Innate defense capability of challenged primary bovine mammary epithelial cells after an induced negative energy balance *in vivo*

K. Danowski[1,2], D. Sorg[1,2], J. Gross[3], H.H.D. Meyer[1,2], H. Kliem[1,2]

[1]Physiology Weihenstephan, Technical University Munich, Freising-Weihenstephan, Germany
[2]ZIEL – Research Centre for Nutrition and Food Sciences, Technical University Munich, Freising-Weihenstephan, Germany
[3]Animal Nutrition, Technical University Munich, Freising-Weihenstephan, Germany

ABSTRACT: Negative energy balance (NEB), if followed by metabolic imbalance, is a common problem in high-yielding dairy cows frequently associated with inflammation of the mammary gland. After entering the teat canal, mammary epithelium is the first line of defense against a pathogen invasion. To investigate the effect of NEB on the innate host defense of the mammary epithelium, primary bovine mammary epithelial cell (pbMEC) cultures were generated by cell extraction of milk derived from energy restricted and control feeding cows. pbMEC were obtained from 8 high-yielding dairy cows affected by induced NEB in mid-lactation due to a reduction to 51 ± 2% of total energy requirement (restriction group) and from 7 control cows (control group). They were exposed to heat-inactivated *Escherichia coli* and *Staphylococcus aureus* for 24 and 72 h to investigate the influence of NEB on gene expression profiles of cytokines, chemokines, genes associated with apoptosis and antimicrobial peptides plus their receptors (AMPR) of the innate immune response. The immune challenge of pbMEC demonstrated an effect of immune capacity and NEB in 15 differential expressed genes. NEB induced a substantial up-regulation in restriction compared to control cells by trend in *E. coli* and a down-regulation in *S. aureus* exposed cells. Our investigations showed that the dietary-induced NEB *in vivo* influenced the immune response of pbMEC *in vitro* and altered the expression of immunological relevant genes due to a difference in energy supply. These results demonstrate that pbMEC are a suitable model for mastitis research, in which even effects of feeding regimes can be displayed.

Keywords: pbMEC; mastitis; energy deficit; *E. coli*; dairy cow; gene expression; innate immune response

Mastitis is the most cost intensive production disease in dairy industry. Medical treatment, reduced fertility, extra labour, and reduced milk yield cause a considerable financial burden. Calculations of annual losses due to mastitis revealed an amount of 10% of total value of farm milk sales, two thirds being a result of reduced milk yield caused by subclinical udder inflammation (Schroeder, 2010). During early lactation, high energy requirements for milk production cannot be adjusted by increasing feed intake and result in negative energy balance (NEB) often followed by metabolic imbalance. Energy deficit leads to extensive mobilization of body fat reserves and may result in increased blood nonesterified fatty acid (NEFA) and β-hydroxybutyrate (BHB) concentrations. Elevated NEFA and BHB levels are considered to have inhibiting effects on immune cells (Suriyasathaporn et al., 2000) and to assist the state of impaired immune system (Loor et al., 2007; Roche et al., 2009). Inflammation of the mammary gland is induced by gram-negative and gram-positive pathogens that cause different appearances of mastitis. The most prevalent gram-negative bacteria, *Escherichia coli* (*E. coli*), is a typical environment-associated pathogen that leads to an acute and severe systemic mastitis. In contrast,

Staphylococcus aureus (*S. aureus*) is among the most prevalent gram-positive bacteria causing a chronical and subclinical form of mastitis (Wellnitz et al., 2006; Tesfaye et al., 2009). Under practical conditions most mastitis incidences are disposed subclinically and remain unnoticed in dairy livestock. Besides their milk secretory function, mammary epithelial cells (MEC) participate in the first line of defense against invading pathogens (Vorbach et al., 2006) and operate together with immune cells during pathogen invasion. Cell culture studies with MEC revealed the expression of host defense mechanisms, e.g., pathogen recognition receptors as well as antimicrobial peptide (Petzl et al., 2008; Griesbeck-Zilch et al., 2009), which enable them to react on pathogen invasion before the acquired immune defense factors intervene. They are also responsible for immune modulatory effects in the udder due to secretion of chemokines (Bournazou et al., 2009) which enables the interaction with immune cells to defend against pathogen invasion.

Most investigated receptors are the transmembrane toll-like receptors (TLR) that mediate pathogen recognition via the pathogen-associated molecule pattern (PAMP) such as lipopolysaccharides (LPS) from *E. coli* and lipoteichoic acid (LTA) of *S. auerus*. In cattle, currently 10 different TLR are described and characterized (Werling et al., 2006). Petzl et al. (2008) demonstrated previously that TLR2 and TLR4 are selectively up-regulated in case of clinical mastitis, whereas TLR9 was not affected. Beside receptor-based defense, mammary epithelial cells secret a wide range of antimicrobial peptides (AMP) (Zasloff, 2002; Roosen et al., 2004; Lutzow et al., 2008; Molenaar et al., 2009). These proteins and peptides react upon all invading pathogens and exhibit strategies of killing. Antiviral, antifungal, and antibiotic mechanisms include membrane disruption, thus perturbing bacterial permeability as well as metabolic inhibition (Almeida and Pokorny, 2009; Bocchinfuso et al., 2009). Additionally, in contrast to the therapeutical problems of increasing antibiotical resistance of pathogens, interest on those potent peptides increases due to minimal resistance development of the pathogens (Kraus and Peschel, 2006). Acute symptoms of mammary infection most often associated with *E. coli* mastitis lead to increasing inflammation parameters. First of all, Tumor necrosis factor alpha (TNFα) and Interleukin 1 beta (IL1β) are to be mentioned. In the acute phase of cytokine release they mediate both local and systemic inflammatory responses. They are most potent endogenous inducers of fever and have both beneficial and injurious properties (Sordillo and Streicher, 2002). Furthermore, TNFα is one of the factors to induce apoptosis in the mammary gland (Bannerman, 2009). During mammary inflammation epithelial cells take part in chemotaxis to recruit immune cells by the release of chemoattractants (Haston and Shileds, 1985). In case of acute mastitis 90% of milk-derived cells are neutrophiles (Mehrzad et al. 2005), which are also supposed to be the first cells to arrive at inflammation due to secretion of growth-related oncogene alpha (Groα) and Interleukin 8 (IL8). Severe mastitis leads to mammary tissue damage and cell death by either apoptosis or necrosis, supported by both bacteria and host defense factors (Zhao and Lacasse, 2008). Apoptosis initiating and regulatory factors are the FAS receptor, the anti-apoptotic B-cell lymphoma 2 (Bcl-2) family members involved in mitochondrial death cascade, and up-stream initiator and down-stream effector cysteine proteases called caspases activated by the death receptor and the mitochondrial cascade (Nunez et al., 1998).

