TECHNISCHE UNIVERSITÄT MÜNCHEN

Lehrstuhl für Physiologie

Functional characterization and comparison
of the intra-mammary immune system
of ancient and modern cattle breeds

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Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

genehmigten Dissertation.

Vorsitzender: Univ.-Prof. Dr. M. Klingenspor

Prüfer der Dissertation:

1. Univ.-Prof. Dr. M. W. Pfaffl
2. Priv.-Doz. Dr. Dr. K. Frölich
   (Universität Hildesheim)

Die Dissertation wurde am 18.12.2012 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 08.04.2013 angenommen.

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Abbreviations

ACTG1	Actin, gamma 1
AMP	Antimicrobial peptide
ANOVA	Analysis of variance
BCA	Bicin chinoninic acid
BCMS	British Cattle Movement Service
BS	Brown Swiss
BSA	Bovine serum albumin
C	Celsius
C1QA	Complement component 1, q subcomponent, A chain (gene)
C3	Complement component 3
C3AR	C3a anaphylatoxin chemotactic receptor (protein)
C3AR1	Complement component 3a receptor 1 (gene)
C5	Complement component 5
C5AR	C5a anaphylatoxin chemotactic receptor (protein)
C5AR1	Complement component 5a receptor 1 (gene)
C6	Complement component 6
C7	Complement component 7
C8	Complement component 8
C9	Complement component 9
CASP1	Caspase 1
CASP8	Caspase 8
CCL2	Chemokine (C-C motif) ligand 2
CCL5	Chemokine (C-C motif) ligand 5
CCL20	Chemokine (C-C motif) ligand 20
CD14	Monocyte differentiation antigen CD14

CD68	Macrosialin
CD163	Scavenger receptor cysteine-rich type 1 protein M130
cDNA	Complementary DNA
Cq	Cycle of quantification
CXCL5	Chemokine (C-X-C motif) ligand 5
DAB	3,3'-diaminobenzidine
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dNTP	Deoxyribonucleotide
ELISA	Enzyme-linked immunosorbent assay
FAO	Food and Agriculture Organization
FBS	Fetal bovine serum
g	Gram
g	Standard gravity
GAPDH	Glyceraldehyd-3-phosphate dehydrogenase
h	Hour
H3F3A	H3 histone, family 3A
HBSS	Hank's balanced salt solution
HLD	Highland
HRP	Horseradish peroxidase
HP	Haptoglobin
IL1B	Interleukin 1, beta (gene)
IL-1b	Interleukin-1, beta (protein)
IL6	Interleukin 6 (gene)
IL-6	Interleukin-6 (protein)
IL8	Interleukin 8 (gene)
IL-8	Interleukin-8 (protein)
IL10	Interleukin 10 (gene)

IL-10	Interleukin-10 (protein)	NF-kB	Nuclear factor kappa B
IRF3	Interferon regulatory factor 3	ng	Nanogram
ITS	Insulin, transferrin, sodium selenite	NLRP1	NLR family, pyrin domain containing 1 (gene); NACHT, LRR and PYD domains-containing protein 1 (protein)
IU	International units		
KRT8	Keratin 8		
l	Litre	NLRP3	NLR family, pyrin domain containing 3 (gene); NACHT, LRR and PYD domains-containing protein 3 (protein)
LAM	Lipoarabinomannan		
LAP	Lingual antimicrobial peptide		
LBP	Lipopolysaccharide binding protein	nm	Nanometre
LF	Lactoferrin	OD	Optical density
LP	Lipoprotein	p65	Transcription factor p65
LPO	Lactoperoxidase	pbMEC	primary bovine mammary epithelial cells
LTA	Lipoteichoic acid		
LY96	Lymphocyte antigen 96	pbMEC I	pbMEC for comparison of tissue and milk extraction
LYZ1	Lysozyme 1, milk isozyme		
mg	Milligram	pbMEC II	pbMEC for comparison of modern and ancient cattle breeds
min	Minute		
MOI	Multiplicity of infection	PBS	Phosphate buffered saline
MYD88	Myeloid differentiation primary response gene (88)	PBST	PBS-Tween20
		PCA	Principal component analysis
MyD88	Myeloid differentiation primary response protein (88)	PGS	Peptidoglycans
		RBST	Rare Breeds Survival Trust
µg	Microgram	RELA	V-rel reticuloendotheliosis viral oncogene homolog A (avian)
µl	Microlitre		
MX1	Myxovirus (influenza virus) resistance 1, interferon inducible protein p78 (mouse) (gene)	RH	Red Holstein
		RNA	Ribonucleic acid
		ROS	Reactive oxygen species
Mx1	Interferon-induced GTP-binding protein Mx1	RT	Reverse transcription
		RT-qPCR	Reverse transcription quantitative polymerase chain reaction
MX2	Myxovirus (influenza virus) resistance 2 (mouse) (gene)		
		S100A9	S100 calcium binding protein A9 (gene)
Mx2	Interferon-induced GTP-binding protein Mx2	S100-A9	S100 calcium binding protein A9
		S100A12	S100 calcium binding protein A12 (gene)
		S100-A12	S100 calcium binding protein A12

