50	1.48	0.072	0.163	0.003	<0.001	0.325
		evening	1.55	1.51					

Abbreviations: BDI = Bureau of Dairy Industry (reference method ISO/TS 22113 standard); NON RESTR = non-restricted feeding treatment; RESTR = restricted feeding treatment.
[1] Traits in reference to ATOL: Animal Trait Ontology for Livestock, https://www.atol-ontology.com/en/erter-2/.
[2] Feeding * Milking.
[3] nd: not determined.
[4] $d_{4,3} = \Sigma(N_i \times d_i^4)/\Sigma(N_i \times d_i^3)$.
[5] $d_{3,2} = \Sigma(N_i \times d_i^3)/\Sigma(N_i \times d_i^2)$.
[6] $s = 6/(\rho \times d_{3,2})$ (with N_i the number of milk fat globules in diameter class d_i and ρ the density of the particle considered (0.92 for fat)).

milk from ewes fed a diet reduced by 65% compared with milk from ewes fed the *ad libitum* diet. This observation contrasts with what we observed in a previous experiment in cows under similar dietary conditions, where increased milk lipolysis value was positively correlated to milk LPL activity (Hurtaud et al., 2023). This may suggest that the lipolytic system – constituted by the lipoprotein lipase, the fat globule (its substrate), and potential regulators in milk – differs markedly between species. As a hypothesis, regulators in milk such as proteins may act to lower lipolysis–related mechanisms following the diet restriction in dairy ewes. For example, we have demonstrated that three proteins (HID1, SURF4 and CUL9) are putative inhibitors of the lipolytic process in cow milk (Delosière et al., 2023). Otherwise, previous studies demonstrate that, in the cow species, the LPL enzyme is mostly associated with casein micelles whereas in the goat, the LPL enzyme is more associated with the MFG (Chilliard et al., 2003) whereas no data are available in ewes. Because the increase of milk lipolysis following feed restriction in cattle is associated with larger MFG in the milk (Hurtaud et al., 2023) while the decrease of lipolysis in milk produced by underfed ewes is associated with larger MFG as well (our study), we can therefore conclude that the size of fat globules cannot be an indicator of the lipolysis level in milk, whatever the species is. The same is true for the casein micelle diameter.

Effect of feeding restriction on milk production and metabolism in ewes

Under feed restriction, milk yield decreased whereas milk fat and protein contents increased conversely to previous data in dairy cows under restriction (Vanbergue et al., 2018; Hurtaud et al., 2023). Otherwise, a specific increase in milk cis9 C18:1, an indicator of adipose tissue mobilization observed when the energy balance is negative (Chilliard, 1987), and of prolactin levels demonstrates physiological and hormonal regulations to sustain the milk synthesis activity. This result is in accordance with the observed increased plasma NEFA. The increase in milk fat and protein content can be explained by both a concentration effect due to the drop in milk yield without any significant reduction in protein or lipid synthesis, as fat mobilization compensates for the reduction in de novo FA synthesis in the mammary gland due to feed restriction.

Furthermore, feed restriction decreased milk urea content (Table 3) conversely to what we observed in dairy cows (Hurtaud et al., 2023). Similarly, plasma urea decreased (Table 4), which could be due to an adaptation of rumen microorganism growth despite a limited intake in protein and energy.

Finally, feed restriction did not affect SCC conversely to the observed SCC increase in the milk from underfed cows (Herve et al., 2019; Hurtaud et al., 2023). The observed increase in SCC in milk from dairy cows has previously been attributed, at least in part, to a change in the integrity of mammary epithelium with a higher rate of mammary epithelial cell exfoliation, resulting in an increase in SCC, as previously observed during feed restriction (Herve et al., 2019). This species difference in SCC response to feed restriction outlines the adaptative response of small ruminants' mammary plasticity to dietary factors.

Effect of milking

The higher milk lipolysis and fat content observed in evening milk are in agreement with data from (Vanbergue et al., 2017, 2018; Hurtaud et al., 2023) and are explained by the difference in the length of the intervals between milkings (14 – 10 h) and by a smaller quantity of milk produced during evening milking. Interestingly, the discrepancy between lipolysis values in morning and evening milk samples was lower when ewes were fed restricted, thus suggesting that regulatory mechanisms may occur in the course of feed restriction with regard to lipolysis (+0.30 and + 0.10 mEq/100 g fat when ewes were fed *ad libitum* or fed restricted, respectively).

The lower milk protein and casein content from evening milking was associated with higher casein micelle diameter conversely to what is observed in dairy cows under similar treatment and with similar milking intervals (Hurtaud et al., 2023) for which milk protein and casein content increased in the evening compared to morning milks and was associated with smaller casein micelle. Otherwise, evening milk is richer in SCC as usually observed in dairy cows due to a concentration effect (Hurtaud et al., 2023;

Green et al., 2006). Regarding milk mineral contents, the observed higher soluble calcium and lower soluble phosphorus contents in evening milk (Supplementary Table S4) are in line with data in dairy cows (Hurtaud et al., 2023). However, total calcium and colloidal calcium were not affected by the treatment conversely to dairy cows, again illustrating differences among species.

Conclusion

The present study concludes that a dietary restriction in Lacaune ewes triggers a decrease with regard to milk lipolysis values, without affecting the activity of the LPL enzyme in ewe milk. This is in sharp contrast to what is actually happening in the cow species where lipolysis in milk is higher when the energy balance is negative (Hurtaud et al., 2023). This could mean that lipolysis-related mechanisms differ in the cow and in the ewe species, a point that needs to be confirmed by a direct interspecies experiment. Further research could also be carried out using different breeds or animals with different milk production potential within the same breed. These data will be enriched with lipidomic and proteomic data collected on ewes' blood and milk samples highly contrasted with regard to lipolysis levels to better understand the regulation of the lipolytic system in the ewe species.

Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.animal.2024.101181.

Ethics approval

The experiment was conducted in accordance with the French legislation on animal experimentation and was approved by the French National Committee for Consideration of Ethics in Animal Experimentation (Authorization: APAFiS n° #23403-2019122016362900 v4 delivered on 05 November 2020).

Data and model availability statement

The data/models were not deposited in an official repository. All data or models that support the findings are available from the authors upon request.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) did not use any AI and AI-assisted technologies.

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Declaration of interest

The authors declare that they have no conflict of interest.

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