However, in most of the above cited works analysis was done in milk or the established cell culture models were generated by mammary biopsy or slaughter after intra mammary infection (Wellnitz and Kerr, 2004; Griesbeck-Zilch et al., 2008; Petzl et al., 2008). Beside its invasive character concerning animal's welfare, the main disadvantage of mammary biopsy is the high risk of contamination with fibroblasts. This fast-growing stroma cells may overgrow the target epithelial cells and might tamper with the results. According to the advice of Boutinaud and Jammes (2002), the establishment of a cell culture model of milk-derived cells was implemented and focus was directed at the immune defense capability of primary bovine mammary epithelial cells (pbMEC) affected by induced *in vivo* NEB. The present investigation should have revealed whether the induced NEB *in vivo* influences also the immune capacity of MEC, for its known inhibiting effect on immune cells (Suriyasathaporn et al., 2000). Therefore cell cultures of pbMEC of energy restricted and control fed cows were generated and an immune challenge was conducted. A set of 15 comprehensive genes involved in the different areas of the innate host defense was selected and the immune response was determined using quantitative reverse transcription polymerase chain reaction (qRT-PCR).

MATERIAL AND METHODS

Animals and dietary-induced NEB

A detailed description of the experimental design and the conduction of the feeding experiment were published in Gross et al. (2011). In brief, Red Holstein cows were housed in a free-stall barn and were evenly assigned to control and restriction feeding according to milk yield, calculated energy balance, and feed intake during the first 85 days postpartum (pp). After re-establishment of metabolic stability and a positive energy balance on day 100 pp, a 51 ± 2% dietary energy deficit of total energy requirements was individually induced for 3 weeks, followed by a re-alimentation period.

Cell culture of primary bovine mammary epithelial cells

Milk samples were taken on the last day of the energy restriction period. One litre of milk was taken from each animal and per quarters subjected to a bacterial milk test to exclude bacterial infection prior to the experiment. Only milk free of bacteria was used to extract pbMEC. The milk was dispersed evenly into four centrifuge cups (250 ml each). The four cups were centrifuged at 1850 *g*, at 20°C for 10 min. Milk was decanted and each cell pellet was re-suspended in 25 ml pre-warmed (37°C) washing medium (HBSS, Sigma-Aldrich, Munich, Germany) containing 200 µg per ml penicillin G, 200 µg/ml of streptomycin, 200 µg/ml gentamicin, and 10 µg/ml amphotericin B (Sigma-Aldrich, Munich, Germany). Two cell solutions were combined into a 50 ml falcon tube, washed by gentle mixing and centrifuged at 500 *g* at room temperature (RT) for 5 min. The pellets were re-suspended in 25 ml HBSS-solution and filtered (Falcon Cell Strainer 100 µm, BD Biosciences, Bedford, USA) into one falcon tube. After centrifugation at 500 *g* for 5 min, the pellet was re-suspended in warm growth medium consisting of DMEM/F12 Ham (Sigma-Aldrich, Munich, Germany), 10% fetal calf serum (FCS) (Gibco, Invitrogen, Carlsbad, USA), ITS supplement (5 mg/ml insulin, 5 mg/ml transferrin, and 0.005 mg/ml sodium selenite; Invitrogen, Carlsbad, USA), 100 µg/ml penicillin, 100 µg/ml streptomycin, 100 µg/ml gentamycin, and 5 µg/ml amphotericin B. The cells were seeded into 25 cm^2 tissue culture flasks (Greiner Bio One, Frickenhausen, Germany) and cultivated at 37°C, 5% $CO_{2,}$ and 90% humidity. The cells were allowed to attach for 24 h. Unattached cells were removed by gentile washing with warm phosphate buffered saline (PBS) of pH 7.4 and the medium was exchanged. Growth medium was changed twice weekly and growth of primary cells was documented until reaching 80% confluence. Due to higher sensibility and higher contamination risk in primary cells compared to cell lines, infected cultures were eliminated at first appearance of bacterial contamination. Additionally, only morphologically healthy cultures were further cultivated and selected for the experiment. The cells were harvested at 80% confluence state in the second passage and stored in DMEM/F12 HAM with 20% FCS and 10% dimethyl sulfoxide (DMSO) (Roth, Karlsruhe, Germany) in liquid nitrogen until all samples were taken. Finally, primary mammary epithelial cell cultures of 8 restriction and 7 control cows were successfully generated.

Immunohistochemistry

Epithelial identity was confirmed by immuno-histological staining of cytokeratins 4, 5, 6, 8, 10, 13, and 18. Concurrently to the seeding of the 48-wells challenge plates, pbMEC were seeded on culture chamber slides (LAB-Tek, Nunc, GmbH, Langenselbold, Germany) in four-times approach. After reaching confluent state, medium was removed and pbMEC were washed twice with PBS. Chambers were removed and attached cells were fixed with ice-cold aceton-methanol mix (1 : 1) for 5 min. Slides were dried at room temperature (RT). Wells were incubated with 1% H_2O_2 (Merk, Darmstadt, Germany) in PBS-Tween (PBST) in the dark at RT for 30 min to block endogenous peroxidases. After triple washing with PBST for 5 min, respectively, the slides were incubated with goat serum (Dako, Glostrup, Denkmark) diluted 1 : 10 in PBST for 30 min at RT. A primary monoclonal mouse IgG anti-pan cytokeratin antibody (F3418, Sigma-Aldrich, St. Louis, USA) was diluted 1 : 50 in PBST, applied to the wells and incubated at 4°C overnight. Goat serum remained on negative controls and was not replaced by primary antibody. On the next day the slides were 3 times washed with PBST for 5 min, respectively, and secondary polyclonal goat anti-mouse antibody (1 : 400; Immunoglobulins HRP, Dako Gostrup, Denmark) was applied. After 1 h incubation at RT the cells were washed 3 times with PBST for 5 min, respec-

tively, and peroxidase was visualized by incubating the wells with 0.01% DAB-dihydrochloride (D-5905, Sigma-Aldrich, Munich, Germany) and 0.01% H_2O_2 in PBST in the dark at RT for 15 min. Afterwards the slides were 3 times washed with PBST for 5 min, respectively, and were dipped in aqua bidets. The cell nuclei were stained with Mayer hemalaun solution (Roth, Karlsruhe, Germany) for 15 s and colour development was obtained by dipping the slides into tap water. The slides were dehydrated in a series of ethanols of increasing concetration (50–100%) for 2 min, respectively, followed by 2 min incubation in xylol (Sigma-Aldrich, Munich, Germany). Cover glasses were fixed with EUKITT (Fluka, Sigma-Aldrich, Steinheim, Germany). Results are shown in Figure 1.

