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Milk yield loss in response to feed restriction is associated with mammary epithelial cell exfoliation in dairy cows

L. Herve,¹ H. Quesnel,¹ M. Veron,¹ J. Portanguen,¹ J. J. Gross,² R. M. Bruckmaier,² and M. Boutinaud^{1*}

¹PEGASE, INRA, Agrocampus Ouest, 35590 Saint-Gilles, France

²Veterinary Physiology, Vetsuisse Faculty, University of Bern, 3012 Bern, Switzerland

ABSTRACT

In dairy cows, feed restriction is known to decrease milk yield by reducing the number of mammary epithelial cells (MEC) in the udder through a shift in the MEC proliferation–apoptosis balance, by reducing the metabolic activity of MEC, or both. The exfoliation of MEC from the mammary epithelium into milk is another process that may participate in regulating the number of MEC during feed restriction. The aim of the present study was to clarify the mechanisms that underlie the milk yield loss induced by feed restriction. Nineteen Holstein dairy cows producing 40.0 ± 0.7 kg/d at 77 ± 5 d in milk were divided into a control group ($n = 9$) and a feed-restricted group ($n = 10$). Ad libitum dry matter intake (DMI) was recorded during a pre-experimental period of 2 wk. For 29 d (period 1), cows were fed either 100 (control) or 80% (feed-restricted) of their ad libitum DMI measured during the pre-experimental period. Then, all cows were fed ad libitum for 35 d (period 2). Milk production and DMI were recorded daily. Blood and milk samples were collected once during the pre-experimental period; on d 5, 9, and 27 of period 1; and on d 5, 9, and 30 of period 2. Mammary epithelial cells were purified from milk using an immunomagnetic method to determine the rate of MEC exfoliation. Mammary tissue samples were collected by biopsy at the end of each period to analyze the rates of cell proliferation and apoptosis and the expression of genes involved in synthesizing constituents of milk. Feed restriction decreased milk yield by 3 kg/d but had no effect on rates of proliferation and apoptosis in the mammary tissue or on the expression of genes involved in milk synthesis. The daily MEC exfoliation rate was 65% greater in feed-restricted cows than in control cows. These effects in feed-restricted

cows were associated with reduced insulin-like growth factor-1 and cortisol plasma concentrations. When all cows returned to ad libitum feeding, no significant difference on milk yield or MEC exfoliation rate was observed between feed-restricted and control cows, but refeeding increased prolactin release during milking. These results show that the exfoliation process may play a role in regulating the number of MEC in the udders of dairy cows during feed restriction without any carryover effect on their milk production.

Key words: dairy cow, feed restriction, mammary epithelial cell, exfoliation

INTRODUCTION

The lactational performance of dairy cows is influenced by numerous management factors. Feeding level is one of the main management factors affecting milk yield and mammary gland metabolism. In lactating dairy cows, feed restriction is known to decrease milk yield (Nørgaard et al., 2005, 2008; Guinard-Flament et al., 2007; Gross et al., 2011; Abdelatty et al., 2017). The extent of milk yield loss depends on the duration and intensity of feed restriction, as well as the lactation stage of dairy cows. A short period of severe feed restriction (cows fed 60% of their ad libitum intakes for 4 d) decreased the milk yield of mid-lactation dairy cows by 20% (Abdelatty et al., 2017), whereas a longer period of moderate feed restriction (cows fed with a control diet vs. a diet providing 80% of the energy and protein digestible in the small intestine provided by the control diet from 2 wk prepartum to 11 wk postpartum) decreased milk yield by 38% (Dessauge et al., 2011). However, there is no clear explanation of the mechanisms that lead to this decrease in milk yield induced by feed restriction.

The milk yield of dairy cows is determined by the secretory activity of mammary epithelial cells (MEC, i.e., cells that synthesize milk; Capuco et al., 2003; Boutinaud et al., 2004a) and by the number of these cells, which is regulated by the balance between cell proliferation and cell death by apoptosis (Knight, 2000;

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*Corresponding author: Marion.Boutinaud@inra.fr

Capuco et al., 2003). Moreover, the exfoliation of MEC, defined as the shedding of MEC from the mammary epithelium into milk, has recently been shown to participate in the regulation of MEC number (Herve et al., 2016). Feed restriction has shown contradictory effects on these factors. For instance, MEC secretory activity has been shown to be reduced in response to long-term global feed restriction (Dessauge et al., 2011), but not to long-term energy restriction (Nørgaard et al., 2005). Similarly, the decreased number of MEC in the mammary gland has been attributed to a decrease in the cell proliferation rate without variation in the apoptosis rate (Nørgaard et al., 2005) but is also ascribed to an increase in the apoptosis rate without variation in the cell proliferation rate (Dessauge et al., 2011). The effect of feed restriction on the MEC exfoliation rate has been investigated in only one study, which showed no variation in the daily rate of MEC exfoliation in response to feed restriction (Herve et al., 2016).

The objective of this study was to better understand the mechanisms underlying the milk yield loss induced by feed restriction. The roles of the factors determining milk yield; namely, metabolic activity and the number of MEC in the mammary gland, were analyzed in feed-restricted cows during and after feed restriction. A special focus was placed on the role of the MEC exfoliation process in regulating the number of MEC in the mammary gland of feed-restricted cows. The carryover effects of feed restriction on the number and secretory activity of MEC were studied during a period in which cows were switched back to ad libitum feeding.

MATERIALS AND METHODS

All procedures performed on animals were approved by the local Ethics Committee in Animal Experiment of Rennes (France) in compliance with French regulations (Decree No. 2001-464; May 29, 2001). The experiment was conducted at the INRA experimental farm Installation Expérimentale en Production du Lait (IEPL, UMR PEGASE, Le Rheu, France).

Animals and Experimental Design

Nineteen Holstein dairy cows (lactations 1 to 4) in early lactation (77 ± 5 DIM) and producing 40.0 ± 0.7 kg of milk/d at the beginning of the experiment were used in this study. The cows were divided into 2 successive blocks of 9 and 10 cows chosen according to their calving dates to include in the study a homogeneous set of cows in terms of stage of lactation. The 2 blocks of cows followed the same experimental design 1 wk apart (Figure 1). Before the beginning of the experiment (the

pre-experimental period), the cows were fed ad libitum for 2 wk. Throughout the pre-experimental period, the ad libitum DMI of each cow was recorded daily. Then, the cows were assigned to either a control group (no feed restriction throughout the experiment, $n = 9$) or a feed-restricted group (feed restriction for 29 d and refeeding for 35 d, $n = 10$) based on parity, DIM, milk yield, SCC, milk fat percentage, milk protein percentage, BW, and ad libitum DMI. For 29 d (period 1), the cows were subjected to 2 different feeding levels. Cows from the control group were fed 100% of their ad libitum DMI measured during the pre-experimental period, whereas cows from the feed-restricted group were fed 80% of their ad libitum DMI measured during the pre-experimental period. Period 1 was preceded by 5 d of feeding transition for feed-restricted cows to gradually switch between ad libitum feeding and feed restriction: feed-restricted cows were fed 90% of their ad libitum DMI for 2 d, 85% for another 2 d, and finally 80% on d 5 of transition. After period 1, cows were switched back to ad libitum feeding for 35 d (period 2). Period 2 was also preceded by a 3-d feeding transition. The cows were weighed 3 times a week throughout the experiment. All cows were milked every day at 0630 and 1630 h in the milking parlor, and milk yield was recorded individually at each milking. Milk samples (50 mL) collected from the entire milking were taken 4 times a week during the morning milking for determination of SCC using flow cytometry and determination of milk composition (fat and protein percentage) using an infrared method (Mylab, Châteaugiron, France). Additional milk samples were collected from the entire morning milking once at the end of the pre-experimental period (d -7 relative to feeding transition), and then 5, 9, and 27 d after the beginning of period 1, and 5, 9, and 30 d after the beginning of period 2 for purification of milk MEC using an immunomagnetic separation technique. The additional milk samples were also used to analyze total N content (Kjeldahl method), NPN content (precipitation at pH 4.6 with trichloroacetic acid and filtration), and noncasein N content (precipitation at pH 4.6 with 10% acetic acid and 1 M sodium acetate; Rowland, 1938). Casein was calculated as total N minus noncasein N, and whey protein was calculated as noncasein N minus NPN.

