Gene expression of six major milk proteins in primary bovine mammary epithelial cells isolated from milk during the first twenty weeks of lactation

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ABSTRACT: The objective of the present study was to refine a previously developed method to isolate primary bovine mammary epithelial cells (pBMEC) from fresh milk. Using this method, it was tested whether the number of pBMEC and the relation of recovered pBMEC to total somatic cell count vary within the individual lactation stages. Furthermore, the expression levels of the milk protein genes during the first twenty weeks of lactation were determined by quantitative PCR method. A total number of 152 morning milk samples were obtained from twenty-four Holstein-Friesian cows during the first 20 weeks of lactation (day 8, 15, 26, 43, 57, 113, and 141 postpartum). Numbers of extracted pBMEC were consistent at all time-points (1.1 ± 0.06 to 1.4 ± 0.03 $\times 10^3$/ml) and an average value of RNA integrity number (RIN) was 6.3 ± 0.3. Percentage of pBMEC in relation to total milk cells (2.0 ± 0.2 to 6.7 ± 1.0%) correlated with milk yield. Expression patterns of the casein genes alpha $(\alpha)_{S1}$, $(\alpha)_{S2}$, beta (β), and kappa (κ) (CSN1S1, CSN1S2, CSN2, CSN3, respectively) and the whey protein genes α-lactalbumin (LALBA) and progestagen-associated endometrial protein (PAEP; known as β-lactoglobulin) were shown to be comparable, i.e. transcripts of all six milk protein genes were found to peak during the first two weeks of lactation and to decline continuously towards mid lactation. However, mRNA levels were different among genes with CSN3 showing the highest and LALBA the lowest abundance. We hypothesized that milk protein gene expression has a pivotal effect on milk protein composition with no influence on milk protein concentration. This paper is the first to describe milk protein gene expression during lactation in pBMEC collected in milk. Future studies will be needed to understand molecular mechanisms in pBMEC including regulation of expression and translation throughout lactation.

Keywords: dairy cow; immunomagnetic cell separation; mammary gland; milk protein gene expression

Six bovine milk proteins, comprising 95% of the total protein, have previously been classified into the four caseins (α_{S1}, α_{S2}, β, and κ), and the two major whey proteins (α-lactalbumin and β-lactoglobulin) (Threadgill and Womack, 1990). All major milk proteins are synthesized in the mammary epithelial cells (MEC). During the milking process, the MEC detach from the alveolar epithelium and are continuously shed into milk during the entire lactational period comprising approximately 2% of total somatic cells (Boutinaud and Jammes, 2002).

The number of mammary secretory cells and their secretory activity are mainly responsible both for the daily produced amount of milk and the fast increase in milk yield during the first weeks of lactation (Boutinaud et al., 2004). After peak lactation, the number of the secretory cells in the mammary gland declines gradually by 8% between days 90 and

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240 of lactation accounting for lower milk yields at the end of lactation (Capuco et al., 2003).

Consequently, typing of different protein variants as well as knowledge about the regulation of expression of the different milk protein genes during lactation is crucial for the genetic improvement of milk composition and milk yield (Groenen and van der Poel, 1994). To study the cellular mechanism responsible for synthesis of milk constituents, especially protein synthesis, and to better understand these molecular events, pBMEC samples need to be harvested either by invasive mammary gland biopsies or by purification from milk. Boutinaud et al. (2008) refined the isolation of viable BMEC directly from fresh milk. Therefore, gene expression studies in pBMEC are frequently repeatable during lactation cycle.

As concerns recent investigations of the expression of the milk protein genes, Bionaz and Loor (2011) determined the expression of the milk protein genes LALBA and CSN3 in bovine mammary gland tissue during lactation cycle. They found the highest expression levels for both on day 60 postpartum (pp) (Bionaz and Loor, 2007, 2011, respectively). In lactating dairy ewe mammary gland expression levels of the caseins (CSN1S1, CSN1S2, CSN2, CSN3) did not change, but during involution and late pregnancy the expression levels lowered (Colitti and Pulina, 2010). Bevilacqua et al. (2006) investigated the expression levels and the translation efficiency of the four caseins in goat, ewe, and cow. In cows, transcripts seemed to be at the same level of abundance.

Research into the molecular mechanisms of milk protein synthesis may also help the improvement of strategies and technologies for enhancing milk protein production of the dairy cow.

MATERIAL AND METHODS

Cows, housing, and feeding

Milk was collected from twenty-four multiparous German Holstein-Friesian cows (parity 2.5 ± 0.1, calving throughout the year). After parturition, cows were machine-milked twice daily (at 4.20 a.m. and 3.40 p.m.). During each milking, milk yield was recorded with electronic milk meters (Metatron P21) and stored electronically (DairyPlan C21; both GEA WestfaliaSurge GmbH, Bönnen, Germany). The partly mixed feed ration was based on corn and grass silage and mixed with concentrates to meet the energy requirements of cows on the basis of a total daily milk production of 22 kg per day. The diet was offered once daily in sufficient amounts to secure ad libitum intake (> 5% residual feed). Additional concentrates were given individually according to the day of lactation. Water was freely available at all times.

Sampling

A total number of 168 morning milk samples was collected. Samples were obtained from each cow at days 8, 15, 26, 43, 57, 113, and 141 after parturition. Whole morning milk samples were separated during milking into a sterile bucket and milk yield was determined with a spring scale. 1800 ml of the total morning milk was filled in autoclaved glass bottles and used for cell isolation immediately. One aliquot was stored at 4°C for a maximum of 7 days with a preservative (acidiol) until analyses of milk composition.

Milk composition analysis

Milk protein, fat, and lactose were analyzed by infrared-spectrophotometric technique – infrared absorption measurement evaluated by Fourier transform (MilkoScan FT6000) and somatic cell count was determined by a fluorescence-optical counting system (Fossomatic FC; both Foss, Hillerød, Denmark) in the laboratories of Milchpruefring Bayern e.V. (Wolnzach, Germany).