Cultivation of *E. coli* and *S. aureus*

S. aureus 1027 and *E. coli* 1303 (Petzl et al., 2008) were donated from Wolfram Petzl (Clinic for Ruminants, Ludwig-Maximilians-University, Munich, Germany). The gram negative pathogen *E. coli* was cultured in lysogeny broth (LB) liquid medium and on LB-agar Lennox (SERVA, Heidelberg, Germany) plates. The cultivation of the gram positive *S. auerus* was conducted in casein-soy-peptone (CASO) broth liquid medium (Fluka, Sigma-Aldrich, Steinheim, Germany) and on blood agar (Blood Agar Base No. 2, Oxoid, Cambridge, UK) plates. The pathogens were thawed and applied to the appropriate agar plates and incubated at 37°C overnight. One colony of each pathogen was picked and applied to 20 ml growth mediums. After overnight incubation at 37°C, *E. coli* was diluted 1 : 1000 and *S. auerus* 1 : 500 into fresh growth medium. Optical density (OD) of 1 ml bacteria solution was measured at 600 nm every 30 min for 4 h to generate a growth curve. Simultaneously with each OD measurement, 5 dilution steps of the pathogens were seeded on respective agar plates and incubated at 37°C. At the beginning, 10^{-4}–10^{-6} dilution steps and with increasing time and pathogen growth 10^{-9}–10^{-10} dilution steps were used. Next day the colonies were counted. According to the assumption that one colony was grown out of one bacterium within the dilution steps the amount of bacteria was calculated. The growth curve was repeated and according to the optimal harvest time the growth was stopped by putting the pathogen tubes on ice for 10 min. The tubes were centrifuged at 1850 *g* twice for 10 min and re-suspended in 50 ml PBS. After the third centrifugation step, the pellet was re-suspended in 5 ml PBS and put into the 63°C water bath for 30 min to inactivate the pathogens. To control the inactivation, respective agar plates were inoculated with the pathogens. Bacteria solutions were aliquoted and stored at –80°C.

Immune challenge of pbMEC with heat-inactivated *E. coli* and *S. aureus*

Cells were thawed in the third passage and seeded into 48 well plates with a concentration of 100 000 cells per a well. Two wells were seeded for *E. coli*, *S. auerus,* and untreated control cells, respectively. Additionally two wells served as counting wells. Those wells were detached prior to treatment and counted twice. The determined mean cell count was assumed for the treatment and the control cell wells to calculate the concentration of applied pathogen. Until 80% confluency was obtained, the growth medium was replaced by 1 ml DMEM/F12 Ham supplied with ITS (challenge medium) solely. The cells in the counting wells were detached, counted, and pathogen concentrations for multiplicity of infection (MOI 30) were calculated. Challenge medium was replaced and the wells were infected with MOI 30 of respective heat-inactivated bacteria solution. Control wells were treated with PBS. A double approach was conducted.

Quantitative reverse transcription PCR (qRT-PCR) for mRNA quantification

After 24 and 72 h the cells were harvested, challenge medium supernatant was removed and stored at –80°C. Total RNA was extracted with the Allprep RNA/Protein kit (Qiagen, Hilden, Germany) as described in the manufacturer's instructions and an additional DNAse digestion (RNase-Free DNase Set, Qiagen, Hilden, Germany) was conducted. RNA integrity was determined with the Agilent Bioanalyzer 2100 and RNA 6000 Nano Assays (Agilent Technologies, Waldbronn, Germany). The reverse transcription was conducted on Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany). For converting the RNA template into cDNA 300 ng of RNA was reverse transcribed with 1 µl of M-MLV Reverse Transcriptase, RNase Minus, Point Mutant (Promega, Mannheim, Germany) using 3 µl of random primers (Invitrogen, Karlsruhe,

Germany) and 3 µl dNTP (Fermentas, St. Leon-Rot, Germany). The protocol started with 10 min at 21°C for optimized primer annealing, followed by 50 min at 48°C for transcription and 2 min at 90°C for inactivation of the enzyme and separation of generated cDNA and RNA template, and a final hold at 5°C. A negative control was added without enzyme for excluding genomic DNA contamination. Primers (Table 1) were designed using open source primer design software Primer 3 and synthesized by Eurofins (MWG GmbH, Ebersberg, Germany). Primer testing and qRT-PCR were conducted on the iQ5 Multicolor real-time PCR detection system (Bio-Rad Laboratories GmbH, Munich, Germany) using twin.tec PCR Plate 96 formats (Eppendorf, Hamburg, Germany). For qRT-PCR reaction 1.5 µl of cDNA equivalent to 7.25 ng of total RNA was amplified in 13.5 µl reaction volume with the MESA Green qPCR MasterMix Plus for SYBR® Assay with fluorescein (Eurogentec Deutschland GmbH, Koln, Germany). 1.5 µl forward and reversed primers were added. The used protocol started with 5 min polymerase activation at 95°C, followed by 40 cycles: denaturation at 95°C for 15 s, primer specific annealing for 20 s, and the elongation at 60°C for 40 s. A melt curve starting from 60°C to 95°C was performed in 10 s with 0.5°C steps per cycle. The size of the PCR products was confirmed by agarose gel electrophoresis after GelRed (Biotium Inc., Hayward, USA) staining.

Data analysis and statistics

Statistical description of the generated gene expression data set was analysed by GenEx software 5.0.1. (MultiD Analyses AB, Gothenburg, Sweden). The *Cq* values were normalized with the arithmetic means of reference genes. The three suitable reference genes – Glyceraldehyde 3-phosphate-dehydrogenase (GAPDH), Ubiquitin (UBQ3), and Actin gamma 1 (Actin γ1) – were selected using GenEx software. To calculate the effects of treatment versus control, $\Delta\Delta Cq$ method according to Livak and Schmittgen (2001) was used and the data transformation with $2^{-\Delta\Delta Cq}$ into relative expression ratio (x-fold regulation) was conducted. Target gene expression is represented as x-fold up-regulation for $x > 1.00$ and down-regulation is represented in values $x < 1.00$ with standard error of means (SEM), respectively. Outliners were identified and excluded using the GenEx function Grubbs' test.

A principal component analysis (PCA) was conducted for ΔCq values to disclose multivariate treatment effects. The PCA is a suitable tool for multidimensional data analysis, which allows recognition of patterns and visualization of treatment information of a heterogeneous data set. Calculation of the two principal components of the measured data for every sample leads to the reduction of dimensions and enables the plotting of samples each as one spot in a two-dimensional room. Therefore, treatment effects can be visualized according to formation of clusters and separation of the samples represented by one spot per sample (Kubista et al., 2006; Riedmaier et al., 2009). The PCA results were further confirmed by comparing the $2^{-\Delta\Delta Cq}$ arithmetic means in a one-way ANOVA (analysis of means) on ranks and subsequent Kruskal-Wallis Test using SPSS (IBM SPSS Statistics 19.0). P-values ≤ 0.05 were considered as significance level.

