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The decrease in milk yield during once daily milking is due to regulation of synthetic activity rather than apoptosis of mammary epithelial cells in goats

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Once daily milking (ODM) is a management practice that can improve working conditions and reduce production costs in dairy farming compared with twice daily milking (TDM). However, ODM is associated with a decrease in milk yield. Previous research indicated that disruption of tight junctions in the mammary gland may be one of the regulatory factors involved in the milk yield decrease observed during ODM. The aim of this study was to investigate the involvement of mammary epithelium disruption in the regulation of the activity and dynamics of mammary epithelial cells (MEC) during 5 weeks of ODM in goats. Twelve alpine goats (producing 3.67 ± 0.64 kg/day and 47 ± 1.6 days in milk) were assigned to two groups that were milked once or twice a day during 5 weeks and then switched back to TDM. Mammary biopsies were collected before and on days 2 and 16 of both ODM and TDM switchback periods. Milk purified epithelial cells were collected before and on days 1, 7, 21 and 28 during ODM as well on days 1 and 7 of the TDM switchback period. The mRNA levels of genes involved in the regulation of synthetic activity and apoptosis were analysed by RT-PCR in milk MEC and mammary biopsies. ODM decreased yields of milk (−23%), lactose (−23%) and casein (−16%). Lactose synthesis was regulated at the transcriptional level by downregulation of α-lactalbumin mRNA levels in both biopsy samples (−30%) and milk MEC (−74%). TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling) staining of mammary gland biopsies did not show any increase in cell apoptosis after 2 and 16 days of ODM (0.8% and 1%, respectively) despite upregulation of Bax mRNA levels in milk MEC. This suggests that the decrease in milk yield observed during ODM is attributable to a decrease in synthetic activity rather than to induction of MEC cell death. ODM induced the disruption of tight junctions in the mammary gland only on the first day of the treatment as indicated by increased blood lactose concentration. This indicates that the decrease in MEC activity observed over the 5 weeks of ODM was not due to disruption of the mammary gland tight junctions. There was no carryover effect of 5 weeks of ODM on milk production. Therefore, it appears that the decrease in milk yield that occurs during ODM in goats is due to regulation of synthetic activity rather than to apoptosis of MEC.

Keywords: milking frequency, mRNA, milk synthesis, mammary gland

Implications

Once daily milking (ODM) is a management practice that improves working conditions and reduces production costs in dairy farming, but decreases milk yield. By identifying the factors that contribute to this phenomenon, it will be possible to develop breeding strategies that can be used to reduce milk loss during ODM in goats. We investigated whether the reduction in milk yield that accompanies ODM could be explained by sustained disruption of the mammary epithelium. We showed that ODM does not affect MEC number but decreases the activity of these cells, and we found that the effect of ODM on milk yield is reversible after 5 weeks with no carryover effect.

Introduction

To maintain the sustainability of dairy farming operations, a reduction in production costs and an improvement in

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working conditions are needed. Once daily milking (ODM) is a management practice that contributes to the attainment of these objectives. However, the decrease in milk yield that is associated with ODM is a major drawback to its adoption. Milk yield is determined by the number of mammary epithelial cells (MEC) and their activity. Few data are available concerning the regulation of MEC activity and number during ODM. However, given that apoptosis is induced during mammary involution (Wilde *et al.*, 1999), the longer interval between milking during ODM should accelerate MEC apoptosis, which, by reducing MEC number, should reduce milk yield. We previously reported a decrease in synthetic activity and suggested that apoptosis of milk MEC may have been induced after 1 and 5 weeks of ODM in goats (Ben Chedly *et al.*, 2011) and after 1 week in cows (Boutinaud *et al.*, 2008). However, in those studies only changes at the transcriptional level were reported.

A decrease in milk yield is observed on the very first day that ODM is implemented. Such a quick response of the mammary gland to ODM suggests the existence of cellular activity regulation. To our knowledge, there are no data describing the early regulation of MEC dynamics and activity during the first days of ODM application. One of the putative regulatory mechanisms is the disruption of mammary epithelium integrity (Stelwagen, 2001). Disruption of mammary tight junctions is associated with a decrease in milk yield in different situations such as psychological stress (Stelwagen *et al.*, 2000), mastitis (Leitner *et al.*, 2004) and longer milking intervals (Stelwagen *et al.*, 1994; Delamaire and Guinard-Flament, 2006). It has recently been shown that the disruption of tight junctions in goat mammary gland can induce cell death and a decrease in mammary activity (Ben Chedly *et al.*, 2009 and 2010). However, there are no data showing the implication of the disruption of mammary epithelium during ODM in the regulatory mechanisms that may control the reduction in milk yield, such as regulation of cell number and activity of MEC.

In order to study regulation of cell number and activity in the mammary gland implied in milk synthesis, we have to sample MEC. There are invasive techniques to recover MEC from the mammary gland such as sampling tissue after slaughter or using a biopsy needle. In order to reduce the invasive effect of the study we should minimise the number of mammary biopsy collection and chose other techniques especially for kinetic studies. The collection of MEC from milk is a non-invasive method that has been used previously to study mammary gene expression in cows (Hayashi *et al.*, 2004; Murrieta *et al.*, 2006; Boutinaud *et al.*, 2008) and in goats (Boutinaud *et al.*, 2002; Ben Chedly *et al.*, 2011). The use of this technique avoids the carryover effect of sampling that may be observed in mammary biopsies.

In the present study, we investigated the involvement of the disruption of mammary epithelium integrity during ODM and the regulation of the synthetic activity and apoptosis of MEC in goats. Another objective of this study was to compare the results obtained using mammary biopsies and MEC collection in the milk.

Material and methods

Animals and experimental design

Twelve alpine goats producing 3.67 ± 0.64 kg/day of milk in their first 47 ± 1.6 days in milk (DIM) were selected on the basis of their well-balanced milk yield and lactose and protein content. Goats were milked in double 12-stall parallel milking parlour using a low line with 38 kPa vacuum pressure. The pulsation rate was 120 pulsations/min and the pulsation ratio was 60 : 40. During the trial on the 48th day of lactation, all the goats were fed according to INRA recommendations (INRA, 2007) with alfalfa hay *ad libitum* and 800 mg concentrate. Before the experiment started, all goats were milked twice a day at 0700 and 1600 h.