Cell isolation

Milk (1800 ml) was defatted by centrifugation at 1800 *g* at 4°C for 30 min in four 450-ml corning tubes and skim milk was removed. Remaining total cell pellets were resuspended in 25 ml of phosphate buffered saline (PBS) and pooled in pairs. After a second centrifugation step (1850 *g*, 15 min at 4°C) the two total cell pellets were resuspended and pooled in 1 ml of PBS containing 1% bovine serum albumin (BSA). Purification of pBMEC was performed applying an immunomagnetic-bead based separation technique. Cell suspension was first incubated for 10 min on a rotary mixer at 4°C with a primary mouse monoclonal antibody against cytokeratin 8 antibody (clone C-43, EXBIO, Prague, Czech Republic), which is specific to bovine epithe-

lial cells. Unbound antibodies were removed from the cell-antibody complex by 8 min of centrifugation at 300 *g* at 4°C. After discarding the supernatant cell-antibody complex was resuspended in 1 ml of 1% BSA-PBS. Dynabeads (25 µl) (PanMouse IgG, Invitrogen, Dynal AS, Oslo, Norway) were added and the suspension was incubated for 20 min on a rotary mixer at 4°C. Antibody-bound cells were collected by placing the sample vials into the Dynal MPC-L (Dynal AS, Oslo, Norway) for 2 min and withdrawing of the supernatant. A second washing including a magnetic separation step was performed with 1 ml of 1% BSA-PBS followed by a suspension of pBMEC in 1 ml of 1% BSA-PBS. A 7 µl aliquot was removed to perform a hematocytometer cell count and a 10 µl aliquot was collected to stain pBMEC immunohistochemically. Purified MEC were obtained by centrifugation of tubes at 1800 *g* at 4°C for 5 min, resuspended in 700 µl Qiazol (Qiagen GmbH, Hilden, Germany), and stored at –80°C until RNA extraction.

Immunohistochemical detection of pBMEC

For immunohistochemical studies, 10 µl of the cell suspension was spread on an object slide, treated with 7 µl of poly-L-Lysine solution (Science Services, Munich, Germany), and cells were fixed with 100% ethanol for 10 min. Addition of methanol (99.8%) for 5 min permeabilized cell surfaces. Thereafter, samples were washed twice with PBS for 5 min. Endogenous peroxidase activity was blocked with 1% hydrogen peroxide (H_2O_2) for 20 min. Following blocking with 10% goat serum, samples were incubated with cytokeratin 8 antibody at 37°C for 45 min. After washing with PBS (twice for 10 min), samples were incubated with a secondary anti-mouse IgG peroxidase-conjugated antibody (2.5 mg/ml, Sigma-Aldrich, Munich, Germany). Next, samples were washed twice with PBS. Binding of antibody was detected by incubation with PBS containing 0.01% diaminobenzene and 0.01% H_2O_2 for 15 min. Cells were counterstained using Mayer's Haemalaun (Carl Roth GmbH, Karlsruhe, Germany).

RNA extraction and reverse transcription

Total RNA was extracted from the purified MEC applying the miRNeasy MiniKit (QIAgen GmbH, Hilden, Germany). In brief, samples, frozen in 700 µl QIAzol were thawed at room temperature (RT) and homogenized by vortexing. To each sample, 140 µl of chloroform was added and the samples were vortexed vigorously for 15 s. After a 2 min incubation at RT, the mixture was centrifuged at 1.5×10^4 *g* for 15 min at 4°C. The aqueous supernatant containing total RNA was recovered and mixed with 1.5 volumes of 100% ethanol. After vortexing, up to 700 µl were pipetted into an RNeasy Mini spin column and centrifuged at 10^4 *g* for 15 s at RT. This step was repeated with the remainder of the sample. To wash the column, 700 µl of Buffer RWT was added and centrifuged at 10^4 *g* for 15 s at RT. Washing was performed twice by adding 500 µl of Buffer RPE followed by centrifugation at 10^4 *g* for 15 s at RT. Preceding dissolving of RNA in 30 µl sterile RNase-free water, the RNeasy Mini spin column membrane was dried by centrifugation (10^4 *g* for 2 min at RT). RNA was quantified by spectrophotometry (BioPhotometer; Eppendorf, Hamburg, Germany). Integrity of the RNA (RNA integrity number; RIN) was measured with the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) connected to the RNA 6000 Nano Assay. Accurate amounts of 250 ng RNA were reversely transcribed to complementary DNA adding the following reverse transcription master mix: 12 µl 5 × Buffer (Promega, Mannheim, Germany), 3 µl Random Hexamer Primers (50mM) (Invitrogen, Carlsbad, USA), 3 µl dNTP Mix (10mM) (Fermentas, St Leon-Rot, Germany), and 200 U of MMLV-H-reverse transcriptase (Promega, Regensburg, Germany). The reverse transcription reaction was carried out according to the manufacturer with a 60 µl reaction volume in a PCR thermocycler (Biometra, Göttingen, Germany) by successive incubations at 21°C for 10 min and at 48°C for 50 min, finishing with enzyme inactivation at 90°C for 2 min. Reverse transcription products were stored at –20°C.

Selected target genes and primer design

The two fractions of milk proteins synthesized in the pBMEC, comprising 95% of the total protein, are namely caseins (80%) and whey proteins (20%). The four caseins are classified into α_{S1}, α_{S2}, β, and κ, and the two major whey proteins into α-lactalbumin (LA) and β-lactoglobulin (LG). Consequently, the genes encoding for the six major milk proteins were in main focus.

Gene sequences for primer design were obtained from the gene bank of the National Center for Biotechnology Information (NCBI). Exon-spanning primers were designed with the help of the NCBI primer tool and synthesized at Eurofins MWG (Ebersberg, Germany). PCR products of primers were sequenced at LGC Genomics (Berlin, Germany). Primers, accession numbers, and product lengths for each gene are listed in Table 1.

Quantitative PCR and PCR amplification efficiency

Quantitative PCR was performed using MESA Green qPCR MasterMix plus for SYBR Assay w/fluorescein (Eurogentec, Cologne, Germany) applying a standard protocol recommended by the manufacturer. All components necessary for real-time qPCR were mixed in the reaction wells. The mastermix was prepared as follows: 7.5 µl 2 × MESA Green qPCR MasterMix, 1.5 µl forward primer (10 pmol/µl), 1.5 µl reverse primer (10 pmol/µl), and 3 µl RNase free water. Per well, 13.5 µl mastermix plus 1.5 µl cDNA was added. The plate was sealed, placed in the iQ5 Cycler (Bio-Rad, Munich, Germany), and the following PCR protocol was started: denaturation step (95°C, 5 min), cycling program (95°C, 3 s; primer specific annealing temperature, 60 s) and melting curve analysis.