RESULTS

Immunohistochemisty

The immuno-histological staining of cytokeratins is presented in Figure 1. Positive brown staining illustrates the purity of the generated cell cultures

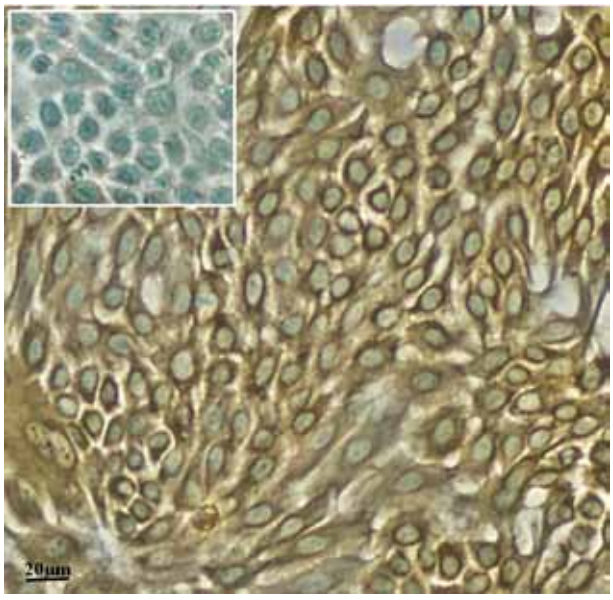

Figure 1. Immuno-histological identification of pbMEC by cytokeratine staining. Positive brown staining of cytokeratines 4, 5, 6, 8, 10, 13, and 18. The insert shows the negative control

Table 1. Primer sequences, PCR product lengths (bp) and sequence references for reference genes and differential expressed target genes

	Genes	Abbreviation	Primer	Sequence (5' to 3')	Size (bp)	Reference
Reference genes	Actin gamma 1	Actin γ1	F	aactccatcatgaagtgtgacg	233	NM_001033618
			R	gatccacatctgctggaagg		
	Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	F	gtcttcactaccatggagaagg	197	Berisha et al. 2002
			R	tcatggatgaccttggccag		
	Ubiquitin 3	UBQ3	F	agatccaggataaggaaggcat	198	NM174133
			R	gctccacttccagggtgat		
Target genes	Toll-like-receptor 2	TLR2	F	cattccctg gcaagtggattatc	202	NM_174197.2
			R	ggaatggccttcttgtcaatgg		
	Toll-like-receptor 4	TLR4	F	tgctggctgcaaaaagtatg	213	NM_174198.6
			R	ttacggcttttgtggaaacc		
	Lactoperoxidase	LPO	F	ccgacaacattgacatctgg	206	NM_173933.2
			R	gtcacagatgaggcgtgaga		
	Defensin beta 1	DEFβ1	F	tgctgggtcaggatttactcaagga	85	NM_175703.3
			R	agggcacctgatcggcacac		
	Interleukin 1 beta	IL1β	F	cagtgcctacgcacatgtct	209	NM_174093.1
			R	aga gga ggtggagagccttc		
	Tumor necrosis factor alpha	TNFα	F	ccacgttgtagccgacatc	108	AF348421
			R	accaccagctggttgtcttc		
	Interleukin 6	IL6	F	caccccaggcagactacttc	182	NM_173923.2
			R	atccgtccttttcctccatt		
	Chemokine (C-C motif) ligand 26/Eotaxin 3	CCL26	F	ctcggagctgccacacgtgg		
			R	tgggcacacactttccggcc	167	XM_002698193.1
	Growth-related oncogene	Groα	F	gctcggacgtgttgaagaac	116	U95812
			R	cctgagccagaggcggactac		
	Chemokine (C-X-C motif) ligand 5	CXCL5	F	ttgtgagagagctgcgttgt	150	NM_174300.2
			R	ccagacagacttcccttcca		
	Interleukin 8	IL8	F	tgctctctgcagctctgtgt	306	NM_173925.2
			R	cagacctcgtttccattggt		
	FAS	FAS	F	agaagggaaggagtacacaga	124	NM_000043
			R	tgcacttgtattctgggtcc		
	B-cell lymphoma 2	Bcl-2	F	cggaggctgggacgcctttg	116	NM_001166486.1
			R	tgatgcaagcgcccaccagg		
	Caspase 6	Casp6	F	ggctcgcggtccaggtgaag	177	NM_001035419.1
			R	ctggtgccaggcctgttcgg		
	Caspase 7	Casp7	F	atccaggccgactcgggacc	235	XM_604643.4
			R	agtgcctggccaccctgtca		

F = forward, R = reverse

and identifies the used cells as pbMEC without contamination of fibroblasts. The calculation revealed 97% of positive stained cells. The proof of quality is provided in the negative control without primary antibody presented in the insert of Figure 1. Unstained cells had an elongated cytoplasm with an oval nucleus and were excluded from the calculation of epithelial cells characterized by typical anti-cytokeratin staining. According to their morphological appearance they might be fibroblasts, which do not stain for cytokeratins (data not shown).

RNA integrity

The integrity of RNA was determined using the Agilent Bioanalyzer 2100 and RNA 6000 Nano Assays and presented as RNA Integrity Numbers (RIN). Mean RIN value was 7.9 ± 0.2 SEM.

qRT-PCR

Antimicrobial peptides and receptors (AMPR). As the first applied statistical tool, the PCA presented in Figure 2A revealed an emigration of *E. coli* treated samples from the general sample cloud. *S. auerus* and control samples are evenly spread and therefore indicate no effect of the *S. aureus* treatment versus control. Differential expressed genes of AMPR (Figure 3A) were influenced by trend by NEB. Significant effects were measured for TLR2 and TLR4, which were significantly up-regulated in *E. coli* infected control cells after 24 compared to 72 h ($P \leq 0.05$). Mean expression levels of TLR4 were low in all treatment groups. Expression levels were the highest in restriction cells exposed to *E. coli* (25–40-fold for Defensin beta 1 (DEFβ1)) and 46-fold for Lactoperoxidase (LPO). *S. aureus* induced an up-regulation from 24 until 72 h within