Diet

Throughout the experiment, cows were fed individually via electronic gating, and DMI and the quantity of feed refusals were recorded daily. The ingredients, chemical composition, and nutritional value of the diet are given in Table 1. The TMR consisted of 60% corn

Table 1. Chemical composition (g/kg of DM unless otherwise noted) and nutritive value of feedstuffs

Item	Corn silage	Energy concentrate ¹	Soybean meal	Dehydrated alfalfa
DM (%)	33.1	91.9	90.5	92.4
OM	961	877	928	882
N	79	102	506	189
Cellulose	201	63	48	252
NDF	420	196	102	420
ADF	247	79	61	299
Starch	286	420	65	39
Fat	29	36	26	29
Ca	2.1	20.1	3.7	22.0
P	1.7	8.7	7.2	2.9
PDIE ²	70	89	255	100
NE _L (MJ/kg of DM)	6.75	7.03	8.53	4.83

¹Energy concentrate on DM basis: 18.3% wheat, 18.3% corn, 18.3% barley, 18.3% beet pulp, 13.2% fine wheat bran, 3.0% cane molasses, 0.9% vegetable oil, 0.9% salts, and 8.8% minerals and vitamins.

²Protein truly digested in the small intestine delimited by energy supply (INRA, 2007).

silage, 15% energy concentrate (composed of 18.3% wheat, 18.3% corn, 18.3% barley, 18.3% beet pulp, 13.2% fine wheat bran, 3.0% cane molasses, 0.9% vegetable oil, 0.9% salts, and 8.8% minerals and vitamins), 15% soybean meal, and 10% dehydrated alfalfa. The diet provided 6.9 Mcal of NE_L and 103 g/kg of DM of MP or PDIE (protein digested in the small intestine supplied by RUP and by microbial protein from rumen-fermented OM; INRA, 2007), its equivalent in the INRA feeding system.

Purification of Mammary Epithelial Cells from Milk

To determine the concentration, number, and viability of MEC in milk, MEC were purified from fresh milk collected from an entire milking (1.8 kg) using the immunomagnetic separation technique described by Herve et al. (2017a). Then, the purified milk MEC suspension was stored in 1 mL of TRIzol (Invitrogen Life Technologies, Carlsbad, CA) at -80°C until total RNA extraction was performed.

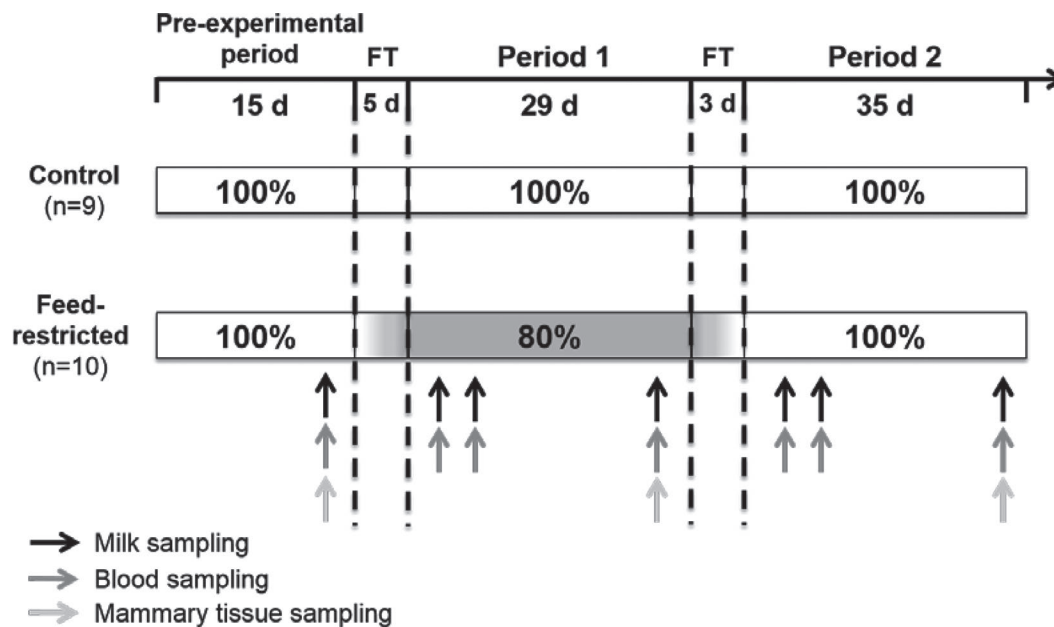


Figure 1. Experimental design of the study. During the pre-experimental period, all cows were fed 100% of their ad libitum DMI. For 29 d (period 1), cows were fed either 100% (control treatment) or 80% (feed-restricted treatment) of their ad libitum DMI. Then, all cows were fed ad libitum for 35 d (period 2). Period 1 was preceded by 5 d of feeding transition (FT), and period 2 was preceded by 3 d of FT for feed-restricted cows.

Blood Sampling and Hormonal and Metabolite Assays

Blood samples (20 mL) were collected from the tail vein 30 min before the beginning of milking once at the end of the pre-experimental period, and then at 5, 9, and 27 d after the beginning of period 1 and 5, 9, and 30 d after the beginning of period 2. Monovette syringes coated with sodium heparin (Sarstedt, Nümbrecht, Germany) were used to collect samples for the measurement of plasma lactose concentrations, and Monovette syringes coated with EDTA (Sarstedt) were used to collect samples for the measurement of plasma prolactin (**PRL**), cortisol (**Cort**), nonesterified fatty acids (**NEFA**), glucose, urea, insulin (**Ins**), and IGF-1 concentrations. On the same days, additional blood samples were collected 15 min after the beginning of milking using Monovette syringes coated with EDTA (Sarstedt) to measure plasma PRL and Cort concentrations. The plasma was separated by centrifugation at 4°C and $2,264 \times g$ for 15 min and stored at -20°C until subsequent analysis. Plasma concentrations of PRL were measured by ELISA. The ELISA plates were coated with 200 μL /well of mouse monoclonal anti-rabbit immunoglobulin antibody (Bertin Pharma, Montigny-le-Bretonneux, France) dissolved at 10 mg/L in 0.05 M phosphate buffer and incubated at 4°C overnight. The plates were then washed, and 300 μL of ELISA buffer (0.1 M phosphate buffer containing 0.15 M sodium chloride and 0.1% BSA, pH 7.4) was added to each well. The plates were stored at 4°C until plasma PRL concentration analysis. After the plates were washed with 300 μL of washing buffer (0.01 M phosphate buffer containing 0.05% Tween 20, pH 7.4) per well, 50 μL of anti-bovine PRL antiserum (Agro-Bio, La Ferté Saint Aubin, France) diluted 1:10,000 in ELISA buffer, 50 μL of standard (ranging from 320 to 2.5 ng/mL) or plasma sample, and 50 μL of PRL-acetylcholinesterase conjugate diluted in ELISA buffer (LERI CEA Saclay, Gif sur Yvette, France) were dispensed into each of the wells. The plates were incubated at 25°C for 24 h and washed twice. Then, 200 μL of Ellman's reagent [5,5'-dithiobis-(2-nitrobenzoic acid); Ellman et al., 1961] was dispensed into each well. When the absorbance of the control wells (that contained no antigen, known as a zero well) at 414 nm reached 0.2 absorbance units (AU), which occurred after approximately 45 to 75 min of incubation, absorbance was read using a plate reader (VersaMax ELISA microplate reader, Molecular Devices, Sunnyvale, CA). The limit of detection was 0.89 ng/mL, the intraassay coefficient of variation (CV) was 4.6%, and the interassay CV was 2.8%. The plasma concentration of Cort was assessed using the ELISA method described by Herve et al. (2017a).

Plasma concentrations of Ins and IGF-1 were assessed using the methods described by Vicari et al. (2008). Plasma glucose, urea, and NEFA concentrations were measured using a multiparameter analyzer (Kone Instrument Corp., Espoo, Finland) with a glucose (HK) kit (Thermo Fisher Scientific, Waltham, MA), a urea kit (Thermo Fisher Scientific), and a NEFA-HR kit (Wako Diagnostics, Mountain View, CA).