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SAA	Serum amyloid A (protein)	TNF	Tumor necrosis factor
SAA3	Serum Amyloid A3 (gene)	U	Units
SCC	Somatic cell count	UBB	Ubiquitine B
sec	Second	VIM	Vimentin
SHRP	Streptavidin horseradish peroxidase	TGRDEU	Central Documentation on Animal Genetic Resources in Germany
TAP	Tracheal antimicrobial peptide		
TGFB1	Transforming growth factor beta 1 (gene)	TMB	3,3,5,5-tetramethylbenzidine
TGFb-1	Transforming growth factor beta 1 (protein)	WP	White Park
TLR	Toll-like receptor	YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide
TLR2	Toll-like receptor 2		
TLR4	Toll-like receptor 4		

Summary

Mastitis, or inflammation of the udder, is a common disease of modern dairy cows. Invading pathogens like *Escherichia coli (E. coli)* and *Staphylococcus aureus (S. aureus)* often cause acute or chronic infections which lead to financial losses for the farmer and diminished welfare of the animal. Beside antibiotic therapy and improved milking and bedding hygiene, one approach is to breed animals that are genetically less susceptible. Ancient cattle breeds that have not intensively been selected for high milk yield are observed to show less symptoms of mastitis and are suspected to be less susceptible to this disease. We chose the ancient Highland (HLD) and White Park (WP) cattle (n=5) and the modern dairy breeds Brown Swiss (BS) and Red Holstein (RH) (n=6) to investigate possible breed differences in the innate immune response of their primary bovine mammary epithelial cells (pbMEC) *in vitro.* These were cultivated from milk and stimulated with heat-inactivated *E. coli* and *S. aureus.* Gene expression of 28 innate immune genes (complement system, inflammatory cytokines, chemokines, TLR pathway, acute phase proteins, scavenger receptors and others) was measured via reverse transcription quantitative polymerase chain reaction (RT-qPCR) on a high-throughput platform. The antimicrobial peptide lactoferrin (LF), the anti-inflammatory cytokine interleukin-10 (IL-10) and the acute phase protein serum amyloid A (SAA) were quantified in the cell protein extract with the enzyme-linked immunosorbent assay (ELISA). The breeds activated generally the same pathways at comparable levels. Differences in basal expression were observed in 16 genes ($P<0.05$), but no consistent ranking of the breeds could be made except for the finding that the TLR pathway components were more highly expressed in the ancient breeds. However, a principal component analysis (PCA) on basal gene expression levels showed a visible separation of the ancient from the modern breeds and from BS and RH. Fold changes of expression were only significantly different in complement component 3 (C3), chemokine (C-C motif) ligand 2 (CCL2), lactoperoxidase (LPO), caspase 8 (CASP8), monocyte differentiation antigen CD14 (CD14), lymphocyte antigen 96 (LY96), transforming growth factor beta 1 (TGFB1) and S100 calcium binding protein A12 (S100A12) ($P<0.05$). While the PCA on the regulation of gene expression after stimulation revealed no visible clustering of the breeds, there was a higher number of up regulated genes ($P<0.05$) in the modern breeds. HLD had higher basal and *E. coli* treated levels of LF protein and higher basal and *S. aureus* treated levels of SAA protein than the modern breeds. In addition to that, there was considerable between-cow variation in gene expression and protein production. Generally, the picture of the immune responses of the different breeds remains heterogeneous and diffuse, but it could be suspected that in the ancient breeds a higher basal level of a part of the innate immune system lead to a less

pronounced response. Breed differences in details of the innate immune system, but also breed-conserved activation of innate immune pathways have been previously described, as well as the large animal-to-animal variation that we found which remains subject to further research.