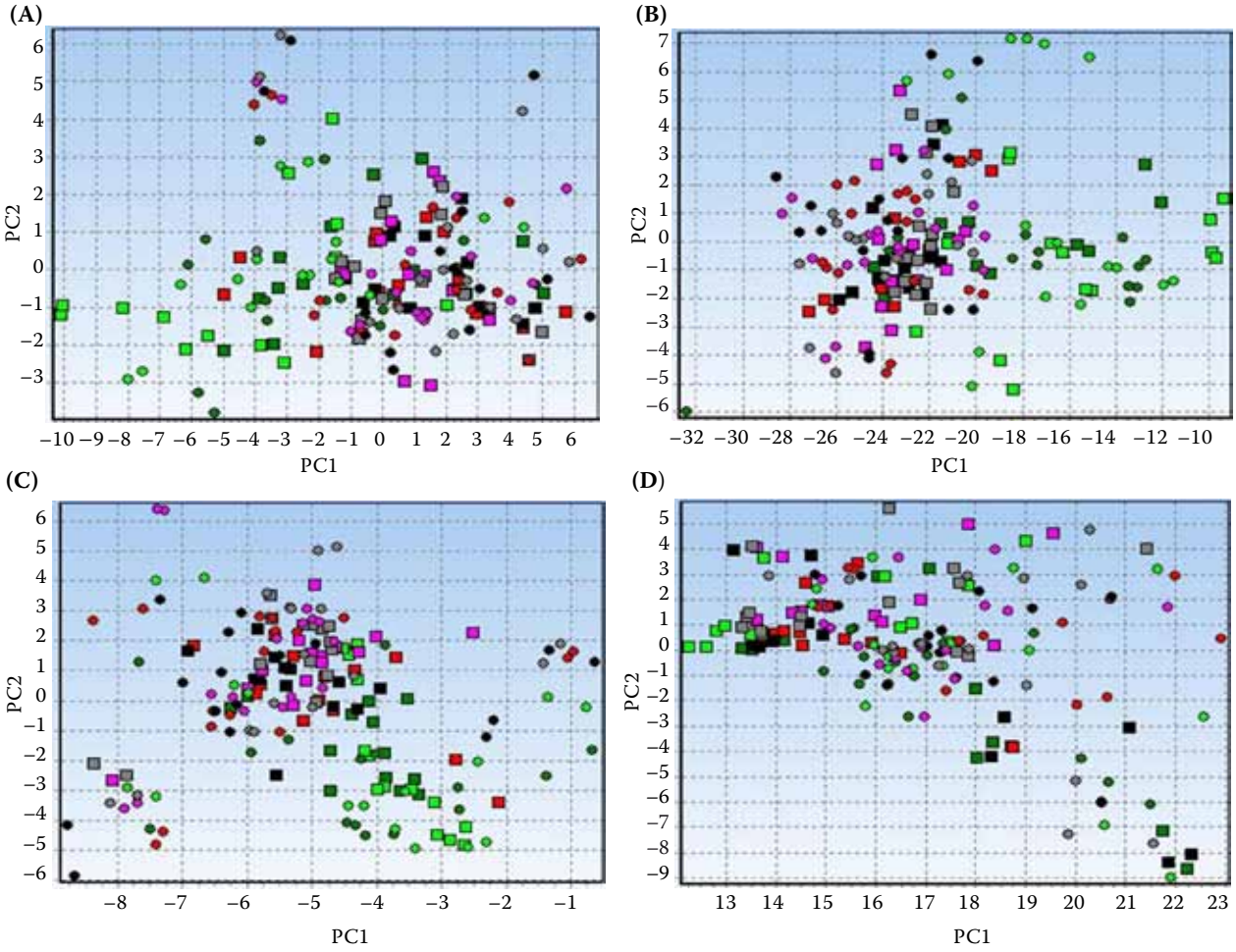

Figure 2. Principal component analysis (PCA) of four different immune functional gene groups presented on Δ*Cq* level: **A** = antimicrobial peptides and receptors (AMPR), **B** = cytokines, **C** = chemokines, **D** = apoptosis. Data sets are arranged according to feeding regime (control = square, restriction = circles), treatment (*E. coli* = green, light green; *S. aureus* = red, pink; control = black, grey), and infection time (24 h = dark colours, 72 h = light colours)