During the experimental period, the goats were assigned to two groups: a control group, which was milked twice daily over the trial, and a once daily milking/twice daily milking (ODM/TDM) group, which was subjected to ODM during 5 weeks and switched back to TDM for the following 5 weeks. The ODM/TDM group was milked at 0700 h during the ODM period. During the ODM period, goats were separated physically into two groups in order to facilitate the access to the milking parlour and to avoid error in milking management of the two groups. During the TDM switchback period, all goats were regrouped in a single pen.

Milk and mammary tissues samples

Milk production was recorded at each milking, 7 days a week. Twice a week, the lactose, fat and protein contents and somatic cell counts (SCC) in milk samples collected at morning and afternoon milkings were determined by a commercial laboratory using infrared analysis (Lillab, Châteaugiron, France).

Milk samples were collected from all goats in the two groups on days −8, 1, 16, 21 and 35 during the ODM period and on days 1 and 16 of the TDM switchback period, to analyse total N (Kjeldahl), non-protein N (precipitation at pH 4.6 with TCA and filtration) and non-casein N (precipitation at pH 4.6 with 10% acetic acid and 1 M sodium acetate) content. Casein was determined as total N minus non-casein N and whey protein as non-casein N minus non-protein N.

Mammary tissue was sampled by collecting mammary biopsies from both half udders with a 12G/10 cm, 22 mm Bard Monopty disposable core biopsy instrument (Bard, Voisins Le Bretonneux, France) as described by Ben Chedly *et al.* (2011). The mammary biopsy samples were collected on day 8 before the start of the ODM period, as well as on days 2 and 16 of ODM and on days 2 and 16 of the TDM switchback period for all goats in both groups. The biopsies were used for total RNA extractions and histochemistry. Three 20 mg biopsy samples were frozen in liquid nitrogen and stored at −80°C until used for RNA extractions. The biopsy sample used for histochemistry was washed in PBS and fixed in 4% paraformaldehyde for immuno-histochemistry. The fixed tissue was cryoprotected by 48 h incubation in a 40% sucrose solution and then coated with OCT Compound Tissue-Tek (Sakura Finetek Europe, LaboNord, Templemars, France), frozen in a cooled bath of isopentane and stored at −80°C until use.

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Blood samples were collected from the jugular vein before morning milking on the 6th day before the start of the trial, on days 1, 2 and 16 of the ODM period, and on days 2 and 16 of the TDM switchback period. These blood samples were used for lactose analysis with a colorimetric reaction (kit for lactose/D-galactose, Roche Diagnostico, Meylan, France) and a multiparameter analyzer (Kone Instrument Corporation, Espoo, Finland).

Purification of MEC

Milk samples (0.9 kg) were collected on the 8th day before the start of the experiment, on days 1, 7, 21 and 28 of the ODM period, and on days 1 and 7 of the TDM switchback period in both groups of goats, in order to perform MEC purification as described by Ben Chedly *et al*. (2011). A Vi-CELL XR analyzer (Beckman Coulter, Roissy, France) to determine the total milk cell count and cell viability.

RNA extraction and real-time RT-PCR

The extraction of total RNA from milk purified MEC and mammary biopsies was performed in Trizol, according to the manufacturer's recommendations. The RNA pellet was suspended in RNase-free water and the total quantity of RNA was determined with an Agilent 2100 bioanalyzer (Agilent Technologies, Massy, France). RNA quality was assessed using the RNA integrity number generated by version B.02 of Agilent 2100 Expert Software (Agilent Technologies). Twelve samples of RNA from a total of 84 milk MEC preparations were eliminated from the analysis due to their poor RNA quality.

Complementary DNA was obtained using the First Strand cDNA kit (Roche Diagnostics), according to the manufacturer's instructions, with 500 ng of total RNA. PCR amplifications of cDNA samples were performed using the primers described by Boutinaud *et al*. (2008) for cyclophilin, α-LA (α-lactalbumin), κ-casein and Bax. The primers used for β-actin, r18S, CLPN-2, MMP-2, occludin, E-cadherin and caspase-3 are described in Ben Chedly *et al*. (2009).

Real-time PCR was performed as described by Ben Chedly *et al*. (2011).

Quantification of RNA

The number of amplified mRNA molecules was determined using the method described by Ben Chedly *et al*. (2009). The mRNA levels of the studied genes were expressed relative to a reference gene. The cyclophilin, β-actin, r18S and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes were evaluated as potential reference genes. The effect of treatments was evaluated in order to identify the gene that showed the least variation. The gene with the most stable expression in both milk MEC and biopsy samples was r18S. Thus, r18S was used as the reference gene during this study. The results for each target gene are expressed as a ratio using the selected reference gene.

Histochemistry

Apoptotic cells were quantified in mammary gland biopsies on the 8th day before the start of the experiment and on days 2 and 16 of the ODM period. This quantification was done by the detection of DNA fragmentation using the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling) method using 7-μm-thick cryosections as described by Ben Chedly *et al*. (2009).

E-cadherin labelling was performed on mammary gland biopsies corresponding to the second day of the ODM period in all goats using 7-μm-thick cryosections as described by Ben Chedly *et al*. (2009).

Proliferating MEC were identified as cells expressing the proliferating cell nuclear antigen (PCNA) in mammary biopsies from days 2 and 16 of the ODM period in all goats using 7-μm-thick cryosections as described by Ben Chedly *et al*. (2009). Alveolar characteristics were determined with propidium iodide staining, which reveals the outlines of the acini. For each acinus, the ImageJ software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA) was used to count the number of epithelial cells and to determine alveolar size.

Statistical analysis

Milk data obtained during the ODM period and during TDM switchback period were analysed separately using the same statistical model. Milk yield and composition and gene expression data were analysed by ANOVA using the MIXED procedure of SAS (SAS Institute, 1999) with the REPEATED statement. Days were used as repeated effects and goats as subjects. Data obtained on day-7 were used as covariates. The effects of group, day and the interaction group $\times$ day were tested. Milk SCC were $\log_{10}$ transformed prior to the analysis. For gene expression data, the ratio of the number of mRNA molecules of the target gene to the corresponding number of molecules of r18S gene was multiplied by 10^6 and $\log_{10}$ transformed and analysed as milk data. Gene expression data obtained from biopsy samples and milk MEC were analysed separately. Total MEC and MEC in milk were analysed as gene expression data. The TUNEL and E-cadherin labelling data were analysed by ANOVA using the MIXED procedure of SAS. The effects of group, day and the interaction group $\times$ day were tested. Goats were used as a RANDOM effect.