Afterwards, the qPCR assays were evaluated by the generation of a standard curve. Calibration curves for each gene were done on the Bio-Rad iQ5 with eight 10-fold serial dilutions (in triplicates) and were calculated by Bio-Rad iQ5 Optical System Software (Version 2.1) with the analysis mode "PCR

Table 1. Primer pairs, product sizes, and accession numbers

Gene[1]	Sequence (5' → 3')	Product size (bp)	GeneBank accession No.	E* (%)
Major milk protein genes				
CSN1S1 for	ATGAAACTTCTCATCCTTACCTGTCTT	179	NM_181029.2	98
CSN1S1 rev	CCAATATCCTTGCTCAGTTCATT			
CSN1S2 for	AGCTCTCCACCAGTGAGGAA	150	NM_174528.2	90
CSN1S2 rev	GCAAGGCGAATTTCTGGTAA			
CSN2 for	GTGAGGAACAGCAGCAAACA	233	NM_181008.2	85
CSN2 rev	AGGGAAGGGCATTTCTTTGT			
CSN3 for	TGCAATGATGAAGAGTTTTTTCCTAG	150	NM_174294.1	87
CSN3 rev	GATTGGGATATATTTGGCTATTTTGT			
LALBA for	CTCTCTGCTCCTGGTAGGCAT	247	NM_174378.2	96
LALBA rev	GTGAGGGTTCTGGTCGTCTT			
PAEP for	AGAAGGTGGCGGGGACTTGG	375	NM_173929.3	100
PAEP rev	TGTCGAATTTCTCCAGGGCCT			
Marker of epithelial cells				
KRT8 for	GCTACATTAACAACCTCCGTC	237	NM_001033610.1	97
KRT8 rev	TCTCATCAGTCAGCCCTTCC			
References genes				
GAPD for	GTCTTCACTACCATGGAGAAGG	197	NM_001034034.1	100
GAPD rev	TCATGGATGACCTTGGCCAG			
H3F3A for	ACTCGCTACAAAAGCCGCTCG	232	NM_001014389.2	94
H3F3A rev	ACTTGCCTCCTGCAAAGCAC			
RPS9 for	CCTCGACCAAGAGCTGAAG	64	NM_001101152.1	100
RPS9 rev	CCTCCAGACCTCACGTTTGTTC			

[1]CSN1S1 = α_{S1}-casein, CSN1S2 = α_{S2}-casein, CSN2 = β-casein, CSN3 = κ-casein, GAPD = glyceraldehyde-3-phosphate dehydrogenase, H3F3A = H3 histone family 3A, KRT8 = keratin 8, LALBA = α-lactalbumin, PAEP = progestagen-associated endometrial protein, better known as β-lactoglobulin, RPS9 = ribosomal protein S9

*efficiency was calculated by the slope of the standard curve by the equation: $E = 10^{(-1/slope)}$

base line substracted". Amplification efficiency (E) of qPCR reactions was calculated with the slope of the log-linear portion of the calibration curve according to the equation: $E = 10^{(-1/slope)}$ (Rasmussen, 2001; Bustin et al., 2009).

Quantification of mRNA

Genes were selected as reference genes using GenEx Pro Software Version 5.2.7.44 (MultiD Analyses, Gothenburg, Sweden). In the present study, the NormFinder algorithm was used. The mean of the three housekeeping genes, glyceraldehyde-3-phosphate dehydrogenase (GAPD), H3 histone family 3A (H3F3A), and ribosomal protein S9 (RPS9) was calculated for the reference index and used for normalization. Quantitative cycle (Cq)-values were calculated by Bio-Rad iQ5 Optical System Software Version 2.1 with the analysis mode "PCR base line substracted curve fit". The ΔCq-values were calculated as $\Delta Cq = Cq_{target\ gene} - meanCq_{refence\ genes}$ (Pfaffl, 2001). In order to avoid negative digits while allowing an estimation of a relative comparison between two time points, data are presented as least square means (LSM) ± standard error of means (SEM) subtracted from the arbitrary value 2 (2 – ΔCq). Thus, a high ΔCq-value resembles high transcript abundance. An increase of one ΔCq represents a two-fold increase of mRNA transcripts.

Statistical analysis

The statistical analysis of the data for milk yield, milk composition, and number of somatic and epithelial cells was assessed by repeated measurements ANOVA using the MIXED Procedure of SAS (Statistical Analysis System, Version 9.1, 2002). The ΔCq-values were normalized individually in relation to the housekeeping gene index of GAPD, H3F3A, and RPS9 before using the MIXED Procedure of SAS with repeated measurements. The ANOVA models used contained the fixed effect DIM and a random cow effect. Results are represented as LSM ± SEM.

RESULTS

Milk yield and milk composition

Detailed findings are presented in Table 2. In summary, the results reflected the well established course of the morning milk yield with peak yields of 21.7 ± 0.7 kg on days 43 and 57 pp. Milk protein concentration decreased to a nadir of 2.99 ± 0.06% on day 43 pp and was followed by an increase until day 141 pp to a value of 3.44 ± 0.09%. Milk fat concentration started with the highest concentrations on day 8 pp (5.48 ± 0.22%), reached the lowest concentrations on day 57 pp (3.94 ± 0.15%), and afterwards it was gradually rising.

Cell isolation

The immunomagnetic cell binding technique using cytokeratin 8 coated antibodies was applicable for specific binding of pBMEC in fresh milk. Quality of extracted mRNA was also sufficient for gene expression studies. An average RIN-value of 6.3 ± 0.3 was obtained.