Estimation of Mammary Epithelium Integrity

Mammary epithelium integrity was estimated by measuring the lactose concentration in plasma and the $\text{Na}^+:\text{K}^+$ ratio in milk. Plasma concentrations of lactose were measured with a lactose/D-galactose assay kit (R-Biopharm, Darmstadt, Germany) in blood samples collected before milking once at the end of the pre-experimental period; at 5, 9, and 27 d after the beginning of period 1; and at 5, 9, and 30 d after the beginning of period 2 using a multiparameter analyzer (Kone Instrument Corp.). A 100- μL sample of milk collected on each of those days was used for total milk Na^+ and K^+ analysis by atomic absorption spectrophotometry (Spectra AA220, Varian, Les Ulis, France) as described by Murthy and Rhea (1967).

Mammary Biopsies

Mammary tissue samples were collected by biopsy 2 h before the afternoon milking once at the end of the pre-experimental period and then 27 d after the beginning of period 1 and 30 d after the beginning of period 2. On the day of the surgery, cows received 165 mg of the antibiotic ceftiofur (Naxcel, Zoetis, Parsippany, NJ) by i.m. injection. Biopsies were taken, as previously described (Boutinaud et al., 2012), from the upper portion of the mammary gland using a 70- \times 4-mm instrument described by Farr et al. (1996). Biopsies were harvested from the same part of the mammary gland, alternating between the right and left rear glands between periods. Mammary tissue (500 mg) was rinsed in sterile saline solution and then cut into 2 parts. One part of the mammary tissue sample was immediately frozen in liquid nitrogen and stored at -80°C until DNA quantification and RNA expression analysis. The other part was used for immunohistochemistry and mammary tissue morphometric analysis. After a wash in PBS, the tissue samples were fixed at room temperature in 4% paraformaldehyde for 2 h and in 0.4% paraformaldehyde for 2 d. The fixed tissue sample used for immunohistochemistry was cryoprotected by incubation for 5 d in a 40% sucrose-PBS solution, embedded in Tissue-Tek O.C.T. compound (Sakura Finetek Europe, LaboNord, Templemars, France), frozen in a cooled bath of isopentane

(Sigma-Aldrich, Lyons, France), and stored at -80°C until use. The fixed tissue sample used for mammary tissue morphological analysis was dehydrated in 70% ethanol and embedded in paraffin.

Detection of Apoptotic and Proliferating Cells by Immunohistochemistry in Mammary Tissue Sections

The determination of the percentage of apoptotic cells in the mammary gland biopsy sections was based on DNA fragmentation detection using terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate (dUTP) nick-end labeling (**TUNEL**) staining, as described below. Cryosections measuring 7 μm in thickness and mounted onto slides treated with 3-aminopropyltriethoxysilane (Sigma-Aldrich, Saint Louis, MO) were thawed and incubated for 20 min at 60°C in a solution of 10 mM citrate sodium (Sigma-Aldrich) and 0.1% Triton (Thermo Fisher Scientific), washed in Tris-buffered saline (**TBS**), and incubated for 20 min at 37°C in 50 ng/ μL proteinase K solution (Promega, Madison, WI). The tissue sections were incubated with 1:2 diluted reagents from the DeadEnd Fluorometric TUNEL System (Promega) according to the manufacturer's instructions.

Proliferating mammary cells were identified as cells expressing the proliferating cell nuclear antigen (**PCNA**), as described below. Although this staining has been shown to overestimate the rate of cell proliferation due to a long half-life, leading to persistence of staining even in cells that have recently left proliferative status (Yu and Filipe, 1993), we observed that it was an efficient method to observe variations in the percentage of proliferative cells as previously reported after prolactin inhibition (Boutinaud et al., 2012), reduction in milking frequency (Boutinaud et al., 2013), or during early involution (Boutinaud et al., 2017). Cryosections (7 μm thick) were mounted onto Superfrost/Plus slides (Prolabo, Bondoufle, France). Mammary gland sections were incubated in TBS with 3% hydrogen peroxide and 10% methanol for 30 min. After several washes in TBS, the sections were permeabilized with TBS containing 1% SDS for 5 min, washed 3 times in TBS, and pre-incubated in TBS containing 1% BSA for 1 h at room temperature. The tissues were then incubated in the presence or absence of a primary antibody (M0879, monoclonal mouse clone PC10, diluted 1:200; DakoCytomation, Glostrup, Denmark) in 1% BSA-TBS overnight at 4°C . After multiple washes in 1% BSA-TBS, the samples were incubated with second antibody (A11059, rabbit anti-mouse AlexaFluor 488-conjugated antibody, Invitrogen) at a dilution of 1:400 for 1 h at room temperature.

After TUNEL or PCNA labeling, the slides were mounted with SlowFade Gold Antifade Mountant (Thermo Fisher Scientific) containing 4',6-diamidino-2-phenylindole (DAPI) and examined by fluorescence microscopy using an Eclipse E400 microscope (Nikon France, Le Pallet, France). The pictures were captured with a DXM 1200 digital still camera (Nikon France) and analyzed with the software ImageJ (National Institutes of Health, Bethesda, MD). Eight microscopic fields (magnification, $200\times$; area, 0.14 mm^2 per microscopic field) were examined for each staining. The percentages of apoptotic and proliferating cells in mammary tissue were determined as the ratio of TUNEL- or PCNA-labeled cells to DAPI-counterstained nuclei.

Mammary Tissue Morphometric Analysis

The paraffin-embedded tissue samples were sectioned at 7 μm thick and mounted onto poly-lysine-coated slides (VWR, Radnor, PA) and subsequently stained with hematoxylin and eosin for morphometric analysis. Slides were scanned with a NanoZoomer imaging system (Hamamatsu Photonics, Hamamatsu City, Japan). One tissue section per cow was subjected to morphometric analysis, including measurements of the percentage of mammary secretory tissue, mean perimeter, mean Ferret's diameter of mammary lobules, and the number of mammary lobule per tissue section, using the software ImageJ (National Institutes of Health).

DNA Quantification

The DNA concentration in mammary tissue samples ($\approx 50\text{ mg}$) was measured as described by Dessauge et al. (2011) with some modifications. Briefly, mammary tissue samples were weighed and homogenized in 1 mL of sodium phosphate extraction buffer. The mixture was ground using an Ultra-Turrax crusher (VWR International, Fontenay-sous-Bois, France), sonicated for 1 min, and centrifuged at $4,000 \times g$ for 1 min. The supernatant was transferred into a new tube, and the pellet was resuspended in 1 mL of the extraction buffer before being subjected once more to the successive grinding, sonication, and centrifugation extraction steps. Then, the DNA mixture was analyzed in triplicate by a fluorescence assay using Hoechst 33258 dye (Sigma-Aldrich) and a Mithras LB940 fluorescence plate reader (Berthold Technologies, Thoiry, France).

RNA Extraction and Reverse Transcription

After defrosting at room temperature, milk MEC and mammary tissue samples were crushed and homogenized in 1 mL of TRIzol by using a Ultra-Turrax crusher

(VWR International). For each sample, 200 μL of chloroform was added and mixed. After 2 min of incubation at room temperature, the mixture was centrifuged at $12,000 \times g$ for 15 at 4°C , and the aqueous supernatant containing total RNA was recovered. Then, to precipitate the RNA, 500 μL of isopropyl alcohol was added and the samples were incubated overnight at -20°C . The samples were centrifuged at $12,000 \times g$ for 10 min at 4°C , and the RNA pellet was recovered after the supernatant was discarded. Cold ethanol (75%, 4°C) was mixed with each sample to rinse the RNA pellet. Finally, after centrifugation at $5,000 \times g$ for 5 min at 4°C , the RNA pellets were dried and dissolved in sterile RNase-free water (Gibco, Invitrogen). The amounts of total RNA extracted from milk MEC or mammary tissue samples were determined using a DeNovix DS-11 Spectrophotometer (DeNovix Inc., Wilmington, DE). Total RNA quality was assessed with a Bioanalyzer (Agilent Technologies, Massy, France) using the RNA Integrity Number (RIN) generated by Agilent 2100 Expert Software, version B.02 (Agilent Technologies).

Complementary DNA was generated from total RNA by using a SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, 1 μL of $10\times$ SuperScript Enzyme mix, 2 μL of $5\times$ VILO reaction mix, 2 μL of diethyl pyrocarbonate (DEPC)-treated water, and 400 ng of RNA in 5 μL were incubated at 25°C for 10 min, followed by at 42°C for 90 min, followed by heating to 85°C for 5 min to inactivate the reaction. Finally, the temperature was reduced to 4°C . Reverse transcription products were stored at -80°C until PCR was performed.