Results

Milk yield and composition

The ODM/TDM goats produced less milk (22%; $P < 0.001$) than control goats during the 5 weeks of ODM (Figure 1). When all goats were milked twice a day during the TDM switchback period, the ODM/TDM group produced a level of milk yield similar to that of the control group (Figure 1). There was an apparent drop in milk production in control goats at the onset of the TDM switchback period. This is related to the biopsies performed on day 2 of the switchback period. The ODM goats were also submitted to the same procedure but the effect is less apparent because milk production was still increasing at that time.

During the ODM period, the goats milked once a day produced less lactose, fat and protein in the milk than the

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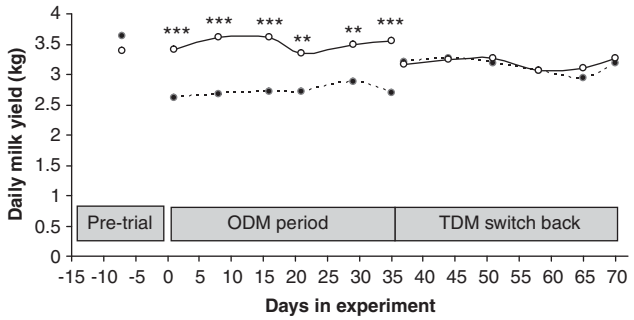


Figure 1 Milk yield of goats milked once a day (once daily milking (ODM)/twice daily milking (TDM)) or twice a day (control). Data are presented as LSM except for day 7, which are means. Statistically different means during a specific time are indicated by $***P < 0.001$ and $**P < 0.01$. —○—, control group; – – ● – –, ODM/TDM group.

control goats (Table 1). Casein yield, but not whey protein yield, was reduced in ODM/TDM goats (Table 1). Milk lactose concentration was similar for the two groups of goats during the ODM period. Protein content was higher, casein content tended to be higher, and whey protein content was higher in ODM/TDM goats than in the control goats. Accordingly, the casein : whey protein ratio was lower in the ODM/TDM group than in the control group (Table 1). There was a treatment × time interaction on milk fat content, with milk of the ODM/TDM group containing less fat during the 1st week of treatment and more fat at the end of the 4th week, as shown in Table 1.

When all animals were on TDM, the lactose, fat, protein and casein yields were similar for the ODM/TDM and control groups (Table 2). Lactose content tended to be lower in the ODM/TDM goats than in the control goats. Fat and protein content were higher in ODM/TDM goats as shown in Table 2. The milk whey protein content remained higher in ODM/TDM goats, while casein content was similar for the two groups of goats. This led to a lower milk casein : whey protein ratio in the ODM/TDM group.

Blood lactose

A higher blood lactose concentration was observed in ODM/TDM goats after the first 24 h of milk accumulation during the ODM period (Figure 2). However, blood lactose concentrations were similar in ODM/TDM and control goats after 2 days and 2 weeks of ODM ($P > 0.10$). Similar blood lactose concentrations were observed for the two groups of goats during the second day and after the second week of TDM (Figure 2).

Milk cells

During the ODM period, SCC was higher for ODM/TDM goats than for control goats (Table 1). The number of MEC per ml of milk recovered and the estimated loss of MEC per day (cells/day) were higher for milk from the ODM/TDM group than from the control group during the ODM period (Table 3). However, the viability of milk MEC was similar for the ODM/TDM group and the ODM group (85.7 ± 2.5% *v.* 83.5 ± 2.8%, $P = 0.58$).

Table 1 *Milk composition of goats milked once a day (ODM/TDM) or twice a day (control) during the once-a-day milking period*

	1st day		7th day		14th day		21st day		28th day		35th day			*P*-values[1]		
Items	ODM/TDM	Control	ODM/TDM	Control	ODM/TDM	Control	ODM/TDM	Control	ODM/TDM	Control	ODM/TDM	Control	s.e.m.	Milking	Time	Milking × time
Lactose																
g/kg	47.8	47.5	47.4	46.5	46.7	46.5	46.9	47.4	46.3	47.0	46.0	47.0	0.46	0.80	0.005	0.03
g/day	123.6**	161.9	125.6***	168.1	126.4***	167.7	127.4**	158.1	132.2**	164.0	123.5***	166.3	6.05	0.001	0.26	0.12
Fat																
g/kg	36.2	39.6	36.6*	42.6	35.7	35.5	36.3	34.8	38.0*	31.6	34.7	33.9	1.57	0.95	0.001	<0.001
g/day	94.2**	131.7	99.3***	149.0	99.2*	126.9	100.6	115.1	110.8	108.6	94.3*	118.6	7.08	0.02	<0.001	<0.001
Protein																
g/kg	28.6	27.5	30.2**	27.6	30.3**	27.5	30.3*	27.9	30.5*	28.5	29.7	28.3	0.59	0.009	0.05	0.29
g/day	74.2*	93.6	80.8*	99.8	82.6*	98.6	83.0	92.9	87.5†	99.6	80.2*	100.1	4.62	0.02	0.001	0.10
Casein																
g/kg	23.3	22.6			23.4†	22.0	23.8	22.6			22.8	21.8	0.48	0.07	0.06	0.82
g/day	61.0**	77.0			64.2**	79.1	65.3†	75.3			61.7*	77.1	3.53	0.01	0.26	0.16
Whey protein																
g/kg	5.3	4.8			6.4**	4.8	5.7**	4.5			6.7***	4.6	0.27	<0.001	0.008	0.009
g/day	13.9	16.0			17.6	17.1	15.7	14.9			18.3	16.3	1.20	0.84	<0.001	0.03
Casein : whey ratio	4.4	4.8			3.7*	4.7	4.2*	5.2			3.5**	4.8	0.24	0.005	0.004	0.12
$\log_{10}$ SCC	5.7	5.4	5.7	5.3	5.7	5.4	5.8	5.5	5.9	5.5	5.7	5.4	0.16	0.04	0.75	0.99

ODM = once daily milking; TDM = twice daily milking; SCC = somatic cell counts.
[1] *P*-values indicate the effect of milking frequency and its interaction with time in a repeated measures analysis.
Statistically different means for ODM/TDM and the control group for a specific time are indicated by: $***P < 0.001$; $**P < 0.01$; $*P < 0.05$ and $^{\dagger}P < 0.10$.