Table 2. Morning milk yield and daily concentrations of protein, fat, and lactose[1] during the first twenty weeks of lactation

	Day of sampling postpartum						
	8	15	26	43	57	113	141
Milk yield (kg)	17.8 ± 0.6^{a}	18.7 ± 0.8^{bf}	20.2 ± 0.7^{ce}	21.7 ± 0.7^{d}	21.7 ± 0.7^{cd}	19.1 ± 0.7^{be}	16.9 ± 0.9^{af}
Protein (%)	3.80 ± 0.07^{a}	3.34 ± 0.07^{bde}	3.08 ± 0.06^{bc}	2.99 ± 0.06^{c}	3.10 ± 0.06^{cd}	3.35 ± 0.07^{de}	3.44 ± 0.09^{e}
Protein (g)	676 ± 24^{a}	620 ± 25^{b}	623 ± 23^{ab}	646 ± 21^{bd}	669 ± 19^{c}	635 ± 21^{bd}	575 ± 27^{cd}
Fat (%)	5.48 ± 0.22^{a}	4.77 ± 0.28^{b}	4.98 ± 0.23^{b}	4.68 ± 0.21^{bc}	3.94 ± 0.15^{ac}	4.48 ± 0.16^{bd}	4.42 ± 0.23^{d}
Fat (g)	969 ± 45^{ab}	884 ± 57^{ad}	1 006 ± 56^{c}	1 024 ± 65^{c}	847 ± 36^{d}	859 ± 48^{d}	664 ± 40^{be}
Lactose (%)	4.62 ± 0.03^{a}	4.82 ± 0.03^{ab}	4.83 ± 0.03^{ab}	4.79 ± 0.03^{a}	4.88 ± 0.03^{b}	4.79 ± 0.03^{a}	4.76 ± 0.03^{a}

[1]values are presented as least square means ± SEM

[a–f]means with different letters within the same row are significantly different

Table 3. Number of samples and least square means ± SEM, number of total milk cells, number of separated MEC, RNA quantity, and cytokeratin mRNA levels

	Day of sampling postpartum						
	8	15	26	43	57	113	141
Milk samples (*n*)	21	22	22	23	22	21	21
No. of total milk cells, $\times 10^3$/ml of milk	83 ± 18^{ac}	89 ± 34^{ac}	77 ± 42^{ac}	38 ± 8^{b}	48 ± 17^{b}	123 ± 80^{c}	108 ± 41^{a}
No. of recovered MEC, $\times 10^3$/ml of milk	1.2 ± 0.04	1.3 ± 0.04	1.1 ± 0.06	1.2 ± 0.04	1.2 ± 0.03	1.4 ± 0.03	1.1 ± 0.06
Recovered pBMEC (%) of total milk cells	2.0 ± 0.2^{a}	3.4 ± 0.4^{b}	3.6 ± 0.3^{b}	5.6 ± 0.8^{c}	6.7 ± 1.0^{c}	4.9 ± 0.9^{c}	2.2 ± 0.3^{a}
RNA quantity (μg)	4.1 ± 0.7	3.3 ± 0.3	2.5 ± 0.5	3.6 ± 0.9	2.6 ± 0.5	3.6 ± 0.6	4.5 ± 0.7
KRT 8 mRNA level, arbitrary value	6.8 ± 0.3	6.7 ± 0.4	6.9 ± 0.4	7.0 ± 0.5	6.6 ± 0.4	7.7 ± 0.5	7.6 ± 0.5

MEC = mammary epithelial cells, pBMEC = primary bovine MEC

$^{a–c}$means with different letters within the same row are significantly different

Ten out of 168 milk samples had a high somatic milk cell count (> 2×10^5 cells/ml) and were discarded without isolation of pBMEC. In addition, total amounts of RNA from six samples were too low for reverse transcription (< 250 ng) and those samples were also excluded from further analysis. The number of total milk cells in the 152 analyzed samples tended to be the lowest on day 43 pp ($38 \pm 8 \times 10^3$ cells/ml milk) and highest on day 113 pp ($123 \pm 80 \times 10^3$ cells/ml milk; Table 3). Totals of pBMEC ($1.2 \pm 0.04 \times 10^3$ cells/ml milk) were comparable at all time points, whereas the percentage of pBMEC in relation to total milk cells differed during lactation ($P < 0.001$) (Table 3). Percentage of pBMEC increased from day 8 pp (2.0 ± 0.2%) to day 43 pp (5.6 ± 0.8%, $P < 0.001$) and day 57 pp (6.7 ± 1.0%). Afterwards, fractions of pBMEC decreased to day 141 pp (2.2 ± 0.3%, $P < 0.001$) (Table 3). Percentage of pBMEC and milk yield were correlated during the first 20 weeks of lactation ($R = 0.79$, $P < 0.05$). Extracted quantity of pBMEC mRNA did not vary during experimental timeframe (Table 3). Expression levels of keratin 8 (KRT8) were used as a marker for epithelial cells. Transcript abundance of this marker was constant at all time points (Table 3).

PCR amplification efficiencies and linearity

Investigated transcripts showed high PCR efficiency rates with high linearity (Pearson's correlation coefficient $r > 0.90$). The calculated average PCR efficiency for the ten genes was 94.7 ± 1.76% and varied between 85 to 100% (Table 1). Since accuracy of qPCR depends highly on PCR efficiency, efficiency should be at least 80% (Ma et al., 2006).

Milk protein gene expression

Transcript abundances of all investigated milk protein genes were different at sampled time points ($P < 0.001$), but all protein genes showed similar expression patterns during the first 20 weeks of lactation (Figure 4). Levels of CSN1S1-mRNA increased by 1.9 fold from day 8 to day 15 pp ($P = 0.03$). After that, transcripts of CSN1S1 decreased gradually by 80% to day 141 pp ($P < 0.01$). Likewise, mRNA levels of CSN1S2 decreased successively after a peak on days 8 and 15 pp, respectively to 0.27 fold on days 113 and 141 pp ($P < 0.01$). Transcripts of CSN2 doubled from days 8 to 15 pp ($P = 0.04$) and lessened after day 15 pp by 80% to day 141 pp ($P < 0.01$). Expression of CSN3-mRNA was the highest on days 8 and 15 pp, respectively and decreased to 2.6 fold until days 113 and 141 pp ($P < 0.001$). The mRNA levels of LALBA were lower compared with those of the other milk proteins. The transcription of LALBA was similar on days8 and 15 pp and declined by 86% to days 113 and 141 pp ($P < 0.001$). After an early peak on day 8 pp, mRNA levels of PAEP decreased subsequently by 0.16 fold until days 113 and 141 pp, respectively ($P < 0.001$).