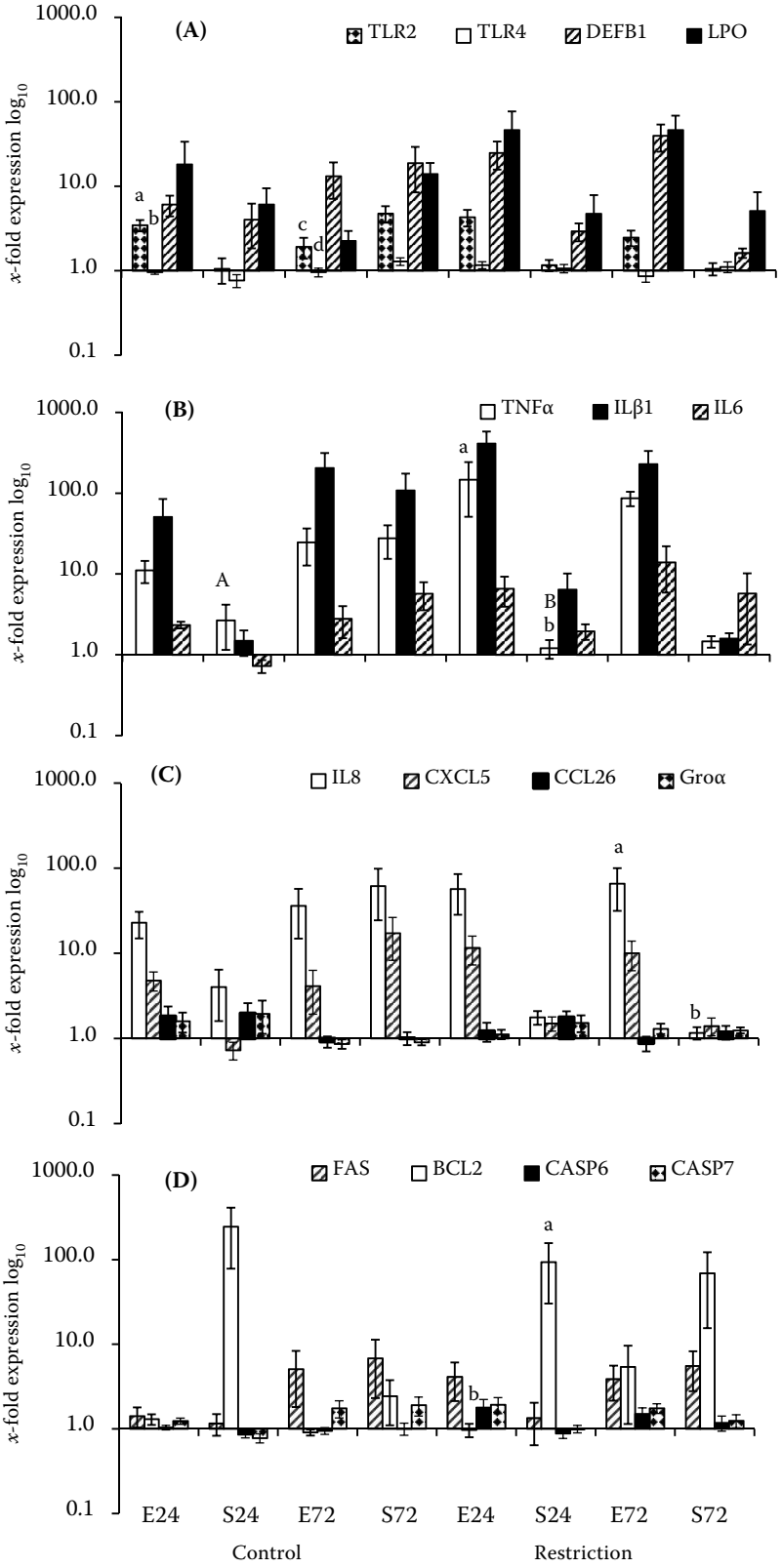

Figure 3. Relative gene expression of means presented as $2^{-\Delta\Delta Cq}$ in $\log_{10}$ scales ± SEM: **A** = antimicrobial peptides and receptors (AMPR), **B** = cytokines, **C** = chemokines, **D** = apoptosis related genes. S24, S72 = *S. aureus* infection for 24 and 72 h; E24, E72 = *E. coli* infection for 24 and 72 h. Significant differences within control or restriction group (E24 vs. E72, S24 vs. S72) are presented by different lowercase letters; significant differences between control and restriction group (E24 vs. E24, E72 vs. E72, S24 vs. S24, S72 vs. S72) are presented by different capitals; significant level $P \leq 0.05$

control fed group, but showed down-regulated expression profiles by trend in the energy restriction group.

Cytokines. The comparison between *E. coli* and *S. aureus* for control feeding and restriction in the PCA analysis for cytokines revealed separation of *E. coli* samples and slight emigration to the left of restriction samples out of the central cloud (Figure 2B). *E. coli* treatment showed a more pronounced transcript increase, especially in IL1β, than *S. aures* (Figure 3B). The combination of *E. coli* and energy restriction induced generally higher expression levels compared to control fed group, but without significance due to high SEM. Expression of all the three genes increased from 24 to 72 h under *S. aureus* influence in the control group, which was not seen in the restriction group. TNFα transcripts decreased significantly ($P \leq 0.05$) in restriction cells after 24 h compared to control cells after exposure to *S. aureus*. *E. coli* induced a higher expression compared to *S. aureus* in the restriction group after 24 h ($P \leq 0.001$). That effect could not be found in the control group. The same regulation pattern, but lower expression levels without significance were found for Interleukin 6 (IL6).

Chemokines. *E. coli* provoked an increased chemokine responses in pbMEC compared to *S. aureus* in the PCA (Figure 2C), which was even higher in the restriction cells (Figure 3C). The highest 125-fold up-regulation was found in IL8 due to restriction feeding and *E. coli* exposure. A significant difference was found between *E. coli* and *S. aureus* for 72 h in the restriction group ($P \leq 0.05$). Gene expressions of chemokine (C-C motif) ligand 26 (CCL26) and Groα in the control group were up-regulated after 24 h and down-regulated after 72 h for both pathogen stimulations. However, low expressions were found in the restriction group. Furthermore, a remarkable effect of the *S. aureus* stimulation was determined in the restriction group compared to the control group. All genes in this group were down-regulated after 24 h as well as 72 h, compared to the control feeding group. But high SEM prevented the calculation from significant differences.

Apoptosis. In contrast to the PCAs of the above mentioned gene classes, no clear clustering of apoptosis genes due to pathogen type could be found (Figure 2D). However, we could assess tendencies for tight clusters of restriction samples. Control feeding samples were arranged in a wide variety indicating a high variation within the data set. Further analysis revealed high SEM and low significant differences. Among apoptosis-related genes (Figure 3D), most pronounced up-regulation was found for the death receptors FAS and Bcl-2. A significant up-regulation was induced by *S. aureus* treatment for anti-apoptotic Bcl-2 compared to *E. coli* infected restriction cells after 24 h. FAS and Bcl-2 were also influenced by NEB and were up-regulated in the restriction group compared to the control feeding group after 24 h for *E. coli* by trend.

DISCUSSION

The accomplished PCAs on Δ*Cq*-level according to the functional gene groups showed a clear separation of *E. coli* infection compared to *S. aureus* and control cells (Figure 2). High variation within the data set is also displayed due to wide arrangement and increased distances of the *E. coli* sample clouds compared to *S. aureus* and control cell arrangements. This is also confirmed by high SEM within the presented bar charts (Figure 3A–D). *S. aureus* samples are arranged around the tight clustering of control samples in the PCA, which was most pronounced in the cytokine and chemokine group. This visualization cluster indicates the lower effect of *S. aureus* treatment compared to *E. coli*. However, the widespread *S. aureus* sample dots indicate high variance and therefore high SEM were calculated, leading to few significant results especially within the AMPR and the apoptosis group (Figure 2A, D). Therefore the calculation of significant differences of infection and feeding confirm the PCA findings and clearly point out that PCA is a suitable tool for the first step statistical analysis to describe treatment effects within the presented heterogeneous data set.

Antimicrobial peptides and receptors were influenced by both pathogens. Furthermore, the restriction additionally increased *E. coli* affected gene expression, but decreased the expression due to *S. aureus* infection, which could be explained by impaired immune capability caused by NEB. Cytokine responses were the highest among the analyzed functional gene groups. IL1β followed by TNFα showed a rapid up-regulation within 24 h indicating the activation of inflammatory action (Figure 3B). In contrast to Wellnitz and Kerr (2004), *E. coli* and not *S. aureus* induced the intensified up-regulations of IL1β and TNFα in our experiment, especially in the energy restriction group. The energy restric-

tion reduced the expression level of TNFα after 24 h in *S. aureus* treated cells and even more, but without significance, after 72 h. Buitenhuis et al. (2011) go in line with our findings. They report up-regulated transcripts of pro-inflammatory genes due to *E. coli* treatment after 24 h. Lower expression of cytokines and other inflammatory mediators after *S. aureus* challenge in our study are also reported in Griesbeck-Zilch et al. (2008) and Bannerman (2009). The latter found higher regulation patterns of pro-inflammatory cytokine induced by *S. aureus* after 1 h by trend. The early responses after *S. aureus* infection may be due to the disease pattern induced by the gram positive pathogen. Although an earlier sampling time than 24 h was not conducted in our experiment, the high magnitude of cytokine expression hypothesized a rapid establishment of cytokine release and showed even further increase of the immune response until 72 h post infection. This is characteristic for the innate immune system as it is poised to react as the first line defense against invading pathogens in the udder. IL1β and TNFα are the most reactive in the case of inflammation and the most potent to induce systemic immune reaction as far as shock, vascular leakage, and multiorgan failure (Bannerman, 2009). In the control fed group the expression of those cytokines rises up until 72 h seen in both bacteria, but is considerably decreased in the restriction group after 72 h for *S. aureus* only. This could indicate an effect of the conducted energy restriction on *S. aureus* infected cells. The measured down-regulation might demonstrate an impaired immune function and therefore may support the manifestation of a chronicle and subclinical *S. aureus* induced mastitis. The reaction of IL1β and TNFα further indicate the potential of our heat-inactivated *E. coli* 1303 used in MOI 30 to simulate an acute mammary infection as well as the defense capacity of the generated pbMEC towards *E. coli* infection (Gunther et al., 2009).