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Table 2 *Milk composition of goats milked once a day (ODM/TDM) or twice a day (control) during the twice-a-day switchback period*

	1st day		7th day		14th day		21st day			*P*-values[1]		
Items	ODM/TDM	Control	ODM/TDM	Control	ODM/TDM	Control	ODM/TDM	Control	s.e.m.	Milking	Time	Milking × time
Lactose												
g/kg	44.4*	46.2	46.4	47.2	45.3*	47.2	45.9	46.5	0.52	0.06	0.004	0.16
Fat												
g/kg	35.1***	28.0	37.4†	33.9	33.6	31.8	35.4	34.9	1.34	0.04	0.002	0.03
Protein												
g/kg	29.4*	27.3	29.0†	27.6	29.0	28.3	29.0	27.9	0.56	0.03	0.87	0.58
Casein												
g/kg	23.1	22.4			23.4	23.1			0.62	0.54	0.36	0.68
Whey												
g/kg	6.3**	5.0			5.5*	4.6			0.28	0.01	0.02	0.33
g/day	20.2*	15.7			17.5	15.1			1.43	0.09	0.12	0.31
Casein : whey ratio	3.8*	4.7			4.3†	5.1			0.28	0.04	0.03	0.58
$\log_{10}$ SCC	5.8*	5.4	5.9*	5.5	5.4	5.2	5.0*	5.5	0.12	0.006	0.001	0.71

ODM = once daily milking; TDM = twice daily milking; SCC = somatic cell counts.
[1]*P*-values indicate the effect of milking frequency and its interaction with time in a repeated measures analysis.
Statistically different means for ODM/TDM and control groups for a specific time are indicated by: ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$ and $^{\dagger}P < 0.10$.

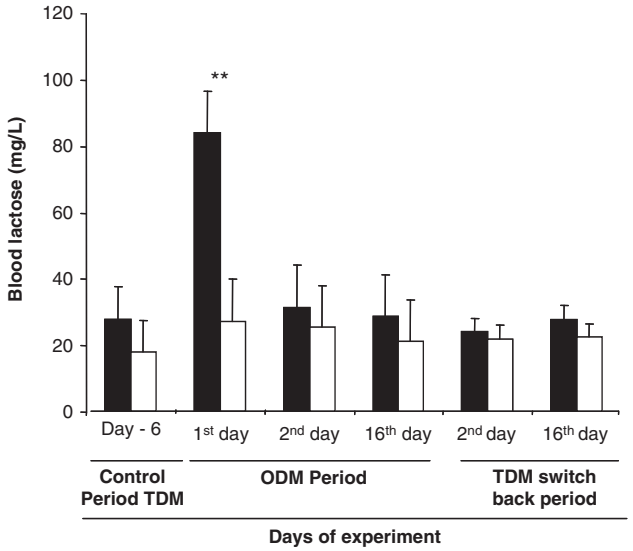


Figure 2 Blood lactose concentration for goats milked once a day (ODM/TDM, ■) or twice a day (control, □). Data are presented as LSM ± s.e.m. except for day 6, which are means. Statistically different means for a specific time are indicated by **$P < 0.01$. ODM = once daily milking; TDM = twice daily milking.

The concentration of milk MEC and the estimated loss of MEC per day remained higher in the ODM/TDM group than in the control group (Table 4) during the TDM switchback period. However, cell viability was similar for the two groups of goats with regard to milk MEC (82.4 ± 3.5% and 88.2 ± 3.8%, respectively for ODM/TDM and Control, $P = 0.29$).

Gene expression

We observed lower levels of α-LA mRNA in ODM/TDM goats compared with control goats during the ODM period in milk MEC sampled after 1, 3 and 4 weeks of ODM application and in mammary biopsies taken after the second day and the second week of the ODM period as shown in Tables 3 and 5. The mRNA level of κ-CN tended to be lower in milk MEC samples from ODM/TDM goats than from control goats in the ODM period (Table 3) but similar levels were found in mammary tissue (Table 5). The mRNA level of galactosyltransferase (GT) did not change with milking frequency.

The expression of gene indicator of cell death varied with milking frequency; however, the results differed according to the indicators used and between milk MEC and mammary tissue. We observed a higher mRNA level of Bax in milk MEC of ODM/TDM goats compared with control goats (Table 3), whereas in the mammary biopsy samples the Bax mRNA level did not change with milking frequency (Table 5). A higher mRNA level of CLPN-2 and a tendency for a higher mRNA level of caspases-3 were observed in mammary biopsies from the ODM/TDM group compared with the control group after 2 weeks of ODM (Table 5). The mRNA levels of MMP-2 in mammary tissue were similar for the ODM/TDM and control groups during the ODM period.

Similar mRNA levels of occludin and E-cadherin in mammary biopsies were observed in ODM/TDM and control goats during the ODM period, as reported in Table 5. The E-cadherin mRNA level in milk MEC was also similar for ODM/TDM and control goats (Table 3).

During the TDM switchback period, similar mRNA levels of α-LA and κ-CN in milk MEC and mammary tissue were observed for the ODM/TDM and control groups, as reported in Table 4 and Table 6. In milk MEC, no carryover effect of ODM was observed during the TDM switchback period for any of the evaluated genes. Nevertheless, mammary tissue from ODM/TDM goats contained a higher mRNA level of caspases-3 and tended to contain a higher mRNA level of Bax in comparison with the control goats. Similarly, E-cadherin mRNA tended to be more expressed in ODM/TDM mammary biopsies than in that of control goats (Table 6).

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Table 3 *MEC recovered in milk and log_{10} of target gene mRNA levels relative to r18S mRNA levels in milk MEC of goats milked once a day (ODM/TDM) or twice a day (control) during the once-a-day milking period*

	1st day		7th day		21st day		28th day			*P*-values		
Items	ODM/TDM	Control	ODM/TDM	Control	ODM/TDM	Control	ODM/TDM	Control	s.e.m.	Milking	Time	Milking × time
Milk MEC (×103 cells/ml)	13	4	55	29	68†	30	63*	23	7	0.02	0.02	0.64
Milk MEC (×106 cells/day)	38	12	149	110	186	104	197*	83	19	0.04	0.007	0.60
α-LA	5.48	5.90	5.47**	6.22	5.37	5.75	5.20**	5.96	0.29	0.01	0.39	0.61
κ-CN	5.72	5.92	5.94	6.45	5.89	6.26	5.72*	6.41	0.33	0.06	0.53	0.77
Bax	3.41	3.15	3.58	3.13	3.62	3.12	4.06	3.56	0.33	0.03	0.10	0.96
Caspase-3	2.29	2.29	2.96	2.68	2.43	2.47	2.71	2.73	0.28	0.73	0.06	0.77
E-cadherin	2.20	2.06	2.77	2.76	2.74	3.05	3.20†	3.02	0.42	0.98	0.04	0.80

MEC = mammary epithelial cells; ODM = once daily milking; TDM = twice daily milking; α-LA = α-lactalbumin; κ-CN = κ-casein.
[1] *P*-values indicate the effect of milking frequency and its interaction with the time of treatment for all data in a repeated measures analysis.
Statistically different means for ODM/TDM and the control group for a specific time are indicated by: $^{**}P < 0.01$; $^{*}P < 0.05$ and $^{\dagger}P < 0.10$.