Zusammenfassung

Mastitis, auch Euterentzündung genannt, ist eine häufige Erkrankung bei modernen Milchkühen. Eindringende Pathogene wie *Escherichia coli (E. coli)* und *Staphylococcus aureus (S. aureus)* rufen oft akute oder chronische Infektionen hervor, die zu finanziellen Einbußen bei den Landwirten und zur Beeinträchtigung des Tierwohls führen. Neben der Behandlung mit Antibiotika und verbesserter Melk- und Stallhygiene ist es ein Ansatz, Tiere zu züchten, die genetisch weniger anfällig gegenüber Mastitis sind. Alte Rinderrassen, die weniger auf hohe Milchleistung selektiert wurden, zeigen weniger Symptome von Mastitis und sind vermutlich weniger anfällig für diese Krankheit. Wir wählten die alten Rassen Schottisches Hochlandrind (HLD) und Englisches Parkrind (WP) (n=5), sowie die modernen Milchrassen Braunvieh (BS) und rot-weißes Holstein (RH), um mögliche Unterschiede in der angeborenen Immunantwort ihrer primären bovinen Euterepithelzellen (pbMEC) *in vitro* zu untersuchen. Diese wurden aus der Milch kultiviert und mit Hitze-inaktivierten *E. coli* und *S. aureus* stimuliert. Die Expression von 28 Genen des angeborenen Immunsystems (Komplementsystem, inflammatorische Zytokine, Chemokine, TLR-Signalübertragung, Akut-Phase-Proteine, Scavenger-Rezeptoren und andere) wurde mittels Reverser Transkriptase-quantitativer Polymerase-Kettenreaktion (RT-qPCR) auf einer Hochdurchsatz-Plattform gemessen. Das antimikrobielle Peptid Lactoferrin (LF), das anti-inflammatorische Zytokin Interleukin-10 (IL-10) und das Akut-Phase-Protein Serum Amyloid A (SAA) wurden im Zellproteinextrakt mit einem Enzyme Linked Immunosorbent Assay (ELISA) quantifiziert. Die Rassen aktivierten generell dieselben Immunsystemkomponenten auf vergleichbaren physiologischen Niveaus. Unterschiede in der Basalexpression wurden in 16 Genen beobachtet (P<0.05), aber es ergab sich keine einheitliche Rangabfolge der Rassen nach diesen Unterschieden, außer dem Befund, dass die Komponenten des TLR-Signalübertragungswegs in den alten Rassen höher exprimiert waren. Eine Hauptkomponentenanalyse (PCA) der basalen Genexpressionsniveaus zeigte jedoch eine sichtbare Trennung der alten von den modernen Rassen und eine Trennung zwischen BS und RH. Die Veränderungen der Expression nach Stimulation unterschieden sich zwischen den Rassen signifikant (P<0.05) nur in den Genen complement component 3 (C3), chemokine (C-C motif) ligand 2 (CCL2), lactoperoxidase (LPO), caspase 8 (CASP8), monocyte differentiation antigen CD14 (CD14), lymphocyte antigen 96 (LY96), transforming growth factor beta 1 (TGFB1) und S100 calcium binding protein A12 (S100A12). Während die PCA der Regulation der Genexpression nach Stimulation keine sichtbare Gruppierung

der Rassen hervorbrachte, fand sich in den modernen Rassen eine größere Anzahl signifikant ($P<0.05$) hochregulierter Gene. HLD hatte höhere Basal- und *E. coli*-behandelte Werte von LF-Protein und höhere Basal- und *S. aureus*-behandelte Werte von SAA-Protein als die modernen Rassen. Ferner gab es eine beträchtliche Variation der Genexpressions- und Proteinwerte zwischen den einzelnen Tieren. Im Allgemeinen bleibt das Bild der Immunantwort der verschiedenen Rassen heterogen und diffus, aber es kann vermutet werden, dass höhere Basalwerte eines Teils des angeborenen Immunsystems in den alten Rassen zu einer weniger ausgeprägten Immunantwort führten. Rassenunterschiede in einzelnen Elementen des angeborenen Immunsystems, aber auch die Rassen-konservierte Aktivierung von Elementen des angeborenen Immunsystems waren bereits vorher beschrieben worden, genau wie die große Tier-zu-Tier-Variation, die wir fanden und die Gegenstand weiterer Forschung bleibt.