Real-Time PCR

To measure mRNA levels of genes encoding κ -CN (*CSN3*), α_{S1} -CN (*CSN1S1*), α -LA (*LALBA*), type 1 glucose transporter (*SLC2A1*), proapoptotic proteins caspase-3 (*CASP3*) and Bcl-2-associated X (*BAX*), transcription factor E74-like factor 5 (*ELF5*), PRL receptor long isoform (*PRLR*), cytokeratin 8 (*KRT8*), ATP binding cassette subfamily G member 2 (*ABCG2*), matrix metalloproteinase-2 (*MMP2*), and ribosomal protein S6 kinase polypeptide 1 (*RPS6KA1*) in purified milk MEC and mammary tissue samples, real-time PCR analyses were performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) as previously described (Boutinaud et al., 2008). The primers used for real-time PCR have been described in previous studies (Schmitz et al., 2004; Boutinaud et al., 2008, 2012, 2013; Ben Chedly et al., 2009; Sciascia et al., 2013; Sigl et al., 2014). The mRNA levels of

the studied genes were expressed relative to the geometric mean of 3 housekeeping genes: peptidylprolyl isomerase A (*PPIA*), 18S ribosomal RNA (*RNA18S*), and ribosomal protein large P0 (*RPLP0*). The primers used for real-time PCR for housekeeping genes have been previously described (Robinson et al., 2007; Ben Chedly et al., 2009; Bonnet et al., 2013). For each gene, the mRNA level was expressed as a semi-absolute number of mRNA molecules using the method previously reported by Boutinaud et al. (2004b).

Calculations and Statistical Analyses

The rate of MEC exfoliation was defined as the number of MEC exfoliated per day and calculated by multiplying the daily milk yield by the MEC concentration measured during the morning milking. The milk somatic cell number was calculated by multiplying the daily milk yield by the SCC. The SCC, milk MEC number, and gene expression data were not normally distributed; thus, a \log_{10} transformation was applied before the analyses. Prolactin delta was determined as the difference between the PRL concentration after milking and the basal concentration of PRL (measured 30 min before milking).

For energy and protein intakes and balances, milk yield and composition, milk somatic cells, plasma metabolites, hormone concentrations, and gene expression in purified milk MEC, data obtained during the feed restriction (period 1) and after the switch back to ad libitum feeding (period 2) were analyzed separately using the same statistical model. Data were analyzed by ANOVA using the repeated-measures PROC MIXED SAS procedure with repeated statements (SAS Institute Inc., Cary, NC). Day was used as the repeated parameter and cow as the subject for repeated measures. The effects of block, day, feeding level (either 100 or 80% of the ad libitum DMI measured during the pre-experimental period), and the interaction between day and feeding level were tested. For traits analyzed in mammary tissue samples (apoptotic and proliferating cells, morphometry, DNA content, zymography, and gene expression in the mammary tissue), data obtained during the 2 periods were analyzed together by ANOVA using the repeated-measures PROC MIXED procedure (SAS Institute Inc.). Period was used as the repeated parameter and cow as the subject for repeated measures. The effects of block, period, feeding level, and the interaction between period and feeding level were tested. The data obtained at the end of the pre-experimental period were used as covariates.

The SAS CORR procedure was used to obtain the Pearson correlation coefficient (r), which measured

Table 2. Average weight, DMI, and energy and protein intake and balance of dairy cows from the feed-restricted group (n = 10) and the control group (n = 9) during a 29-d period of feed restriction (period 1) and a subsequent 35-d period of ad libitum feeding (period 2)

Item	Period 1				Period 2			
	Control	Restricted	SEM	<i>P</i> -value	Control	Restricted	SEM	<i>P</i> -value
Weight (kg)	662	626	1.1	<0.001	676	662	1.3	<0.001
DMI (kg/d)								
Total	24.1	19.2	0.09	<0.001	24.5	24.6	0.10	0.37
As forage	16.9	13.4	0.07	<0.001	17.0	17.1	0.07	0.48
As concentrate	7.2	5.9	2.76	<0.001	7.4	7.5	3.20	0.28
Intake								
NE _L (MJ/d)	158.5	123.8	1.11	<0.001	160.6	158.5	1.33	0.30
PDIE ¹ (g/d)	2,525	1,974	17.4	<0.001	2,563	2,526	21.7	0.26
Balance								
NE _L (MJ/d)	2.2	-21.4	1.15	<0.001	6.1	7.2	1.34	0.59
PDIE (g/d)	273	-33	19.3	<0.001	321	331	21.1	0.76

¹Protein truly digested in the small intestine delimited by energy supply (INRA, 2007).

the strength of association between pairs of variables (hormones, mammary epithelium integrity, and the MEC exfoliation rate at milking) without specifying dependencies.

All data were expressed as the means ± standard errors of the mean. The statistical significance threshold was set at $P < 0.05$, and the trend-level significance was defined as $0.05 \leq P < 0.10$.

RESULTS

DMI, BW, Energy and Protein Balances, and Plasma Metabolites

By design, during the feed restriction period (period 1), the total DMI was lower in feed-restricted than in control cows (19.2 vs. 24.1 kg/d; Table 2), with a 20% decrease in total DMI. The reduced total DMI in feed-restricted cows was due to reduced intakes of forage and concentrates (minerals and vitamins included) compared with control cows (13.4 vs. 16.9 and 5.9 vs. 7.2 kg/d, respectively; Table 2). Consequently, the NE_L and PDIE intakes were 22% lower in feed-restricted than in control cows ($P < 0.001$; Table 2). During

feed restriction, NE_L and PDIE balances were therefore negative in feed-restricted cows (-21.4 MJ/d and -33 g/d) and positive in control cows (2.2 MJ/d and 273 g/d), and feed-restricted cows were on average 5% lighter than control cows (average BW during period 1: 626 vs. 662 kg; Table 2). During the feed restriction period, the plasma NEFA concentration was greater in feed-restricted cows than in control cows ($P < 0.001$, Table 3), but no difference in plasma glucose or urea concentration was observed between control and feed-restricted cows ($P > 0.10$, Table 3).

During period 2, no difference in total DMI, forage intake, or concentrate intake was observed between feed-restricted and control cows ($P = 0.27$; Table 2). The NE_L and PDIE intakes and balances were also similar in both groups ($P = 0.25$; Table 2). During period 2, however, a difference was observed in the BW of cows, feed-restricted cows still being 2% lighter than control cows ($P < 0.001$; Table 2). Regarding plasma metabolites, no difference in plasma NEFA or glucose concentration was observed during period 2 ($P = 0.86$; Table 3), but plasma urea tended to be greater in feed-restricted than in control cows ($P = 0.06$; Table 3).

Table 3. Plasma metabolite concentrations of dairy cows from the feed-restricted group (n = 10) and the control group (n = 9) during a 29-d period of feed restriction (period 1) and a subsequent 35-d period of ad libitum feeding (period 2)

Item ¹	Period 1				Period 2			
	Control	Restricted	SEM	<i>P</i> -value	Control	Restricted	SEM	<i>P</i> -value
NEFA ² (μmol/L)	95	432	27.8	<0.001	93	91	7.7	0.87
Glucose (mg/L)	752	764	12.1	0.50	764	766	11.3	0.87
Urea (mg/L)	279	263	6.6	0.11	282	308	8.9	0.06

¹Plasma samples were collected on d 5, 9, and 27 after the beginning of period 1 and on d 5, 9, and 30 after the beginning of period 2 for determination of the average metabolite concentrations in plasma.

²Nonesterified fatty acids.