Table 4 *Mammary epithelial cells (MEC) recovered in milk and log10 of target gene mRNA levels relative to r18S mRNA levels obtained in milk MEC of goats milked once a day (ODM/TDM) or twice a day (control) during the twice-a-day switchback period*

	1st day		7th day.			*P*-values		
Items	ODM/TDM	Control	ODM/TDM	Control	s.e.m.	Milking	Time	Milking × time
Milk MEC (×103 cells/ml)	45	28	60*	22	7	0.03	0.62	0.29
Milk MEC (×106 cells/day)	140	89	186*	71	21	0.002	0.63	0.28
α-LA	5.82	5.66	5.58	5.99	0.23	0.63	0.85	0.21
κ-CN	6.30	6.15	5.80	6.40	0.30	0.51	0.67	0.22
Bax	3.33	3.27	3.63	3.57	0.19	0.75	0.13	0.98
Caspase-3	2.64	2.39	2.55	2.65	0.26	0.77	0.73	0.50
E-cadherin	3.27	2.87	3.25	3.62	0.42	0.98	0.37	0.35

MEC = mammary epithelial cells; ODM = once daily milking; TDM = twice daily milking; α-LA = α-lactalbumin; κ-CN = κ-casein.
[1] *P*-values indicate the effect of milking frequency and its interaction with the time of treatment for all data in a repeated measures analysis.

Table 5 *Log_{10} of target gene mRNA levels relatively to r18S mRNA levels obtained in mammary tissue of goats milked once a day (ODM/TDM) or twice a day (control) during the once-a-day milking period*

	2nd day		16th day			*P*-values[1]		
Items	ODM/TDM	Control	ODM/TDM	Control	s.e.m.	Milking	Time	Milking × time
α-LA	7.00*	7.22	7.17	7.26	0.06	0.04	0.12	0.28
GT	4.94	5.06	5.18	5.16	0.05	0.35	0.008	0.19
κ-CN	6.86	6.88	6.99	6.96	0.06	0.99	0.10	0.67
Caspase-3	2.43	2.49	2.61†	2.50	0.05	0.57	0.11	0.07
Bax	2.51	2.52	2.50	2.47	0.23	0.96	0.92	0.92
CLPN-2	4.05	4.06	4.14*	3.99	0.05	0.13	0.88	0.12
MMP-2	2.67	2.73	3.62	3.49	0.16	0.83	<0.001	0.55
Occludin	2.67	2.65	2.63	2.56	0.06	0.53	0.32	0.70
E-cadherin	4.03	3.95	4.16	4.14	0.08	0.54	0.07	0.74

ODM = once daily milking; TDM = twice daily milking; GT = galactosyl-transferase; α-LA = α-lactalbumin; κ-CN = κ-casein.
[1] *P*-values indicate the effect of milking frequency and its interaction with time of treatment for all data in a repeated measures analysis.
Statistically different means for ODM/TDM and control groups for a specific time are indicated by: $^{*}P < 0.05$ and $^{\dagger}P < 0.10$.

Histochemical analyses of mammary tissue

The proportion of apoptotic cells in mammary tissue measured after TUNEL staining was similar for the ODM and control goat groups after 2 and 16 days during the ODM period ($P = 0.79$), as shown in Figure 3a. The TUNEL labelled apoptotic cells were more abundant after 16 days of experiment than after 2 days ($P = 0.02$) without a substantial difference between the milking frequencies ($P = 0.10$).

The proliferation of MEC in the mammary tissue, evaluated by the count of PCNA labelled cells, was similar for the

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Table 6 *Log_{10} of target gene mRNA levels relatively to r18S mRNA levels obtained in mammary tissue of goats milked once a day (ODM/TDM) or twice a day (control) during the twice-a-day switchback period*

	2nd day		16th day			*P*-values		
Items	ODM/TDM	Control	ODM/TDM	Control	s.e.m.	Milking	Time	Milking × time
α-LA	7.32	7.23	7.33	7.25	0.06	0.20	0.87	0.88
GT	5.10	5.01	5.30	5.25	0.06	0.28	0.006	0.79
κ-CN	6.87	6.88	6.90	6.79	0.07	0.45	0.67	0.41
Caspase-3	2.53*	2.36	2.50	2.43	0.05	0.04	0.74	0.36
Bax	2.61	2.49	2.87†	2.62	0.09	0.06	0.06	0.46
CLPN-2	4.05	4.04	3.97	3.90	0.05	0.39	0.05	0.56
MMP-2	3.13	3.13	3.12*	2.88	0.08	0.14	0.14	0.16
Occludin	2.49	2.53	2.57	2.48	0.07	0.66	0.86	0.36
E-cadherin	4.03	3.92	3.96	3.86	0.06	0.09	0.45	0.92

ODM = once daily milking; TDM = twice daily milking; GT = galactosyl-transferase; α-LA = α-lactalbumin.
[1]*P*-values indicate the effect of milking frequency and its interaction with time of treatment for all data in a repeated measures analysis.
Statistically different means for ODM/TDM and control groups for a specific time are indicated by: $^{*}P < 0.05$ and $^{\dagger}P < 0.10$.