1. Introduction

1.1. Bovine Mastitis

Bovine mastitis is the inflammation of the udder of the cow. This disease not only compromises the welfare of the animal, but it is also one of the most frequent and costly diseases dairy farmers have to deal with worldwide (Halasa *et al.*, 2007). Losses occur through direct costs such as decreased milk yield, decreased milk quality, discarded milk, medical treatment costs, penalties for antibiotic contaminated milk and excess labour demand. But also indirect costs such as premature killing of cows and rearing of new heifers to replace them have been taken into account. It is even expected that mastitis increases susceptibility to other diseases (Heikkilä *et al.*, 2012).

Mastitis comprises the ducts and alveoli of the milk producing parenchyma plus the milk collecting cistern and the teat. It happens when the inflammation response of the immune system is triggered. This is mostly done by invading pathogens. Known udder pathogens are found amongst bacteria, viruses and fungi, of which bacteria are by far the most frequent. Clinical mastitis is generally marked by acute inflammation symptoms that range from mild to severe and from local to systemic. These can be pain, swelling and hardening of the infected udder quarter, elevated body temperature, decreased milk synthesis, altered milk texture (flakiness), loss of rumen motility, general sickness of the animal and even death (Marek and Mócsy, 1956, Schulz, 1994). This type of mastitis is mostly elicited by gram-negative bacteria, amongst which the most frequent are coliforms (Hogan and Smith, 2003). These are *Escherichia*, *Klebsiella* and *Enterobacter* (Koneman *et al.*, 1983). They have an environmental reservoir in the intestinal tract, soil and bedding material (Schukken *et al.*, 2011). The other type of mastitis is the subclinical form that often remains without visible symptoms except elevated somatic cell count (SCC) and can become chronic (Schulz, 1994). It is often caused by the gram-positive *Staphylococcus aureus (S. aureus)* which is present on the skin surface and can be transferred from animal to animal by contact or milking machine and even into raw milk products for human consumption (Sutra and Poutrel, 1994). Many studies have shown the difference in bovine mammary immune responses to the gram-negative *Escherichia coli* (*E. coli*) and gram-positive *S. aureus in vivo* (Bannerman, 2004, Lee *et al.*, 2006, Petzl *et al.*, 2008) and *in vitro* (Griesbeck-Zilch *et al.*, 2008, Yang *et al.*, 2008, Gunther *et al.*, 2011).

1.2. Innate immune response

The innate immune system responds to a specific set of conserved pathogen patterns while the adaptive immune system adapts to recognize any pathogenic antigen throughout life (Vivier and Malissen, 2005). The innate immune system is the first and more or less unspecific response to invading pathogens in the udder. The cellular defence part consists of leucocytes (macrophages, neutrophils, natural killer cells and dendritic cells) and mammary epithelial cells, while the humoral part involves antimicrobial peptides (AMPs), acute phase proteins and the complement system (Rainard and Riollet, 2006).