Table 4. Milk composition of dairy cows from the feed-restricted group (n = 10) and the control group (n = 9) during a 29-d period of feed restriction (period 1) and a subsequent 35-d period of ad libitum feeding (period 2)

Item ¹	Period 1				Period 2			
	Control	Restricted	SEM	<i>P</i> -value	Control	Restricted	SEM	<i>P</i> -value
Milk fat (%)	3.42	3.63	0.032	<0.001	3.58	3.59	0.032	0.86
Milk protein (%)	2.93	2.79	0.011	<0.001	3.08	3.02	0.014	<0.01
Milk lactose (%)	5.03	4.93	0.017	<0.001	4.99	4.94	0.017	0.07
Fat yield (g/d)	1,324	1,278	12.1	<0.05	1,311	1,302	13.5	0.68
Protein yield (g/d)	1,132	989	8.4	<0.001	1,131	1,097	8.2	<0.05
Whey protein (g/kg)	5.5	5.2	0.08	0.06	5.8	5.9	0.10	0.37
Casein (g/kg)	23.7	22.3	0.24	<0.01	24.6	24.4	0.36	0.64
Lactose yield (g/d)	1,979	1,728	37.3	<0.001	1,813	1,787	30.3	0.56
Log ₁₀ SCC	4.38	4.50	0.032	<0.05	4.50	4.69	0.043	<0.05
Log ₁₀ somatic cell number	8.97	9.03	0.032	0.18	9.07	9.23	0.043	<0.05

¹Milk samples were collected 4 times a week throughout the experiment for determination of milk composition and SCC.

Milk Yield and Composition

Milk yield of feed-restricted cows began to decrease as early as the feeding transition period and continued to decrease at the beginning of the feed restriction (Figure 2). During feed restriction, feed-restricted cows produced, on average, 3.5 kg of milk/d less than control cows, representing a milk yield loss of 9% compared with control cows ($P < 0.001$; Figure 2). The milk of feed-restricted cows had a slightly higher percentage of fat (3.63 vs. 3.42%, $P < 0.001$; Table 4), a lower percentage of protein (2.79 vs. 2.93%, $P < 0.001$; Table 4), and a lower percentage of lactose (4.93 vs. 5.03%, $P < 0.001$; Table 4) than the milk of control cows, resulting in significant reductions in the amounts of fat (−3.5%), protein (−12.6%), and lactose (−12.6%) produced per

day ($P < 0.05$; Table 4). The decrease in milk protein percentage from feed-restricted cows was due largely to a reduced milk casein concentration ($P < 0.01$) but also to a tendency toward a reduced milk whey protein concentration ($P = 0.06$; Table 4).

During the first and second days of period 2, milk yield was still greater in control than in feed-restricted cows ($P < 0.05$; Figure 2), but milk yield was similar in feed-restricted and control cows for the rest of the period (Figure 2). During this period of ad libitum feeding, average milk fat percentage and yield were similar in both groups ($P = 0.67$; Table 4), but average milk protein percentage and yield were still lower in feed-restricted cows than in control cows ($P < 0.01$; Table 4). However, milk casein and whey protein concentrations were similar in feed-restricted and control cows

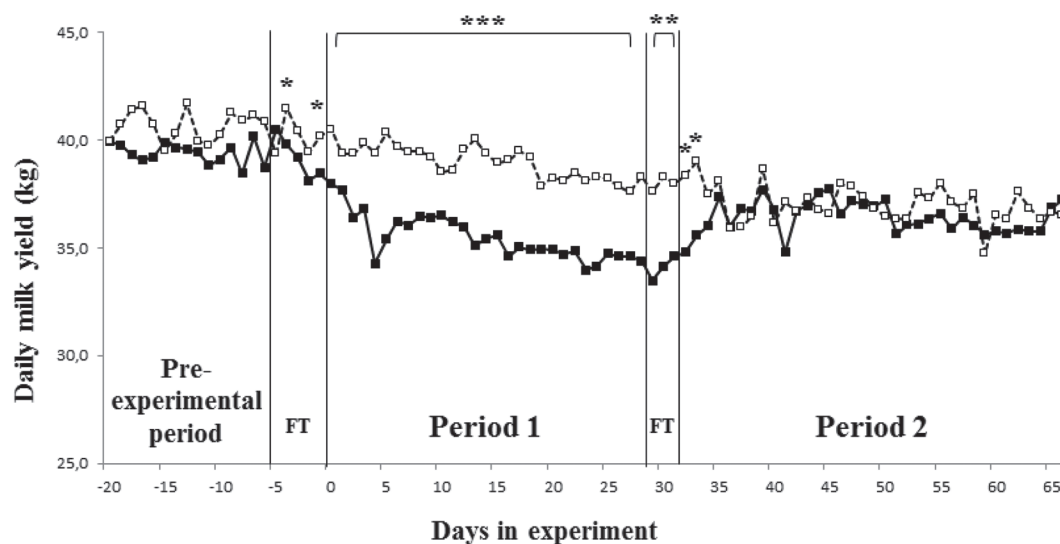


Figure 2. Milk yield of dairy cows in the feed-restricted group (n = 10, ■) and the control group (n = 9, □) during a 29-d period of feed restriction (period 1) and a subsequent 35-d period of ad libitum feeding (period 2); FT = feeding transition. The data are presented as the means estimated by the statistical model. Statistically different means are indicated by *** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$.

Table 5. Concentration and viability of milk mammary epithelial cells (MEC) in dairy cows from the feed-restricted group (n = 10) and the control group (n = 9) during a 29-d period of feed restriction (period 1) and a subsequent 35-d period of ad libitum feeding (period 2)

Item ¹	Period 1				Period 2			
	Control	Restricted	SEM	<i>P</i> -value	Control	Restricted	SEM	<i>P</i> -value
Milk MEC concentration (10 ³ cells/mL)	3.6	4.7	0.69	0.28	7.2	10.3	2.5	0.42
Milk MEC viability (%)	53.7	59.7	2.13	0.08	52.1	55.6	2.05	0.26

¹Milk samples were collected on d 5, 9, and 27 after the beginning of period 1 and on d 5, 9, and 30 after the beginning of period 2 for the purification of MEC and determination of the concentration, number, and viability of milk MEC.

(*P* = 0.36; Table 4). During this period, average lactose percentage still tended to be lower in feed-restricted cows than in control cows (4.94 vs. 4.99%, *P* = 0.07; Table 4) but lactose yield was similar in both groups (*P* = 0.56; Table 4).

Milk Somatic Cells

During feed restriction, the SCC was higher in feed-restricted cows than in control cows (+32%, *P* < 0.05; Table 4), but the somatic cell number did not differ between groups. The average milk MEC concentration was similar in both groups, but the average rate of MEC exfoliation was 65% greater in feed-restricted than in control cows (98.7 vs. 59.9 × 10⁶ cells/d, *P* < 0.05; Table 5, Figure 3). The proportion of exfoliated MEC that were viable tended to be greater in feed-restricted cows than in control cows (*P* = 0.08; Table 5).

When feed-restricted cows switched back to ad libitum feeding, the SCC and the somatic cell number were greater in feed-restricted cows than in control

cows (+194% and +45%, respectively, *P* < 0.05; Table 4) but no difference in milk MEC concentration, rate of MEC exfoliation, or milk MEC viability was observed (*P* > 0.10; Table 5, Figure 3).

Hormones

During feed restriction, the average concentrations of IGF-1 and Ins were lower in feed-restricted cows than in control cows (96.7 vs. 111.4 ng/mL, *P* < 0.05, and 12.7 vs. 19.7 μU/mL, *P* < 0.01, respectively; Table 6). During this period, the basal concentration of Cort was similar in both groups but the concentration of Cort after milking was lower in feed-restricted cows than in control cows (6.4 vs. 8.7 ng/mL, respectively, *P* < 0.05; Table 6). Regarding PRL, neither the basal concentration nor the concentration after milking differed between groups (*P* = 0.69; Table 6). Consequently, PRL delta was similar in both groups (*P* = 0.99; Table 6).