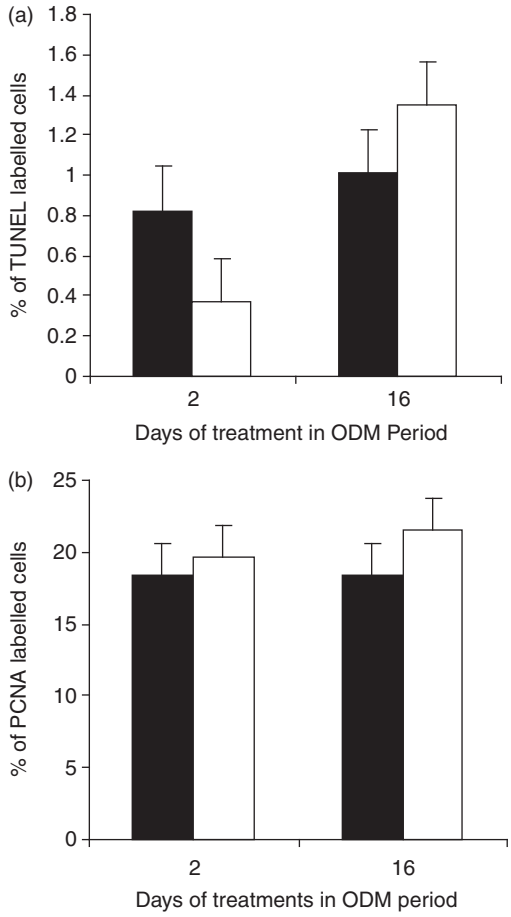


Figure 3 Percentage of TUNEL (a) or PCNA (b) labelled cells in mammary tissue from goats milked once a day (■) or twice a day (□) for 2 and 16 days. Data are presented as LSM ± s.e.m. TUNEL = terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling; PCNA = proliferating cell nuclear antigen; ODM = once daily milking.

ODM/TDM and control groups after 2 and 16 days in the ODM period ($P = 0.46$, Figure 3b). The PCNA labelled cells were similar after 2 and 16 days of experiment ($P = 0.47$) without any difference between the milking frequencies ($P = 0.44$).

Alveolar size (24 300 ± 2100 and 20 700 ± 2100 μm^2, respectively for ODM/TDM and Control, $P = 0.27$) and the number of cells per alveolus cross section (33.4 ± 1.5 and 32.2 ± 1.5, respectively for ODM/TDM and Control, $P = 0.51$) were similar for the ODM/TDM and control goats during ODM period. Similarly, the number of alveoli per area unit did not differ significantly between the milking frequencies (5.4 ± 0.3 and 5.6 ± 0.3, respectively for ODM/TDM and Control, $P = 0.79$). Only the effect of time was observed with higher cell number per alveoli and number of alveoli per area unit as lactation advanced ($P = 0.02$ and $P = 0.002$, respectively).

Similar labelling intensity of E-cadherin was observed in both groups of goats after 2 days of ODM ($P = 0.88$, data not shown).

Discussion

Decrease in milking frequency, especially ODM, is generally associated with a decline in milk yield (Davis *et al.*, 1999; Pomiès *et al.*, 2008). The decline in milk yield varies between 6% and 40% due to different factors, such as the stage of lactation, breed of animals and duration of the treatment (Marnet and Komara, 2008). The 22% decrease in milk yield observed over the 5 weeks of ODM in this trial was in agreement with a previous study reporting a 20% milk loss in goats milked once a day (Salama *et al.*, 2003). In the present trial, we did not observe a time-dependent effect, as milk yield loss was found to be constant over the 5-week ODM period. This is consistent with the observations of Komara *et al.* (2009), but not with our previous observations (Ben Chedly *et al.*, 2011) or those of Salama *et al.* (2003), in which milk yield loss was greater during the first weeks of ODM. This difference is likely attributable to the longer experimental period in those studies (15 and 24 weeks, respectively).

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The decrease in milk yield observed during ODM was associated with a decrease in milk lactose yield. Loss of lactose through disrupted tight junctions would decrease the amount of lactose recovered in milk (Stelwagen *et al.*, 1994). However, ODM only induced transient tight junction disruption, which was limited to the first day of ODM. This is consistent with an acute regulation of milk synthesis through tight junction disruption followed by metabolic adaptations probably involving changes in milk secretion after actin skeleton modifications as proposed by Knight *et al.* (1988). Thus, the reduction of milk lactose yield is essentially due to a reduction of its synthesis by the mammary gland. The decrease in lactose synthesis during ODM seems to be regulated at the transcriptional level since a lower mRNA level of α-LA was observed over the 5 weeks of the ODM period. Downregulation of the α-LA transcripts in ODM was observed in both milk MEC and tissue samples. The decrease in α-LA mRNA levels observed in the present experiment is consistent with our previous observations in goats (Ben Chedly *et al.*, 2011) and cows (Boutinaud *et al.*, 2008) in milk MEC and in bovine mammary tissue (Boutinaud *et al.*, 2009; Littlejohn *et al.*, 2010; Grala *et al.*, 2011). Manipulation of α-LA transcript levels in mice by deletion and replacement has been linked to changes in milk volume (Stacey *et al.*, 1995). Therefore, we believe, as suggested by Kuhn *et al.* (1980), that lactose synthesis and, consequently, milk volume, is regulated by the synthesis of α-LA.

The higher milk protein content observed in the ODM/TDM group compared with the control group during the ODM period indicates that protein synthesis decreased less than milk volume. Accordingly, only a trend toward lower κ-CN mRNA levels was detected in MEC. Downregulation of κ-CN was previously reported during ODM in cows (Boutinaud *et al.*, 2008) and also in goats (Ben Chedly *et al.*, 2011).

The increase in milk whey protein content can be attributed, in part, to a leakage of serum protein into milk through disrupted tight junctions (Stelwagen *et al.*, 1994). However, tight junction disruption was transient and cannot explain our observations. The increase in milk whey protein during ODM might be attributable to higher levels in serum albumin and IgG still observed after the closure of the tight junctions probably associated with the higher level of SCC (Stelwagen and Lacy-Hulbert, 1996; Auldist and Prosser, 1998). However, it can also be due to an increase in casein hydrolysis, which could generate small protein fragments quantified as whey proteins. An increase in proteases such as plasmin was previously reported in cows during ODM (Stelwagen *et al.*, 1994). In addition, the synthesis of some whey proteins such as β-lactoglobulin might be less sensitive to inhibition of milk accumulation.

The results obtained in this study reveal time-dependent regulation of milk fat synthesis, which must be confirmed through further studies. However, the variation in milk fat content observed over the different weeks of the ODM period can explain the contradictory effects reported in various studies. Some studies reported an increase in milk fat content (Stelwagen *et al.*, 1994; Lacy-Hulbert *et al.*, 1999; O'Brien *et al.*, 2002) but others reported no variation (Rémond *et al.*, 2002; Marnet and Komara, 2008) or a decrease (Ben Chedly *et al.*, 2011) in milk fat content during ODM. Our finding suggests that the inhibition of milk fat synthesis by ODM may gradually disappear beginning in the second week of treatment.