Pathogens are recognised by a set of toll-like receptors (TLRs) on the cell membrane of macrophages and epithelial cells (Figure 1) (Beutler, 2009). This is enhanced by specific co-factors - lipopolysaccharide binding protein (LBP), monocyte differentiation antigen (CD14) and lymphocyte antigen 96 (LY96) (Manukyan et al., 2005). Upon binding to these receptors or receptor co-factor complexes, a signalling cascade inside the cells involving myeloid differentiation primary response protein (88) (MyD88) and interferon regulatory factor 3 (IRF3) leads to activation of the transcription factor nuclear factor kappa B (NF-kB) that activates the expression of a range of proinflammatory genes (Hatada et al., 2000, Karin and Lin, 2002, Tian et al., 2005). Additionally, TLR2 signalling directly activates apoptosis inducing caspase 8 (CASP8) (Aliprantis et al., 2000). Many of the NF-kB activated genes are inflammatory cytokines, comprising interleukin 1 beta (IL-1b), interleukin 6 (IL-6), interleukin 10 (IL-10), tumor necrosis factor (TNF), transforming growth factor beta 1 (TGFb-1), and chemokines such as interleukin 8 (IL-8), the chemokines (C-C motif) ligand 2 (CCL2), 5 (CCL5), 20 (CCL20) and (C-X-C motif) ligand 5 (CXCL5). Cytokines and chemokines are local signalling molecules which attract and activate immune cells (Kuby, 1992, Gangur et al., 2002). In the innate immune response in the mammary gland the most important step is the infiltration of polymorphonuclear neutrophil (PMN) leukocytes from the blood vessel into the alveolar lumen, called diapedesis, where they exert their function as phagocytes together with the macrophages (Paape et al., 2002). Mammary epithelial cells not only play a major role in signalling and initiating the cellular response but also by producing a range of defence molecules: Lactoferrin (LF) binds free iron to make it unavailable to bacteria (Kutila et al., 2003) and is able to directly attack the bacterial membrane (Ellison et al., 1988). Lactoperoxidase (LPO) forms highly reactive hypothiocyanate (OSCN-) from thiocyanate (SCN-) present in milk using H_2O_2 as an oxidant to damage bacterial cell membranes (De Spiegeleer et al., 2005). Lysozyme (LYZ1) cleaves peptidoglycans in the cell wall of gram-
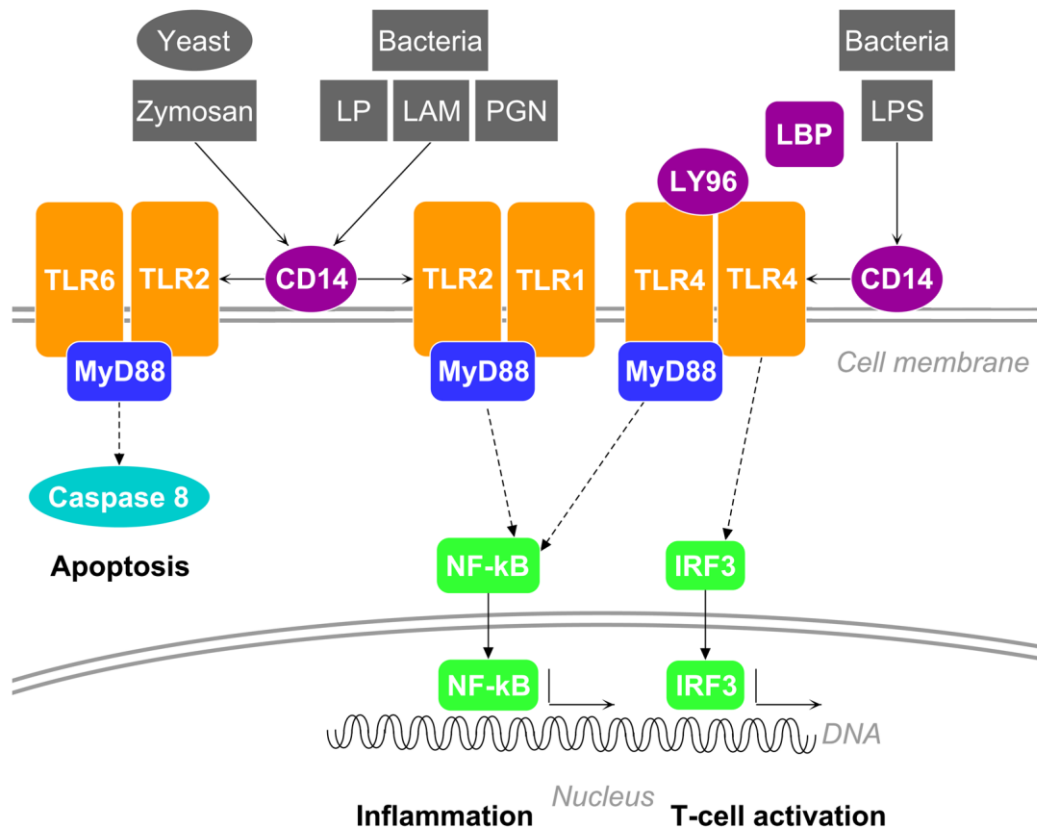
Fig. 1: Toll-like receptor 1, 2, 4 and 6 (TLR) signalling, simplified after bovine TLR pathway on KEGG database (Kanehisa et al., 2004). Pathogenic compounds bind to TLRs and co-factors on the cell surface of mammary epithelial cells and activate caspase 8 and transcription of immune genes. Solid arrows: direct interaction, dotted arrows: indirect interaction, CD14= monocyte differentiation antigen CD14; IRF3 = interferon regulatory factor 3; LAM = lipoarabinomannan; LBP = lipopolysaccharide binding protein; LP = lipoprotein; LPS = lipopolysaccharide; LY96 = lymphocyte antigen 96; MyD88 = myeloid differentiation primary response protein (88); NF-kB = nuclear factor kappa B; PGN = peptidoglycan. Created with PathVisio 2.0.11 (van Iersel et al., 2008).