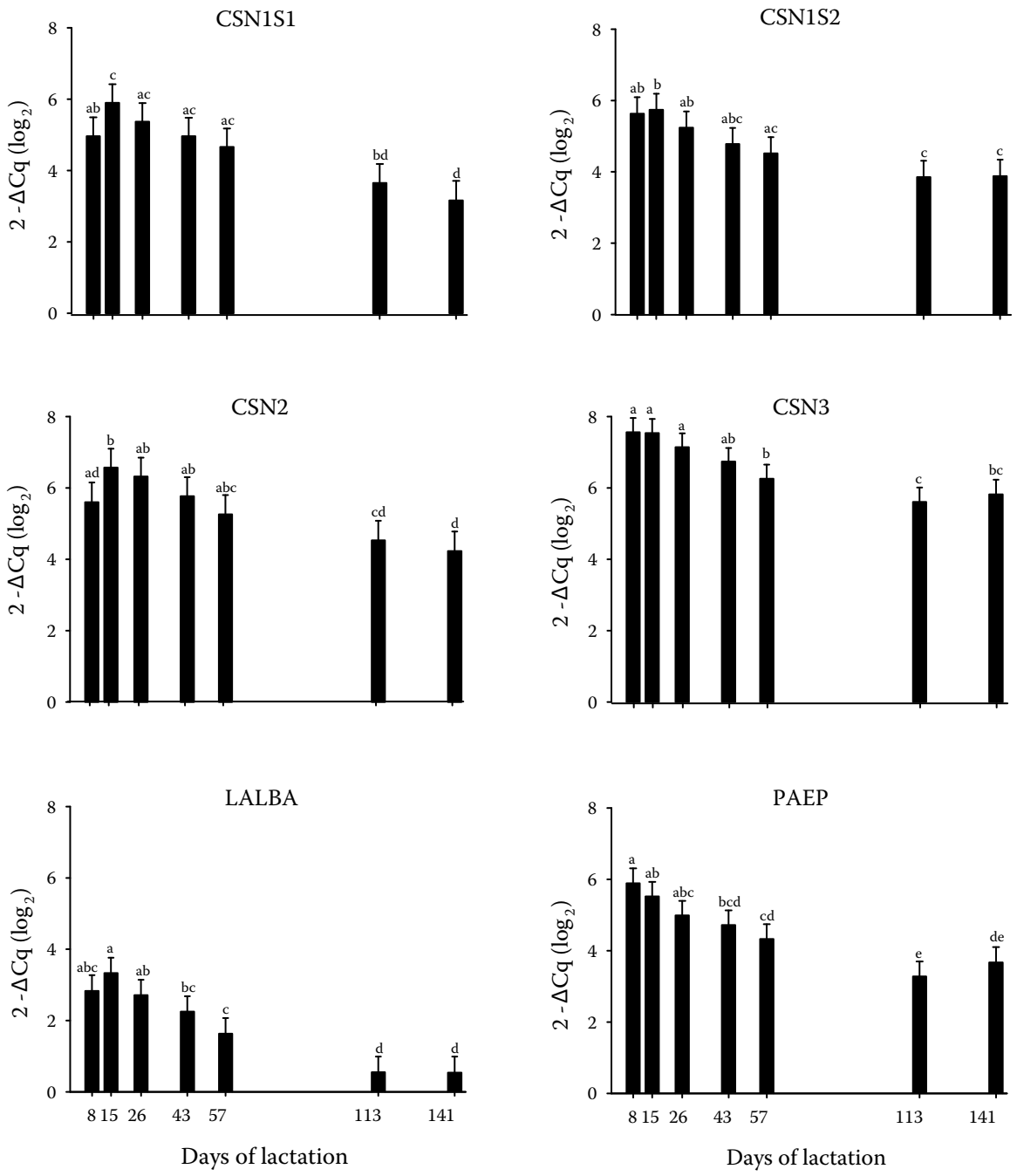

Figure 1. Transcript abundance of α_{S1}-, α_{S2}-, β-, κ-casein, α-lactalbumin, and β-lactoglobulin during the first 20 weeks of lactation in primary bovine mammary epithelial cells purified from milk. ΔCq was calculated as $Cq_{target\ gene}$ – $meanCq_{refence\ genes}$. Results are shown as 2 – ΔCq ± SEM. Letters indicate significant differences ($P < 0.05$)

DISCUSSION

Milk yield and milk composition

Milk yield increased rapidly during the first six weeks, plateaued and lowered towards the end of the experimental period roughly around mid lactation. As a result, although only morning milk yield which is higher than evening milk yields (Quist et al., 2008) was evaluated, the estimated shape of lactation curves during the first 20 weeks of lactation were consistent with those reported by numerous previous studies (e.g. Wood, 1969; Walsh et al., 2007). Decline of milk protein and milk fat concen-

tration during the first two months after parturition followed by a respective increase of values is in agreement with findings of Friggens et al. (2007). The authors examined milk composition of Danish Holstein-Friesian cows during 301-day lactation periods and found that milk protein and fat concentration reached their lowest points approximately on days 40 and 60 pp, respectively. In the present study, proportion of milk lactose was constant during the first 20 weeks of lactation, but tended to be higher on day 57 pp. These results coincide with data from Gáspárdy et al. (2004) which show that lactose concentration of Israeli Holstein-Friesian cows peaked on day 66 of lactation. Total somatic cell count was constant during the experimental timeframe but tended to be higher at the end of the study. Our results agree with those from Sheldrake et al. (1983) and Hagnestam-Nielsen et al. (2009), who reported a constant amount of somatic cells during early and mid lactation.