It has been suggested that the decrease in milk yield during ODM can be attributed to a loss of MEC in the gland caused by apoptosis. In this study, similar apoptosis and proliferation rates were observed in both groups after 2 and 16 days in the ODM period. Accordingly, similar size of alveoli and number of cells per alveolus were observed for ODM/TDM and control goats. This suggests that apoptosis is not involved in the decrease in milk yield observed during ODM in goats. A previous study reported similar apoptotic rates in mammary tissue after 3 weeks of unilateral ODM in goats (Boutinaud *et al.*, 2003). An increase in cell death in mammary tissue was reported after 4 weeks of unilateral ODM in goats (Li *et al.*, 1999). We found that the milk yield of ODM/TDM goats reached that of the control goats as soon as TDM was resumed. The carryover effect of ODM seems to be dependent on the duration of its application (Pomiès *et al.*, 2008). According to Li *et al.* (1999), apoptosis induction during ODM is a time-dependent phenomenon with high DNA laddering in mammary tissue after 4 weeks, but not after 10 weeks, in glands subjected unilaterally to ODM. However, our data obtained with milk cells suggested a higher loss of epithelial cells through the milk during ODM as already observed in a previous study (Ben Chedly *et al.*, 2011). Further studies should be investigated in order to know whether the higher exfoliation of MEC could account for the reduction in milk yield. The absence of any carryover effect of ODM during the TDM period support our histological data and suggests that ODM for 5 weeks did not significantly affect the number of MEC in goat mammary gland.

Despite the lack of an observable increase in cell apoptosis, we observed an increase in pro-apoptotic transcripts in MEC during ODM, which agrees with previous observations related to milk MEC after 1 and 5 weeks of ODM in goats and cows (Boutinaud *et al.*, 2008; Ben Chedly *et al.*, 2011). We can hypothesise that milk accumulation during 24 h in mammary gland induces cellular regulation, which initiated the apoptosis process by overexpressing pro-apoptotic transcripts. However, maintaining the lactogenic stimulus by milking may inhibit the apoptotic process, which may explain the limited detection of the last steps in apoptosis by TUNEL after 2 weeks of ODM in goats. Nevertheless, pro-apoptotic transcripts in mammary tissue biopsies were not affected by the treatment in contrast with results obtained in cows (Boutinaud *et al.*, 2009; Grala *et al.*, 2011). The higher pro-apoptotic gene expression in milk MEC could result from a longer stay of MEC in the cistern of the gland due to 24 h milk accumulation, and that could constitute an artefact of this method of cell collection. Since the viability of milk MEC was similar between the two groups of animals, we believe that milk cells are freshly exfoliated after myoepithelial

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contraction during the milking. Further investigations on anti-apoptotic transcripts and on the characterisation of apoptosis in milk MEC will give us more information on the mammary apoptotic process during ODM.

In the present experiment, we also investigated the implication of tight junction disruption in the decrease of milk yield during ODM. The disruption of mammary epithelium integrity is a phenomenon associated with a decrease in milk yield (Stelwagen *et al.*, 2000; Leitner *et al.*, 2004; Delamaire and Guinard-Flament, 2006). The accumulation of milk in mammary gland during less than 18 h for cows and 21 h for goats induces a disruption of tight junctions (Stelwagen *et al.*, 1994 and 1997). In our experiment, we showed that ODM induced a disruption of tight junctions as suggested by the studies reporting longer milk accumulation in mammary gland. However, our finding revealed that the disruption of tight junctions occurs only on the first day of ODM application and disappears thereafter. The disruption of mammary epithelium has been associated with a decrease in the expression of constitutive tight and adherens junctions protein such as occludin and E-cadherin respectively (Ben Chedly *et al.*, 2010). The similar labelling intensity of E-cadherin observed for the ODM/TDM and control groups after 2 days of the ODM period suggests that adherens junctions was not affected by ODM after 2 days of treatment. The analysis of blood lactose concentrations confirmed that tight junctions had already returned to a tight state on the second day of ODM. The similar mRNA levels of E-cadherin and occludin observed in the ODM/TDM group and the control group after the second day and week of ODM is consistent with the similar expression levels of E-cadherin in that period of ODM application. Although the disruption of tight junctions was transient, the decrease in milk yield persisted over the 5 weeks of ODM. This indicates that the disruption of tight and adherens junctions was not essential for the inhibition of milk synthesis during ODM. Nevertheless, we cannot rule out the possibility that the disruption of tight and adherens junctions observed at the onset of ODM may initiate cellular regulation that induces an inhibition of milk synthesis.

The results obtained in this experiment indicate that the decrease in milk yield was largely attributable to a decrease in MEC activity as evidenced by the decrease in milk lactose and protein associated with a downregulation of transcripts involved in milk synthesis. Comparison of gene expression of mammary biopsies and milk cells harvested on consecutive days (days 1 and 2 of each period), shows good level of agreement between the methods for all gene, except for Bax suggesting that milk cells constitute an alternative method for studying gene expression in mammary cells. However, we were unable to demonstrate that ODM affects MEC number. We found no evidence that the disruption of tight junctions was involved in the decrease in milk yield during the ODM period. The inhibition of milk synthesis observed during ODM is attributed to a decrease in MEC synthetic activity rather than to a decrease in their number.

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References

Auldist MJ and Prosser CG 1998. Differential effects of short-term once daily milking on milk yield, milk composition and concentration of selected blood metabolites in cows with high or low pasture intake. Proceedings of the New Zealand Society of Animal Production 58, 41–43.

Ben Chedly H, Boutinaud M, Bernier-Dodier P, Marnet PG and Lacasse P 2010. Disruption of cell junctions induces apoptosis and reduces synthetic activity in lactating goat mammary gland. Journal of Dairy Science 93, 2938–2951.

Ben Chedly H, Lacasse P, Marnet PG, Wiart-Letort S, Finot L and Boutinaud M 2009. Cell junction disruption after 36 h milk accumulation was associated with changes to mammary secretory tissue activity and dynamics in lactating dairy goats. Journal of Physiology and Pharmacology 60 (suppl. 3), 105–111.

Ben Chedly H, Lacasse P, Marnet PG, Komara M, Marion S and Boutinaud M 2011. Use of milk epithelial cells to study regulations of cell activity and apoptosis during once daily milking in goats. Animal 5, 572–579.

Boutinaud M, Galio L and Devinoy E 2009. Transcripts in milk purified mammary epithelial cells can reveal the effect of once daily milking. 6th International Symposium of Milk Genomics and Human Health, Paris, France.