Immune challenge also activated the chemotaxis pathway in pbMEC. The highest expressions for IL8 and chemokine (C-X-C motif) ligand 5 (CXCL5) were found in the present work and confirm the findings of Pareek et al. (2005) using microarray technology on LPS stimulated bMEC, even though RANTES (regulated upon activation, normal T-cell expressed and secreted) was measured but not expressed in our experiment. Results by trend show a down-regulation of those chemokines by energy restriction of the *S. aureus* stimulated cells. GROα showed only low regulation changes due to treatments. This is in contrast to Lahouassa et al. (2007) who reported a 30-fold up-regulation of GROα after 24 h *E. coli* infection. Again, as found in the cytokine group, a further up-regulation was found in the pbMEC of energy restricted cows compared to control fed cows due to *E. coli* infection whereas a down-regulation of the chemokine expression was found due to *S. aureus* infection. The differences were not significant though because of high SEM.

The comparatively small effects of the dietary-induced energy deficit could also be explained by the metabolic screening results published in Gross et al. (2011). Cows were able to overcome induced NEB without suffering from metabolic instability and metabolic disorders even though only 51 ± 2% of total energy requirement was covered. This might be a reason for the existing, but low reaction of the pbMEC upon the feeding regime. However, our results by trend indicate an effect of the conducted dietary energy restriction. In the present study, *E. coli* exposed an immune stimulus and led to up-regulations of 15 innate immune system genes from 24 to 72 h and additional increase in the restriction group. *S. aureus* also induced effects on target genes with mostly increasing gene expressions from 24 to 72 h. In the restriction group, however, expression decreased considerably at both time points which might indicate a delayed immune function against *S. aureus* due to energy restriction. These findings are also reported in other studies. By means of the induced clinical signs of *S. aureus* caused mastitis, which remains subclinical and even chronicle, the activation of the immune response occurs within the very first hours post infection (Lahouassa et al., 2007; Griesbeck-Zilch et al., 2009) but remains generally at low levels. This strategy enables *S. aureus* strains to persist concealed by the immune system and develop lifelong infections. In our study no earlier time points than 24 h were sampled but the reaction due to *S. aureus* penetration was at lower levels than that due to *E. coli*. Ongoing infection activated the immune response against *S. aureus* and led to higher expression than *E. coli* in 72 h in the control fed group (Figure 3A, C). This late immune function seems to be blocked and decreased in the situation of induced NEB, which might enable *S. aureus*-induced mastitis to establish and persist. Concomitantly, anti-apoptotic Bcl-2 (Akbar et al., 1996) was considerably up-regulated by additional low regulation levels of the death receptor FAS for *S. aureus*-infected cells in 24 h. The

up-regulation of Bcl-2 might be a reaction on the restraining impact of NEB in order to overcome and protect the cells. By this assumption, the impact of the conducted dietary energy restriction could be indirectly confirmed.

CONCLUSION

In the present work, the immune challenge of *E. coli* and *S. aureus* induced expression changes of the determined AMPR, cytokine, chemokines, and apoptotic genes by time. Moreover, the accomplished energy restriction until 51 ± 2% of total energy requirement influenced the immune capacity of the generated cell cultures visibly, but with marginal significances. The immune responses in *E. coli*-infected cells increased in the restriction compared to the control feeding group, whereas *S. aureus*-infected cells seemed to be immune impaired by induced NEB, which led to down-regulations of the determined target genes.

Furthermore, our results approve the capability of pbMEC as a model for mastitis research. Physiological effects of metabolic challenges conducted to the animals seem to be transmitted into cell culture situation and even measurable in the immune response of primary cell cultures in the third passage. Additionally, we approve the capability of the principal component analysis (PCA) for visualization of treatment related differences within a heterogeneous data set.

Acknowledgement

We want to thank Prof. Dr. Michael Pfaffl, Dr. Irmgard Riedmaier, Dr. Ales Tichopad, and Jakob Müller Physiology Weihenstephan, for their expertise and help in primer design and data analysis. We appreciate the help of Dr. Wolfram Petzl, Clinic for Ruminants, Ludwig-Maximilians-University, Munich, Germany, who provided the mastitis-inducing pathogens.

REFERENCES

Akbar A.N., Borthwick N.J., Wickremasinghe G.R., Panayiotidis P., Pilling D., Bofill M., Krajewski S., Reed J.C., Salmon M. (1996): Interleukin-2 receptor common *y*-chain signaling cytokines regulate activated T cell apoptosis in response to growth factor withdrawal: Selective induction of anti-apoptotic (*bcl-2, bcl-x_L*) but not pro-apoptotic (*bax, bcl-x_S*) gene expression. European Journal of Immunology, 26, 294–299.

Almeida P.F., Pokorny A. (2009): Mechanisms of antimicrobial, cytolytic, and cell-penetrating peptides: from kinetics to thermodynamics. Biochemistry, 48, 8083–8093.

Bannerman D.D. (2009): Pathogen-dependent induction of cytokines and other soluble inflammatory mediators during intramammary infection of dairy cows. Journal of Animal Science, 87, 10–25.

Bocchinfuso G., Palleschi A., Orioni B., Grande G., Formaggio F., Toniolo C., Park Y., Hahm K.S., Stella L. (2009): Different mechanisms of action of antimicrobial peptides: insights from fluorescence spectroscopy experiments and molecular dynamics simulations. Journal of Peptide Science, 15, 550–558.

Bournazou I., Pound J.D., Duffin R., Bournazos S., Melville L.A., Brown S.B., Rossi A.G., Gregory C.D. (2009): Apoptotic human cells inhibit migration of granulocytes via release of lactoferrin. The Journal of Clinical Investigation, 119, 20–32.

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

Buitenhuis B., Rontved C.M., Edwards S.M., Ingvartsen K.L., Sorensen P. (2011): In depth analysis of genes and pathways of the mammary gland involved in the pathogenesis of bovine *Escherichia coli*-mastitis. BMC Genomics, 12, 130.

Griesbeck-Zilch B., Meyer H.H., Kuhn C.H., 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.

Griesbeck-Zilch B., Osman M., Kuhn C., Schwerin M., Bruckmaier R.H., Pfaffl M.W., Hammerle-Fickinger A., Meyer H.H., Wellnitz O. (2009): Analysis of key molecules of the innate immune system in mammary epithelial cells isolated from marker-assisted and conventionally selected cattle. Journal of Dairy Science, 92, 4621–4633.