Cell isolation

In numerous previous studies on mRNA expression, mammary gland tissue was obtained at one or at few time points via intricate biopsies (Farr et al., 1996; Finucane et al., 2008) or once after slaughtering of precious experimental animals (Capuco et al., 2001; Colitti and Pulina, 2010). In those samples, mRNA was extracted directly from all cells or after a preceding step of pBMEC cultivation (Talhouk et al., 1990; Griesbeck-Zilch et al., 2008). Also, techniques to culture pBMEC from milk were described (Buehring, 1990). Next to the disadvantage of a potential influence of cell culture condition on pBMEC mRNA expression, cell cultivation from mammary gland tissue partly resulted in samples that included a large fraction of non-pMEC, like fibroblasts and adipocytes. To circumvent that drawback, Gomm et al. (1995) described the isolation of pure pMEC from human mammary tissue applying an immunomagnetic separation technique. Boutinaud et al. (2008) refined that method further to extract pBMEC directly from milk. During milking, the pBMEC detach from the alveolar epithelium and discard the mammary gland within the milk. Moreover, milk is a noninvasive source of viable pBMEC (Boutinaud and Jammes, 2002). The number of pBMEC (2.1×10^6) purified from a similar volume of milk (1800 ml) did not vary among sampled time points in our study reflecting a constant renewal during lactation. Boutinaud et al. (2008) isolated approximately 162 days pp comparable 2.7×10^6 pBMEC from 1750 ml of milk of Holstein-Friesian cows which comprised 2% of total milk cells. Despite the constant discharge of pBMEC, it is well established that fraction of pBMEC of total milk cells is low (Miller et al., 1991; Boutinaud and Jammes 2002). In our study, pBMEC represented about 2–6.7% of total milk cells and that proportion was the highest during peak lactation. These results are supported by Capuco et al. (2001) who found a peak of MEC number in the udder during early lactation with a subsequent decrease during the following lactation. They concluded that the proportion of pBMEC is influenced by the stage of lactation. However, milk somatic cell count (SCC) depends mainly on immune status of the udder and only cows with a total somatic cell count below 2×10^5/ml were included in the study. Therefore, a varying proportion of pBMEC in the milk is expected in cases of clinical mastitis due to increased number of immune cells with or without increased shedding of pBMEC. Contrary to that of SCC, the number of pBMEC depends predominantly on the structure of the mammary epithelium, stage of lactation, and milking methods (Boutinaud and Jammes, 2002).

Contrary to the direct cell isolation method described by Boutinaud et al. (2008), a method of indirect cell purification was established in the present work. Total cells were first coated with the monoclonal antibody directed against cytokeratin 8, and afterwards cells-antibody complexes were incubated with the immunomagnetic particles resulting in a comparable number of recovered MEC. Previously, it was postulated that milk yield depended primarily on the size of the mammary gland (Linzell, 1966; Sorensen et al., 1998). However, it has been demonstrated more recently that milk yield is regulated by the quantity of mammary secretory cells and their secretory activity (Capuco et al., 2001). According to this, in our study the ratios of pBMEC of total milk cells and milk yield were found to be correlated during the experimental timeframe ($R = 0.79$), whereas correlation of milk yield and total somatic milk cells was lower ($R = 0.62$). Earlier studies revealed that the number of pBMEC found in milk is correlated with milk yield. Annen et al. (2007) supported the hypothesis that increased milk yield during early lactation is associated with an increased accumulation of new pBMEC during late gestation and increased pBMEC shedding during early lactation.

Milk protein gene expression

The relative expression of the six major milk protein genes (CSN1S1, CSN1S2, CSN2, CSN3, LALBA, and PAEP) showed similar patterns during the first 20 weeks of lactation. Maxima of mRNA abundances were reached during the first two weeks of lactation followed by respective declines towards the end of the experimental period. Due to applying the immunomagnetic isolation method of pBMEC from fresh milk, it was possible for the first time to determine milk protein gene expression profiles in the very same animal over the course of lactation. Nonetheless, major milk protein gene expression patterns in mammary tissue of other species like common brushtail possum or mouse during pregnancy, lactation, and dry period did exist (Demmer et al., 1998; Stein et al., 2004; Anderson et al., 2007). Colitti and Farinacci (2009) examined gene expression of LALBA in mammary tissue in dairy ewes, collected after slaughtering, during peak (day 30 pp), mid (day 60 pp), and end of lactation (day 150 pp). In contrast to our findings in lactating cows, the relative expression level of LALBA in ewes reached the highest value only at the end of lactation. Furthermore, Colitti and Pulina (2010) analyzed transcripts of the four caseins CSN1S1, CSN1S2, CSN2, and CSN3 in mammary tissue after slaughter in dairy ewes. Respective to the study of Colitti and Pulina (2010), gene expression of the four caseins was up-regulated during peak, mid-, and late lactation but down-regulated during pregnancy and involution. Those findings correspond to ovine milk protein composition during lactation. Concentrations of caseins, total albumins, and β-lactoglobulin in whole milk increased significantly over the course of lactation (Poulton and Ashton, 1970). No milk protein fractions were analyzed in the present study, but previous studies in dairy cows showed different composition during lactation by contrast with ewes. Early reports stated peak concentrations of total caseins and serum proteins approximately five days after calving followed by a decline during the remaining 310-day lactation period, except for a slight increase during the time of peak yield (Larson and Kendall, 1957). In contrast, Ng-Kwai-Hang et al. (1987) determined a decline in concentrations of major milk proteins only between days 30 and 90 pp during peak milk yield followed by a marked increase until day 365 pp concomitant to lowering volumes of milk. Nevertheless, the highest total protein production was found, as described previously, during the first months of lactation (Friggens et al., 2007). The ratio of total caseins to whey proteins does not vary depending on the stage of lactation, reflecting no changes in the rates of synthesis for both main fractions (Coulon et al., 1998). Yet, relations between specific caseins differed depending on the stage of lactation (Kroeker et al., 1985; Çardak, 2009). During the first two months a marked decrease of α-casein and a reciprocal systematical increase of β-casein as a proportion of the casein fraction were demonstrated. The relative amount of κ-casein remained constant during the whole lactation cycle. Concentrations of β-lactoglobulin were on their minimum level during the second month of lactation, whereas proportions of α-lactalbumin, which is involved in milk lactose synthesis, decreased with progress in lactation as a result of lowering milk yields. In the present study, transcripts of all six milk protein genes were found to peak during the first two weeks of lactation and to decline continuously towards mid lactation. We hypothesized that milk protein gene expression has a pivotal effect on milk protein composition whereas milk protein concentration was not influenced. This assumption is confirmed by Bionaz and Loor (2007).

In this context, the translational efficiency of milk protein transcripts also has to be taken into account. Bevilacqua et al. (2006) measured equal proportions of casein gene transcripts which is roughly comparable to our findings. However, the four casein mRNAs were not translated with the same efficiency. They showed that CSN1S1 and CSN2 were translated 3 to 4-fold more efficiently in comparison with CSN1S2 and CSN3 and explained their findings with differences in the mRNA leader region. Due to those differences in translational efficiency, the differences in quantities of milk proteins could be explained. Milk proteins α_{S1}- and β-casein account for the major part of milk proteins (15 and 11 g/l, respectively), whereas α_{S2}- and κ-casein represent only a minor part (both 4 g/l) in skim milk (Farrell et al., 2004). In addition, whey proteins only amount to 5.5 g/l (1.5 g/l for α-lactalbumin and 4 g/l for β-lactoglobulin). However, no data are available on translational efficiency throughout lactation which could be influenced by different factors such as genetics, epigenetics, nutrition, milking frequency, hormonal status, or diseases.