During the switch back to ad libitum feeding, average concentrations of IGF-1 and Ins no longer differed

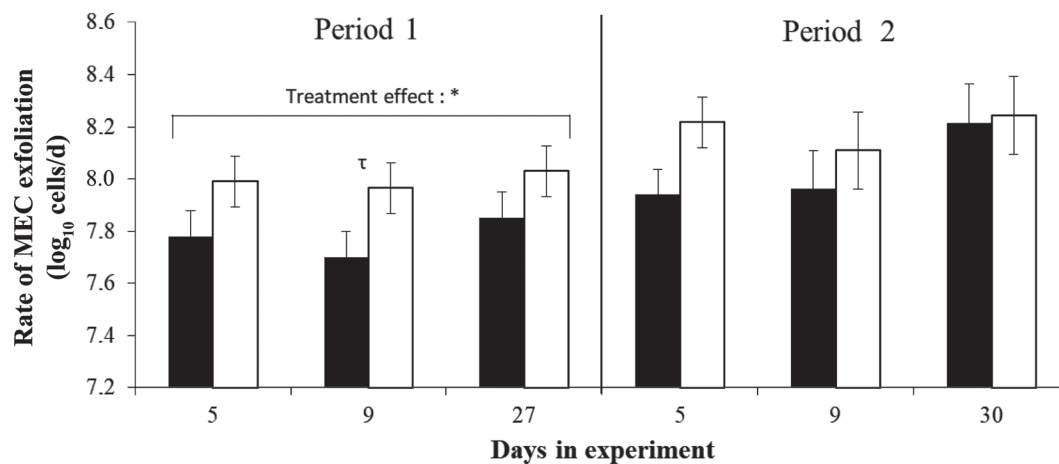


Figure 3. Rate (±SEM) of exfoliation of mammary epithelial cells (MEC) in dairy cows from the feed-restricted group (n = 10, ■) and the control group (n = 9, □) during a 29-d period of feed restriction (period 1) and a subsequent 35-d period of ad libitum feeding (period 2). The data are presented as the means estimated by the statistical model. For period 1, treatment (*P* = 0.02), day (*P* = 0.56), day × treatment (*P* = 0.91), and for period 2, treatment (*P* = 0.25), day (*P* = 0.42), day × treatment (*P* = 0.72). Within day, a trend for treatment effect is indicated by τ for 0.05 ≤ *P* ≤ 0.10.

Table 6. Plasma concentrations of IGF-1, insulin (Ins), cortisol (Cort), and prolactin (PRL) in dairy cows from the feed-restricted group (n = 10) and the control group (n = 9) during a 29-d period of feed restriction (period 1) and a subsequent 35-d period of ad libitum feeding (period 2)

Item ¹	Period 1				Period 2			
	Control	Restricted	SEM	<i>P</i> -value	Control	Restricted	SEM	<i>P</i> -value
IGF-1 (ng/mL)	111.4	96.7	4.26	<0.05	117.3	122.8	6.37	0.60
Ins (μU/mL)	19.7	12.7	1.43	<0.01	18.5	20.1	1.66	0.53
Cort basal (ng/mL)	10.5	8.4	0.90	0.14	14.3	8.4	1.39	<0.05
Cort after milking (ng/mL)	8.7	6.4	0.51	<0.05	7.0	8.0	0.52	0.26
PRL basal (ng/mL)	106.2	102.5	6.38	0.70	90.0	75.7	4.25	<0.05
PRL after milking (ng/mL)	149.2	149.8	5.59	0.94	110.3	133.8	5.15	<0.01
PRL delta ² (ng/mL)	45.0	45.0	7.45	0.99	26.9	51.1	5.79	<0.05

¹Plasma samples were collected on d 5, 9, and 27 after the beginning of period 1 and on d 5, 9, and 30 after the beginning of period 2 for the determination of the average concentrations of IGF-1, Ins, Cort, and PRL.

²Difference after correction between the PRL concentration after milking and the basal concentration of PRL (measured 30 min before milking).

between feed-restricted and control cows ($P = 0.52$; Table 6). During this period, the basal concentration of Cort was lower in feed-restricted cows than in control cows (8.4 vs. 14.3 ng/mL, respectively, $P < 0.05$; Table 6), but the concentration of Cort after milking was similar in both groups ($P = 0.26$; Table 6). Regarding PRL, although the basal concentration was lower ($P < 0.05$; Table 6), the PRL concentration after milking was greater in feed-restricted cows than in control cows ($P < 0.01$; Table 6). Consequently, PRL delta was higher in feed-restricted cows than in control cows (51.1 vs. 26.9 ng/mL, $P < 0.05$; Table 6).

Mammary Tissue Morphometric Analyses, DNA Concentration, and Apoptotic and Proliferative Rates

No difference in the percentage of apoptotic or proliferative cells was observed between feed-restricted

and control cows during the feed restriction period or the subsequent ad libitum feeding period ($P = 0.17$, Table 7). Feed restriction did not affect the percentage of mammary secretory tissue, the mean perimeter, the mean Feret's diameter, or the mean area of the mammary lobules; however, feed-restricted mammary tissue tended to have a greater number of mammary lobules per mm² ($P = 0.08$; Table 7). The DNA concentration in mammary tissue was, on average, 11% greater in feed-restricted cows than in control cows ($P < 0.05$; Table 7).

During the switch back to ad libitum feeding period, the percentage of secretory tissue and the DNA concentration were similar in both groups ($P = 0.38$, Table 7), whereas feed-restricted cows tended to have larger mammary lobules, as indicated by the greater mean perimeter, mean Feret's diameter, and mean area of the mammary lobules ($P \leq 0.10$; Table 7). Consequently, the number of mammary lobules per mm² tended to be

Table 7. Percentages of apoptotic and proliferative cells and DNA concentration and morphometric analysis of mammary tissue collected from dairy cows in the feed-restricted group (n = 10) and the control group (n = 9) at the end of a 29-d period of feed restriction (period 1) and at the end of a subsequent 35-d period of ad libitum feeding (period 2)

Item ¹	Period 1				Period 2			
	Control	Restricted	SEM	<i>P</i> -value	Control	Restricted	SEM	<i>P</i> -value
Apoptotic cells (%)	0.16	0.27	0.052	0.18	0.51	0.47	0.117	0.83
Proliferative cells (%)	29.5	29.8	1.47	0.85	21.4	21.0	2.06	0.90
DNA concentration (mg/g of mammary tissue)	2.59	2.87	0.087	<0.05	2.98	3.00	0.161	0.93
Mammary secretory tissue (%)	71.0	74.1	3.55	0.56	64.9	71.2	4.74	0.39
Mean perimeter of the mammary lobules (mm)	3.0	2.6	0.22	0.23	2.4	3.0	0.21	0.08
Mean Feret's diameter of the mammary lobules (mm)	0.9	0.9	0.06	0.37	0.8	1.0	0.06	0.08
Number of mammary lobules (no./mm ²)	1.8	2.2	0.16	0.08	2.3	1.8	0.19	0.09
Mean area of mammary lobules (mm ²)	0.5	0.4	0.05	0.32	0.3	0.5	0.07	0.07

¹Mammary tissue samples were collected by biopsy on d 27 after the beginning of period 1 and on d 30 after the beginning of period 2 for the determination of the percentages of apoptotic and proliferative cells and for morphometry analyses.

Table 8. Abundance of mRNA quantified by real-time PCR in mammary tissue collected from dairy cows in the feed-restricted group (n = 10) and the control group (n = 9) at the end of a 29-d period of feed restriction (period 1) and at the end of a subsequent 35-d period of ad libitum feeding (period 2)

Item ¹	Period 1				Period 2			
	Control	Restricted	SEM	P-value	Control	Restricted	SEM	P-value
<i>CSN3</i>	5.9	6.4	0.38	0.42	5.9	5.4	0.63	0.63
<i>CSN1S1</i>	4.4	5.1	0.45	0.27	4.1	4.4	0.33	0.53
<i>LALBA</i>	8.4	9.2	0.49	0.26	8.3	7.9	0.89	0.75
<i>SLC2A1</i>	3.1	3.3	0.13	0.32	3.1	2.8	0.28	0.52
<i>CASP3</i>	2.2	2.3	0.09	0.33	2.3	2.6	0.13	0.29
<i>BAX</i>	0.6	0.5	0.05	0.64	0.6	0.6	0.10	0.99
<i>ELF5</i>	5.6	5.5	0.15	0.76	5.7	5.4	0.28	0.41
<i>PRLR</i>	2.9	2.8	0.22	0.79	3.1	2.5	0.38	0.36
<i>KRT8</i>	3.0	3.2	0.17	0.48	3.5	3.4	0.19	0.85
<i>ABCG2</i>	2.7	3.2	0.16	<0.05	2.7	2.7	0.32	0.98
<i>MMP2</i>	3.0	3.1	0.18	0.64	2.6	3.0	0.19	0.19
<i>RPS6KA1</i>	2.9	2.6	0.32	0.48	3.1	3.0	0.34	0.78
Mean of reference genes	7.3	7.6	0.36	0.48	7.0	7.3	0.32	0.63

¹Mammary tissue samples were collected by biopsy on d 27 after the beginning of period 1 and on d 30 after the beginning of period 2 for the analysis of gene expression of κ -casein (*CSN3*), α_{S1} -casein (*CSN1S1*), α -lactalbumin (*LALBA*), the proapoptotic proteins caspase-3 (*CASP3*) and Bcl-2-associated X (*BAX*), the transcription factor E74-like factor 5 (*ELF5*), prolactin receptor long isoform (*PRLR*), cytokeratin 8 (*KRT8*), ATP binding cassette subfamily G member 2 (*ABCG2*), the type 1 glucose transporter (*SLC2A1*), matrix metalloproteinase-2 (*MMP2*), and ribosomal protein S6 kinase polypeptide 1 (*RPS6KA1*).

lower in feed-restricted cows than in control cows ($P = 0.09$; Table 7).