Gross J., van Dorland H.A., Bruckmaier R.M., Schwarz F.J. (2011): Performance and metabolic profile of dairy cows during a lactational and deliberately induced negative energy balance with subsequent realimentation. Journal of Dairy Science, 94, 1820–1830.

Gunther J., Koczan D., Yang W., Nurnberg G., Repsilber D., Schuberth H.J., Park Z., Maqbool N., Molenaar A., Seyfert H.M. (2009): Assessment of the immune capac-

ity of mammary epithelial cells: comparison with mammary tissue after challenge with *Escherichia coli*. American Journal of Veterinary Research, 40, 31.

Haston W.S., Shileds J.M. (1985): Neutrophil leucocyte chemotaxis: a simplified assay for measuring porlarizing responses to chemotactic factors. Journal of Immunological Methods, 81, 229–237.

Kraus D., Peschel A. (2006): Molecular mechanisms of bacterial resistance to antimicrobial peptides. Current Topics in Microbiology and Immunology, 306, 231–250.

Kubista M., Andrade J.M., Bengtsson M., Foroota A., Jonak J., Lind K., Sindelka R., Sjoback R., Sjogreen B., Strombom L., Stahlberg A., Zoric N. (2006): The real-time polymerase chain reaction. Molecular Aspects of Medicine, 27, 95–125.

Lahouassa H., Moussay E., Rainard P., Riollet C. (2007): Differential cytokine and chemokine responses of bovine mammary epithelial cells to *Staphylococcus aureus* and *Escherichia coli*. Cytokine, 38, 12–21.

Livak K.J., Schmittgen T.D. (2001): Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods, 25, 402–408.

Loor J.J., Everts R.E., Bionaz M., Dann H.M., Morin D.E., Oliveira R., Rodriguez-Zas S.L., Drackley J.K., Lewin H.A. (2007): Nutrition-induced ketosis alters metabolic and signaling gene networks in liver of periparturient dairy cows. Physiological Genomics, 32, 105–116.

Lutzow Y.C., Donaldson L., Gray C.P., Vuocolo T., Pearson R.D., Reverter A., Byrne K.A., Sheehy P.A., Windon R., Tellam R.L. (2008): Identification of immune genes and proteins involved in the response of bovine mammary tissue to *Staphylococcus aureus* infection. BMC Veterinary Research, 4, 18.

Mehrzad J., Duchateau L., Burvenich C. (2005): Influence of resident milk neutrophils chemiluminescence and viability on the severity of bovine coliform mastitis. Veterinary Research, 36, 101–116.

Molenaar A.J., Harris D.P., Rajan G.H., Pearson M.L., Callaghan M.R., Sommer L., Farr V.C., Oden K.E., Miles M.C., Petrova R.S., Good L.L., Singh K., McLaren R.D., Prosser C.G., Kim K.S., Wieliczko R.J., Dines M.H., Johannessen K.M., Grigor M.R., Davis S.R., Stelwagen K. (2009): The acute-phase protein serum amyloid A3 is expressed in the bovine mammary gland and plays a role in host defence. Biomarkers, 14, 26–37.

Nunez G., Benedict M.A., Hu Y., Inohara N. (1998): Caspases: the proteases of the apoptotic pathway. Oncogene, 17, 3237–3245.

Pareek R., Wellnitz O., Van Dorp R., Burton J., Kerr D. (2005): Immunorelevant gene expression in LPS-challenged bovine mammary epithelial cells. Journal of Applied Genetics, 46, 171–177.

Petzl W., Zerbe H., Gunther J., Yang W., Seyfert H.M., Nurnberg G., Schuberth H.J. (2008): *Escherichia coli*, but not *Staphylococcus aureus* triggers an early increased expression of factors contributing to the innate immune defense in the udder of the cow. Veterinary Research, 39, 18.

Riedmaier I., Becker C., Pfaffl M.W., Meyer H.H. (2009): The use of omic technologies for biomarker development to trace functions of anabolic agents. Journal of Chromatography A, 1216, 8192–8199.

Roche J.R., Friggens N.C., Kay J.K., Fisher M.W., Stafford K.J., Berry D.P. (2009): Invited review: Body condition score and its association with dairy cow productivity, health, and welfare. Journal of Dairy Science, 92, 5769–5801.

Roosen S., Exner K., Paul S., Schroder J.M., Kalm E., Looft C. (2004): Bovine beta-defensins: identification and characterization of novel bovine beta-defensin genes and their expression in mammary gland tissue. Mammalian Genome, 15, 834–842.

Schroeder J.W. (2010): Bovine mastitis and milking management. Mastitis control programs, North Dakota State University and U.S. Department of Agriculture, NDSU Extension Service Circular AS-1129. Available from http://www.ag.ndsu.edu/pubs/ansci/dairy/as1129.pdf

Sordillo L.M., Streicher K.L. (2002): Mammary gland immunity and mastitis susceptibility. Journal of Mammary Gland Biology and Neoplasia, 7, 135–146.

Suriyasathaporn W., Heuer C., Noordhuizen-Stassen E.N., Schukken Y.H. (2000): Hyperketonemia and the impairment of udder defense: a review. Veterinary Research, 31, 397–412.

Tesfaye G.Y., Regassa F.G., Kelay B. (2009): Milk yield and associated economic losses in quarters with subclinical mastitis due to *Staphylococcus aureus* in Ethiopian crossbred dairy cows. Tropical Animal Health and Production, 42, 925–931.

Vorbach C., Capecchi M.R., Penninger J.M. (2006): Evolution of the mammary gland from the innate immune system? BioEssays, 28, 606–616.

Wellnitz O., Kerr D.E. (2004): Cryopreserved bovine mammary cells to model epithelial response to infection. Veterinary Immunology and Immunopathology, 101, 191–202.

Wellnitz O., Reith P., Haas S.C., Meyer H.H.D. (2006): Immune relevant gene expression of mammary epithelial cells and their influence on leukocytechemotaxis in response to different mastitis pathogens. Veterinarni Medicina, 51, 125–132.

Werling D., Piercy J., Coffey T.J. (2006): Expression of toll-like receptors (TLR) by bovine antigen-presenting cells – potential role in pathogen discrimination? Veterinary Immunology and Immunopathology, 112, 2–11.

Zhao X., Lacasse P. (2008): Mammary tissue damage during bovine mastitis: causes and control. Journal of Animal Science, 86, 57–65.

Zasloff M. (2002): Antimicrobial peptides of multicellular organisms. Nature, 415, 389–395.

Received: 2011–09–16
Accepted after corrections: 2011–12–19

Corresponding Author

Dr. Heike Kliem, Physiology Weihenstephan, Technische Universität München, Weihenstephaner Berg 3, D-85354 Freising-Weihenstephan, Germany
Tel. +49 816 198 684 42, fax +49 816 180 609 09, e-mail: kliem@wzw.tum.de