Furthermore, milk protein synthesis may be regulated at multiple levels within the mammary epithelial cells including transcription, post-transcription,

translation, and amino acid supply (Menzies et al., 2009). The genes encoding these proteins are regulated by the complex interplay of peptide and steroid hormones, predominantly the lactogenic hormones prolactin, insulin, and hydrocortisone, and cell-cell and cell-substratum interactions. Moreover, the uptake of amino acids from feed and their metabolic conversion are important preludes to milk protein synthesis. Therefore, Shennan and Peaker (2000) reported that the transport rate of amino acids seems to be the limited factor for milk protein synthesis. In this context, many signalling pathways in the lactating pBMEC are known, i.e. the janus kinase/signal transducer and activator of transcription cascade (Darnell, 1997), the growth hormone effects in the mammary mammalian target of rapamycin signalling pathway (Cui et al., 2003), the interaction of insulin and the major milk protein transcription factor E74-like factor 5 (Menzies et al., 2009), and the amino acids and glucose transporters (Zhao et al., 1996, 2005). Future research in that field could provide valuable information on improved lactation performance of dairy cows.

CONCLUSION

The indirect immunomagnetic bead-based method was appropriate to isolate pBMEC directly from fresh milk for further quantitative PCR analysis. The percentage of shed pBMEC in relation to somatic milk cells was highly correlated to milk yield. Expression patterns of the six major milk protein genes in twenty-four Holstein-Friesian cows were comparable during the first 20 weeks of lactation and respective proportions were comparable to previous findings on casein and whey protein concentrations in milk. Milk proteins are of great importance to the dairy industry. Therefore, further studies are likely to include investigations on regulation of milk protein gene expression and translation efficiency during the course of lactation.

REFERENCES

Anderson S.M., Rudolph M.C., McManaman J.L., Neville M.C. (2007): Secretory activation in the mammary gland: it's not just about milk protein synthesis! Breast Cancer Research, 9, 204–218.

Annen E.L., Fitzgerald A.C., Gentry P.C., McGuire M.A., Capuco A.V., Baumgard L.H., Collier R.J. (2007): Effect of continuous milking and bovine somatotropin supplementation on mammary epithelial cell turnover. Journal of Dairy Science, 90, 165–183.

Bevilacqua C., Helbling J.C., Miranda G., Martin P. (2006): Translation efficiency of casein transcripts in the mammary tissue of lactating ruminants. Reproduction Nutrition Development, 5, 567–578.

Bionaz M., Loor J.J. (2007): Identification of reference genes for quantitative real-time PCR in the bovine mammary gland during the lactation cycle. Physiological Genomics, 29, 312–319.

Bionaz M., Loor J.J. (2011): Gene networks driving bovine mammary protein synthesis during the lactation cycle. Bioinformatics and Biology Insights, 5, 83–98.

Boutinaud M., Jammes H. (2002): Potential uses of milk epithelial cells: a review. Reproduction Nutrition Development, 42, 133–147.

Boutinaud M., Guinard-Flament J., Jammes H. (2004): The number of activity of mammary epithelial cells, determining factors for milk production. Reproduction Nutrition Development, 44, 499–508.

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

Buehring G.C. (1990): Culture of mammary epithelial cells from bovine milk. Journal of Dairy Science, 73, 956–963.

Bustin S.A., Benes V., Garson J.A., Hellemans J., Huggett J., Kubista M., Mueller R., Nolan T., Pfaffl M.W., Shipley G.L., Vandesompele J., Wittwer C.T. (2009): The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical Chemistry, 55, 611–622.

Capuco A.V., Wood D.L., Baldwin R., Mcleod K., Paape M.J. (2001): Mammary cell number, proliferation, and apoptosis during a bovine lactation: relation to milk production and effect of bST. Journal of Dairy Science, 84, 2177–2187.

Capuco A.V., Ellis S.E., Hale S.A., Long E., Erdman R.A., Zhao X., Paape M.J. (2003): Lactation persistency: insights from mammary cell proliferation studies. Journal of Animal Science, 81 (Suppl. 3), 18–31.

Çardak A.D. (2009): Environmental effects on protein composition of milk from Holstein-Friesian and Simmental cattle. Milchwissenschaft – Milk Science International, 64, 3–6.

Colitti M., Farinacci M. (2009): Cell turnover and gene activities in sheep mammary glands prior to lambing to involution. Tissue and Cell, 41, 326–333.

Colitti M., Pulina G. (2010): Short communication: Expression profile of caseins, estrogen and prolactin

receptors in mammary glands of dairy ewes. Italian Journal of Animal Science, 9, 285–289.

Coulon J.B., Verdier I., Pradel P., Montserrat A. (1998): Effect of lactation stage on the cheesemaking properties of milk and the quality of Saint-Nectaire-type cheese. Journal of Dairy Research, 65, 295–305.

Cui X., Zhang P., Deng W., Oesterreich S., Lu Y., Mills G.B., Lee A.V. (2003): Insulin-like growth factor-I inhibits progesterone receptor expression in breast cancer cells via the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin pathway: Progesterone receptor as a potential indicator of growth factor activity in breast cancer. Molecular Endocrinology, 17, 575–588.

Darnell Jr. J.E. (1997): STATs and gene regulation. Science, 277, 1630–1635.

Demmer J., Ross I.K., Ginger M.R., Piotte C.K., Grigor M.R. (1998): Differential expression of milk protein genes during lactation in the common brushtail possum (*Trichosurus vulpecula*). Journal of Molecular Endocrinology, 20, 37–44.

Farr V.C., Stelwagen K., Cate L.R., Molenaar A.J., McFadden T.B., Davis S.R. (1996): An improved method for the routine biopsy of bovine mammary tissue. Journal of Dairy Science, 79, 543–549.