Gene Expression in Mammary Tissue and Purified Milk MEC

Feed restriction had no effect on the expression of selected genes in the mammary tissue ($P = 0.26$; Table 8), except for an increase in the expression of *ABCG2* ($P < 0.05$; Table 8). During the switch back to ad libitum feeding period, no difference in gene expression in the mammary tissue was observed between feed-restricted and control cows ($P = 0.18$; Table 8).

In MEC purified from milk, feed restriction tended to decrease the expression of *LALBA* ($P = 0.07$; Table 9). During the switch back to ad libitum feeding period, the expression of *LALBA* and *CASP3* tended to be lower in feed-restricted cows than in control cows ($P = 0.07$ and $P = 0.09$, respectively; Table 9).

Indicators of Mammary Epithelium Integrity

Feed restriction had no effect on plasma lactose concentration, milk K^+ concentration, or milk $Na^+ : K^+$ ratio ($P > 0.16$; Table 10) but it did increase milk Na^+ concentration ($P < 0.05$; Table 10). When cows were switched back to ad libitum feeding, no difference in indicators of mammary epithelium integrity was observed between feed-restricted and control cows ($P > 0.22$, Table 10).

Association Among Exfoliation Rate During Milking, Hormonal Status, and Mammary Epithelium Integrity

The rate of MEC exfoliation was negatively correlated with IGF-1 concentration ($r = -0.38$; $P < 0.001$) and with basal and postmilking concentrations of Cort ($r = -0.20$ and -0.20 , respectively; $P < 0.05$). No correlation was found between MEC exfoliation rate and the concentration of PRL before and after milking or between MEC exfoliation rate and concentration of Ins.

The rate of MEC exfoliation was positively correlated with several indicators of mammary epithelium integrity; namely, concentration of plasma lactose measured before milking ($r = 0.41$, $P < 0.001$), Na^+ concentration ($r = 0.48$, $P < 0.001$), and the $Na^+ : K^+$ ratio in milk ($r = 0.44$, $P < 0.001$).

DISCUSSION

In the present study, feed restriction decreased forage and concentrate intakes, which decreased overall DMI by 20%. This decrease in DMI resulted in negative energy and protein balances in feed-restricted cows. In response to these deficiencies, cows mobilized their body lipid reserves as indicated by an increase in plasma NEFA concentration and a decrease in BW. These findings are in accordance with previous experiments in which feed restriction was shown to trigger lipomobilization (Chilliard et al., 1998; Gross et al., 2011; Schütz et al., 2013). This lipomobilization should allow maintenance of milk production to a certain extent (Schütz et al.,

Table 9. Abundance of mRNA quantified by real-time PCR in mammary epithelial cells (MEC) purified from the milk of dairy cows in the feed-restricted group (n = 10) and the control group (n = 9) during a 29-d period of feed restriction (period 1) and a subsequent 35-d period of ad libitum feeding (period 2)

Item ¹	Period 1				Period 2			
	Control	Restricted	SEM	P-value	Control	Restricted	SEM	P-value
<i>CSN3</i>	5.2	5.4	0.22	0.66	5.1	4.7	0.29	0.50
<i>CSN1S1</i>	4.3	4.3	0.14	0.99	5.3	4.6	0.34	0.25
<i>LALBA</i>	6.7	5.3	0.45	0.07	7.5	5.8	0.50	0.07
<i>SLC2A1</i>	4.3	4.4	0.09	0.52	4.0	3.8	0.21	0.64
<i>CASP3</i>	2.1	2.1	0.11	0.81	2.2	2.0	0.05	0.09
<i>BAX</i>	1.8	1.9	0.09	0.62	1.9	1.9	0.08	0.62
<i>ELF5</i>	4.5	4.2	0.11	0.17	4.5	3.9	0.22	0.14
<i>PRLR</i>	1.8	1.6	0.14	0.30	2.1	1.6	0.22	0.26
<i>KRT8</i>	3.5	3.5	0.13	0.89	3.8	3.4	0.18	0.20
<i>ABCG2</i>	3.2	3.1	0.10	0.36	3.4	3.1	0.21	0.40
<i>MMP2</i>	1.2	1.4	0.29	0.67	0.7	NE ²	0.18	0.34
<i>RPS6KA1</i>	3.9	4.0	0.08	0.36	3.8	3.8	0.08	0.64
Mean of reference genes	6.9	7.0	0.09	0.33	NE	7.0	0.10	0.90

¹Milk samples were collected on d 5, 9, and 27 after the beginning of period 1 and d 5, 9, and 30 after the beginning of period 2 for the purification of MEC and the analysis of gene expression of κ -casein (*CSN3*), α_{S1} -casein (*CSN1S1*), α -lactalbumin (*LALBA*), proapoptotic proteins caspase-3 (*CASP3*) and Bcl-2-associated X (*BAX*), transcription factor E74-like factor 5 (*ELF5*), prolactin receptor long isoform (*PRLR*), cytokeratin 8 (*KRT8*), ATP binding cassette subfamily G member 2 (*ABCG2*), type 1 glucose transporter (*SLC2A1*), matrix metalloproteinase-2 (*MMP2*), and ribosomal protein S6 kinase polypeptide 1 (*RPS6KA1*).

²Not estimated because of missing values.

2013). Nevertheless, the 4-wk feed restriction was associated with a 9% decrease in milk yield. The negative effect of feed restriction on milk production has been reported in many studies in dairy cows (Andersen et al., 2004; Radcliff et al., 2006; Guinard-Flament et al., 2007; Gross et al., 2011). As expected, feed restriction also affected the composition of milk by reducing milk protein and lactose percentages, and protein, lactose, and fat yields, as previously reported (Guinard-Flament et al., 2007; Dessauge et al., 2011; Gross et al., 2011; Abdelatty et al., 2017). Probably as a consequence of lipomobilization, the decrease in fat yield (−3.5%) was smaller than the decrease in milk yield.

A 4-wk period of 20% feed restriction had almost no effect on the metabolic activity of MEC, as indicated by the absence of variation in the expression of major genes involved in milk synthesis in the mammary tissue and in purified milk MEC. Consistent with the decrease

in lactose yield and percentage, only the expression of *LALBA*, a gene coding for one of the major co-enzymes involved in lactose synthesis, tended to be lower in purified milk MEC of feed-restricted cows than in those of control cows. The absence of significant variation in expression of *CSN3* and *LALBA* in mammary tissue is in accordance with a previous study where a 30% feed restriction for 3 wk did not modify the expression of these 2 genes (Boutinaud et al., 2008). Similarly, in another study, a 6-mo period of feed restriction did not modify the expression of genes encoding proteins indicative of secretory activity; namely, *LALBA* and *ACACA* (Nørgaard et al., 2008). These results contrast, however, with the significant decrease in expression of *CSN3* and *LALBA* in response to 13 wk of 20% feed restriction (Dessauge et al., 2011) or when glucose availability is reduced (Gross et al., 2015). As previously reported (Boutinaud et al., 2015), more variation has

Table 10. Indicators of mammary epithelium integrity (plasma concentration of lactose, milk concentrations of Na⁺ and K⁺, and milk Na⁺:K⁺ ratio) in dairy cows from the feed-restricted group (n = 10) and the control group (n = 9) during a 29-d period of feed restriction (period 1) and a subsequent 35-d period of ad libitum feeding (period 2)

Item ¹	Period 1				Period 2			
	Control	Restricted	SEM	P-value	Control	Restricted	SEM	P-value
Plasma lactose (mg/L)	8.3	8.5	0.85	0.87	7.0	9.1	1.43	0.32
Milk Na ⁺ (mg/kg)	302	315	3.9	<0.05	320	332	6.6	0.23
Milk K ⁺ (mg/kg)	1,726	1,748	17.0	0.40	1,729	1,758	19.3	0.32
Milk Na ⁺ :K ⁺ ratio	0.18	0.18	0.003	0.17	0.19	0.19	0.004	0.50

¹Milk and plasma samples were collected on d 5, 9, and 27 after the beginning of period 1 and d 5, 9, and 30 after the beginning of period 2 for the analysis of mammary epithelium integrity indicators.

been observed in MEC purified from milk than in mammary tissue, indicating that analysis of gene expression in milk MEC would be a more sensitive method for detecting small changes. This could be due to the purification of MEC alone from milk, whereas mammary tissue contains different cell types that may dilute the RNA of interest and then hide the effect of treatment on gene expression. Another explanation could be that MEC with reduced synthetic activity are those targeted for exfoliation. The absence of significant variation in the expression of the gene encoding one of the main glucose transporters—*SLC2A1*—also contrasts with the decrease observed after 30% feed restriction (Boutinaud et al., 2008). The moderate intensity and short duration of the feed restriction in the present study may explain this absence of significant effects on the expression of genes involved in milk synthesis.