Boutinaud M, Ben Chedly MH, Delamaire E and Guinard-Flament J 2008. Milking and feed restriction regulate transcripts of mammary epithelial cells purified from milk. Journal of Dairy Science 91, 988–998.

Boutinaud M, Rousseau C, Keisler DH and Jammes H 2003. Growth hormone and milking frequency act differently on goat mammary gland in late lactation. Journal of Dairy Science 86, 509–520.

Boutinaud M, Rulquin H, Keisler DH, Djiane J and Jammes H 2002. Use of somatic cells from goat milk for dynamic studies of gene expression in the mammary gland. Journal of Animal Science 80, 1258–1269.

Davis SR, Farr VC and Stelwagen K 1999. Regulation of yield loss and milk composition during once-daily milking: a review. Livestock Production Science 59, 77–94.

Delamaire E and Guinard-Flament J 2006. Longer milking intervals alter mammary epithelial permeability and the udder's ability to extract nutrients. Journal of Dairy Science 89, 2007–2016.

Grala TM, Phyn CVC, Kay JK, Rius AG, Littlejohn MD, Snell RG and Roche JR 2011. Temporally alterations to milking frequency, immediately post calving, modified the expression of genes regulating milk synthesis and apoptosis in the bovine mammary gland. Proceedings of the New Zealand Society of Animal Production 71, 3–8.

Hayashi AA, McCoard SA, Roy NC, Barnett MPG, Mackenzie DDS and Mcnabb WC 2004. Gene expression in bovine mammary somatic cells isolated from milk. Journal of Animal and Feed Sciences 13, 401–404.

INRA 2007. Ruminant nutrition: recommended allowances & feed tables. Quae, Versailles, France.

Knight CH, Wilde CJ and Peaker M 1988. Manipulation of milk secretion. In Nutrition and lactation in the dairy cow (ed. PC Garnsworthy), pp. 3–14. Anchor-Brendon Ltd, Tiptree, UK.

Komara M, Boutinaud M, Ben Chedly H, Guinard-Flament J and Marnet PG 2009. Once daily milking effects in high yielding alpine dairy goats. Journal of Dairy Science 92, 5447–5455.

Kuhn NJ, Carrick DT and Wilde CJ 1980. Lactose synthesis: the possibilities of regulation. Journal of Dairy Science 63, 328–336.

Lacy-Hulbert SJ, Woolford MW, Nicholas GD, Prosser CG and Stelwagen K 1999. Effect of milking frequency and pasture intake on milk yield and composition of late lactation cows. Journal of Dairy Science 82, 1232–1239.

Leitner G, Chaffer M, Shamay A, Shapiro F, Merin U, Ezra E, Saran A and Silanikove N 2004. Changes in milk composition as affected by subclinical mastitis in sheep. Journal of Dairy Science 87, 46–52.

https://doi.org/10.1017/S1751731112001176 Published online by Cambridge University Press

Li P, Rudland PS, Fernig DG, Finch LM and Wilde CJ 1999. Modulation of mammary development and programmed cell death by the frequency of milk removal in lactating goats. Journal of Physiology 519 (Pt 3), 885–900.

Littlejohn MD, Walker CG, Ward HE, Lehnert KB, Snell RG, Verkerk GA, Spelman RJ, Clark DA and Davis SR 2010. Effects of reduced frequency of milk removal on gene expression in the bovine mammary gland. Physiological Genomics 41, 21–32.

Marnet PG and Komara M 2008. Management systems with extended milking intervals in ruminants: regulation of production and quality of milk. Journal of Animal Science 86, 47–56.

Murrieta CM, Hess BW, Scholljegerdes EJ, Engle TE, Hossner KL, Moss GE and Rule DC 2006. Evaluation of milk somatic cells as a source of mRNA for study of lipogenesis in the mammary gland of lactating beef cows supplemented with dietary high-linoleate safflower seeds. Journal of Animal Science 84, 2399–2405.

O'Brien B, Ryan G, Meaney WJ, McDonagh D and Kelly A 2002. Effect of frequency of milking on yield, composition and processing quality of milk. Journal of Dairy Research 69, 367–374.

Pomiès D, Marnet PG, Cournut S, Barillet F, Guinard-Flament J and Rémond B 2008. Simplified milking methods in dairy herds: the end of twice-a-day milking constraints. Productions Animales 21, 59–70.

Rémond B, Aubailly S, Chilliard Y, Dupont D, Pomiès D and Petit M 2002. Combined effects of once-daily milking and feeding level in the first three weeks of lactation on milk production and enzyme activities, and nutritional status, in Holstein cows. Animal Research 51, 101–117.

Salama AA, Such X, Caja G, Rovai M, Casals R, Albanell E, Marin MP and Marti A 2003. Effects of once versus twice daily milking throughout lactation on milk yield and milk composition in dairy goats. Journal of Dairy Science 86, 1673–1680.

SAS Institute 1999. Statistical analysis system release 8.01. SAS Institute Inc., Cary, NC, USA.

Stacey A, Schnieke A, Kerr M, Scott A, McKee C, Cottingham I, Binas B, Wilde C and Colman A 1995. Lactation is disrupted by alpha-lactalbumin deficiency and can be restored by human alpha-lactalbumin gene replacement in mice. Proceedings of the National Academy of Science 92, 2835–2839.

Stelwagen K 2001. Effect of milking frequency on mammary functioning and shape of the lactation curve. Journal of Dairy Science 84, E204–E211.

Stelwagen K and Lacy-Hulbert SJ 1996. Effect of milking frequency on milk somatic cell count characteristics and mammary secretory cell damage in cows. American Journal of Veterinarian Research 57, 902–905.

Stelwagen K, Hopster H, Van der Werf JTN and Blokhuis HJ 2000. Short communication: effects of isolation stress on mammary tight junctions in lactating dairy cows. Journal of Dairy Science 83, 48–51.

Stelwagen K, Davis SR, Farr VC, Prosser CG and Sherlock RA 1994. Mammary epithelial cell tight junction integrity and mammary blood flow during an extended milking interval in goats. Journal of Dairy Science 77, 426–432.

Stelwagen K, Farr VC, McFadden HA, Prosser CG and Davis SR 1997. Time course of milk accumulation-induced opening of mammary tight junctions, and blood clearance of milk components. American Journal of Physiology 273, R379–R386.

Wilde CJ, Knight CH and Flint DJ 1999. Control of milk secretion and apoptosis during mammary involution. Journal of Mammary Gland Biology and Neoplasia 4, 129–136.

https://doi.org/10.1017/S1751731112001176 Published online by Cambridge University Press