Farrell Jr. H.M., Jimenez-Flores R., Bleck G.T., Brown E.M., Butler J.E., Creamer L.K., Hicks C.L., Hollar C.M., Ng-Kwai-Hang K.F., Swaisgood H.E. (2004): Nomenclature of the proteins of cows' milk – sixth revision. Journal of Dairy Science, 87, 1641–1674.

Finucane K.A., McFadden T.B., Bond J.P., Kennelly J.J., Zhao F.Q. (2008): Onset of lactation in the bovine mammary gland: gene expression profiling indicates a strong inhibition of gene expression in cell proliferation. Functional and Integrative Genomics, 8, 251–264.

Friggens N.C., Ridder C., Løvendahl P. (2007): On the use of milk composition measures to predict the energy balance of dairy cows. Journal of Dairy Science, 90, 5453–5467.

Gáspárdy A., Schwartz Z., Zöldág L., Veresegyházy T., Fekete S. (2004): Changes in daily energy amounts of main milk components (lactose, protein and fat) during the lactation of high-yielding dairy cows. Acta Veterinaria Hungarica, 52, 457–467.

Gomm J.J., Browne P.J., Coope R.C., Liu Q.Y., Buluwela L., Coombes R.C. (1995): Isolation of pure populations of epithelial and myoepithelial cells from the normal human mammary gland using immunomagnetic separation with dynabeads. Analytical Biochemistry, 226, 91–99.

Griesbeck-Zilch B., Meyer H.H.D., Kühn C., Schwerin M., Wellnitz O. (2008): *Staphylococcus aureus* and *Escherichia coli* cause deviating expression profiles of cytokines and lactoferrin messenger ribonucleic acid in mammary epithelial cells. Journal of Dairy Science, 91, 2215–2224.

Groenen M.A.M., van der Poel J.J. (1994): Regulation of expression of milk protein genes: a review. Livestock Production Science, 38, 61–78.

Hagnestam-Nielsen C., Emanuelson U., Berglund B., Strandberg E. (2009): Relationship between somatic cell count and milk yield in different stages of lactation. Journal of Dairy Science, 92, 3124–3133.

Kroeker E.M., Ng-Kwai-Hang K.F., Hayes J.F., Moxley J.E. (1985): Effects of environmental factors and milk protein polymorphism on composition of casein fraction in bovine milk. Journal of Dairy Science, 68, 1752–1757.

Larson B.L., Kendall K.A. (1957): Protein production in the bovine. Daily production of the specific milk proteins during the lactation period. Journal of Dairy Science, 40, 377–386.

Linzell J.L. (1966): Measurement of udder volume in live goats as an index of mammary growth and function. Journal of Dairy Science, 49, 307–311.

Ma H., Shieh K.J., Chen G., Qiao X.T., Chuang M.Y. (2006): Application of real-time polymerase chain reaction (RT-PCR). Journal of American Science, 2, 1–15.

Menzies K.K., Lefèvre C., Macmillan K.L., Nicholas K.R. (2009): Insulin regulates milk protein synthesis at multiple levels in the bovine mammary gland. Functional and Integrative Genomics, 9, 197–217.

Miller R.H., Paape M.J., Fulton L.A. (1991): Variation in milk somatic cells of heifers at first calving. Journal of Dairy Science, 74, 3782–3790.

Ng-Kwai-Hang K.F., Hayes J.F., Moxley J.E., Monardes H.G. (1987): Variation in milk protein concentrations associated with genetic polymorphism and environmental factors. Journal of Dairy Science, 70, 563–570.

Pfaffl M.W. (2001): A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research, 29, 2002–2007.

Poulton S.G., Ashton W.M. (1970): A study of the composition of Clun Forest ewe's milk. IV. The proteins of ewe's milk and their variation with stage of lactation. Journal of Agricultural Science, 75, 245–250.

Quist M.A., LeBlanc S.J., Hand K.J., Lazenby D., Miglior F., Kelton D.F. (2008): Milking-to-milking variability for milk yield, fat and protein percentage, and somatic cell count. Journal of Dairy Science, 91, 3412–3423.

Rasmussen R. (2001): Quantification on the LightCycler. In: Meuer S., Wittwer C., Nakagawara K. (eds): Rapid Cycle Real-Time PCR: Methods and Applications. Springer Verlag Press, Heidelberg, Germany, 21–34.

Sheldrake R.F., Hoare R.J.T., McGregor G.D. (1983): Lactation stage, parity, and infection affecting somatic cells, electrical conductivity, and serum albumin in milk. Journal of Dairy Science, 66, 542–547.

Shennan D.B., Peaker M. (2000): Transport of milk constituents by the mammary gland. Physiological Reviews, 80, 925–951.

Sorensen A., Alamer M., Knight C.H. (1998): Physiological characteristics of high genetic merit and low genetic merit dairy cows: a comparison. In: Proc. British Society of Animal Science. Scarborough, UK, 4.

Stein T., Morris J.S., Davies C.R., Weber-Hall S.J., Duffy M.A., Heath V.J., Bell A.K., Ferrier R.K., Sandilands G.P., Gusterson B.A. (2004): Involution of the mouse mammary gland is associated with an immune cascade and an acute-phase response, involving LBP, CD14 and STAT3. Breast Cancer Research, 6, R75–R91.

Talhouk R.S., Neiswander R.L., Schanbacher F.L. (1990): *In vitro* culture of cryopreserved bovine mammary cells on collagen gels: synthesis and secretion of casein and lactoferrin. Tissue and Cell, 22, 583–599.

Threadgill D.W., Womack J.E. (1990): Genomic analysis of the major bovine milk protein genes. Nucleic Acids Research, 18, 6935–6942.

Walsh S., Buckley F., Berry D.P., Rath M., Pierce K., Byrne N., Dillon P. (2007): Effects of breed, feeding system, and parity on udder health and milking characteristics. Journal of Dairy Science, 90, 5767–5779.

Wood P.D.P. (1969): Factors affecting shape of lactation curve in cattle. Animal Production, 11, 307–316.

Zhao F.Q., Dixon W.T., Kennelly J.J. (1996): Localization and gene expression of glucose transporters in bovine mammary gland. Comparative Biochemistry and Physiology – Part B: Biochemistry and Molecular Biology, 115, 127–134.

Zhao F.Q., Zheng Y.C., Wall E.H., McFadden T.B. (2005): Cloning and expression of bovine sodium/glucose cotransporters. Journal of Dairy Science, 88, 182–194.

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