Feed restriction had no effect on the proportion of secretory tissue in the mammary tissue or on the size of the mammary lobules (perimeter, Feret's diameter, or area) but increased the DNA concentration in mammary tissue by 11%. This effect is in accordance with the effect of a longer and more severe feed restriction, which induced a 25% increase in mammary DNA concentration (Dessaige et al., 2011). In that study, the effect on DNA concentration was associated with a decrease in the number of MEC in the mammary gland due to an increase in the apoptosis rate (Dessaige et al., 2011), as previously observed in dairy ewes (Colitti et al., 2005). In our study, feed restriction had no effect on the balance of cell proliferation and apoptosis in the mammary tissue. Consistent with the lack of effect on the percentage of apoptotic cells, feed restriction in the present study did not affect expression of *CASP3* or *BAX*, 2 genes involved in apoptotic signaling. Similarly, Nørgaard et al. (2008) reported no effect of feeding level on the expression of proapoptotic genes or on the balance between cell proliferation and apoptosis, despite using a longer treatment duration than that in the present study. The discrepancies between studies regarding the balance between cell proliferation and apoptosis may be due not only to differences in duration and intensity of the feed restriction but also to differences in the stage of lactation of dairy cows, because the strongest effect was observed in early lactation (Nørgaard et al., 2005; Dessaige et al., 2011). Another explanation for the absence of variation in cell proliferation and apoptosis in our study may be the time of sampling. Specifically, mammary tissue was collected at the end of the feed restriction period, when the difference in milk yields between feed-restricted and control cows was stabilized. Indeed, the difference in milk yield in response to feed restriction progressively increased

during the transition period and for approximately 5 d during the feed-restriction period and then stabilized. The cell proliferation–apoptosis balance might have been more influenced by feed restriction during the first 5 d of feed restriction.

Another indicator of MEC number variation in mammary tissue is MEC exfoliation. In the present study, the decrease in milk yield induced by feed restriction was associated with a 65% increase in the MEC exfoliation rate. In dairy ruminants, lower milk yield has been previously associated with a higher MEC exfoliation rate in response to various physiological, environmental, or breeding factors (Herve et al., 2016). Moreover, the proportion of viable MEC exfoliated into milk tended to be higher in feed-restricted cows than in control cows. This finding, together with the increased DNA concentration in mammary tissue, suggests a decrease in the number of MEC in the tissue of feed-restricted cows. We previously suggested that PRL and Cort might regulate the MEC exfoliation process by limiting it (Lollivier et al., 2015; Herve et al., 2017a). In the present study, however, it is likely that the increase in the exfoliation rate did not depend on PRL concentrations, because PRL concentrations were not affected by feed restriction. Nevertheless, decreased Cort secretion at milking was observed in response to feed restriction. An increase in postprandial concentrations of Cort in plasma has been observed in response to feeding (Willett and Erb, 1972). In contrast, severe feed restriction of 50% has been shown to induce an increase in Cort concentration (Stumpf et al., 2013). The MEC exfoliation rate was negatively correlated with Cort concentration before and after milking, as previously reported (Herve et al., 2017a), but whether a causal relationship exists remains to be determined.

As expected, reduced plasma concentrations of IGF-1 and Ins were observed in feed-restricted cows. Indeed, feed restriction has been shown to induce a decrease in IGF-1 and Ins concentrations (Andersen et al., 2004; Dessaige et al., 2011; Ferraretto et al., 2014). It is well known that IGF-1 acts as a survival factor for MEC (Flint and Knight, 1997) and that both IGF-1 and Ins can stimulate MEC proliferation (Turkington, 1970; Shamay et al., 1988; Baumrucker and Stemberger, 1989). In the present study, the average circulating concentration of IGF-1 during feed restriction was negatively correlated with the MEC exfoliation rate. Therefore, we suggest that lower concentrations of IGF-1 may favor MEC exfoliation.

In dairy ruminants, a high MEC exfoliation rate has been associated with disruption of the mammary epithelium (Herve et al., 2016). In the present study, integrity of the mammary epithelium was assessed

by measuring plasma lactose concentrations and the $\text{Na}^+:\text{K}^+$ ratio in milk. Feed restriction had no effect on plasma lactose concentration or milk $\text{Na}^+:\text{K}^+$ ratio but increased milk Na^+ concentration; thus, we could not verify a clear effect on mammary epithelial integrity. During more severe feed restrictions (75% or less of control feed intake), in contrast, increases in mammary epithelium permeability have been observed (Stumpf et al., 2013; Herve et al., 2017b). In the present study, the MEC exfoliation rate at milking was positively correlated with the concentration of lactose measured before milking, milk Na^+ concentration, and the $\text{Na}^+:\text{K}^+$ ratio in milk, suggesting again that MEC exfoliation is associated with disruption of the mammary epithelium.

After the end of the feed restriction, cows were returned to ad libitum feeding to study the carryover effect of feed restriction. As early as 3 d after the return to ad libitum feeding, the milk yield of feed-restricted and control cows was similar, showing that feed restriction did not affect the milk production potential of dairy cows. Feed restriction had no carryover effect on the composition of milk except for protein percentage and protein yield, which remained slightly lower (−2 and −3%, respectively) in feed-restricted cows than in control cows after the switch back to ad libitum feeding. This carryover effect was not associated with reductions in the expression of *CSN3* and *CSN1S1* genes or the casein content. Concerning the metabolic activity of MEC, we observed no significant carryover effect of feed restriction. Feed restriction also had no carryover effect on the mechanisms determining the number of MEC in the mammary gland; that is, the MEC exfoliation rate and balance between cell proliferation and apoptosis. Nevertheless, as during the feed restriction period, variations in milk yield occurred during the feeding transition period and the first 2 d after the switch back to ad libitum feeding. We cannot exclude the possibility that changes in the balance between cell proliferation and apoptosis occurred in these few days. In contrast to the lack of carryover effects on other traits, the switch back to ad libitum feeding had a significant effect on plasma PRL and Cort concentrations. Specifically, feed-restricted cows had a greater PRL release at milking (a greater concentration after milking and a greater delta) and a lower basal concentration of Cort than control cows. Because PRL is known to increase MEC proliferation (Olazabal et al., 2000), limit MEC apoptosis (Accorsi et al., 2002), and increase MEC metabolic activity (Boutinaud et al., 2012), the absence of a carryover effect on milk yield may be due to the increased release of the galactopoietic hormone PRL at milking after the switch back to ad libitum feeding.

CONCLUSIONS

The decreased milk yield induced by feed restriction was not associated with any significant change in the expression of genes involved in the metabolic activity of MEC. Feed restriction also did not affect the balance between cell proliferation and apoptosis. In the present study, however, feed restriction was associated with an elevated rate of MEC exfoliation. The potential roles of IGF-1 and Cort in the increased exfoliation rate need to be investigated. Our results suggest that the exfoliation of MEC from the mammary epithelium may play a role in regulating milk production of dairy cows during feed restriction with no carryover effect after refeeding. During the switch back to ad libitum feeding period, other mechanisms such as the modulation of PRL release at milking may have limited the negative effect of MEC exfoliation on subsequent milk production.

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