positive bacteria and in the outer membrane of gram-negative bacteria (Hettinga *et al.*, 2011).The lingual antimicrobial peptide (LAP) and tracheal antimicrobial peptide (TAP) belong to the group of beta-defensins that are a large part of the AMPs present in the bovine genome (Cormican *et al.*, 2008). These multifunctional molecules have anti-bacterial, anti-viral and anti-fungal activity as well as chemotactic quality to T-lymphocytes, dendritic cells and monocytes (Yang *et al.*, 2011).

Additional signals come from the complement system (Figure 2). Briefly, after activation of alternative, classic and lectin pathway, the complement component 3 (C3) is cleaved to the active form C3b which binds to and marks bacteria for enhanced phagocytosis. It also cleaves complement component 5 (C5) into C5a and C5b. The latter and complement components 6, 7, 8 and 9 (C6, C7, C8, C9) form a ring to break through bacterial cell membranes, the so called membrane attack complex. The anaphylatoxins C3a and C5a bind to their receptors C3aR and C5aR on leucocytes, T-cells and antigen presenting cells, inducing phagocytosis, chemotaxis and inflammation (as reviewed by (Peng *et al.*, 2009).

Another pathogen recognition and response system, apart from the TLRs, is found in the intracellular inflammasomes. These are intracellular receptor complexes that recognise whole pathogens as well as pathogen components and stress signals. Amongst them are NACHT, LRR and PYD domains-containing protein 1 (NRPL1) and 3 (NRPL3) (Figure 3) which self-oligomerize to form high-molecular weight complexes and activate caspase 1 (CASP1) to mature the proinflammatory cytokine IL-1b (Franchi *et al.*, 2012).

Furthermore, a whole line-up of other molecules with lesser known functions such as acute phase proteins is increased in inflammation. The most studied one of these is serum amyloid A (SAA). It is often considered as a marker for inflammation because it is increased in blood plasma and milk (Gronlund *et al.*, 2003) and pbMEC (Molenaar *et al.*, 2009) during mastitis. It enhances phagocytosis by opsonising gram-negative bacteria (Shah *et al.*, 2006). Haptoglobin, the other major acute phase protein (Gronlund *et al.*, 2003), binds free haemoglobin to avoid tissue damage through its oxidative and toxic iron-containing haem and is itself bound by the scavenger receptor cysteine-rich type 1 protein M130 (CD163) (Kristiansen *et al.*, 2001). Another scavenger receptor is macrosialin (CD68) which clears cell debris and promotes phagocytosis by macrophages. For a long time is has been regarded as a macrophage marker but has recently been found in other cell types as well (Gottfried *et al.*, 2008). The S100 calcium binding proteins S100-A8, S100-A9 and S100-12, also referred to as calgranulins, exert a range of protective and anti-infective functions (Hsu *et al.*, 2009). Another group of protective agents is the Mx proteins belonging to the large GTPases. They confer protection from different viruses and are triggered by virus recognition and interferon (Lee and Vidal, 2002).
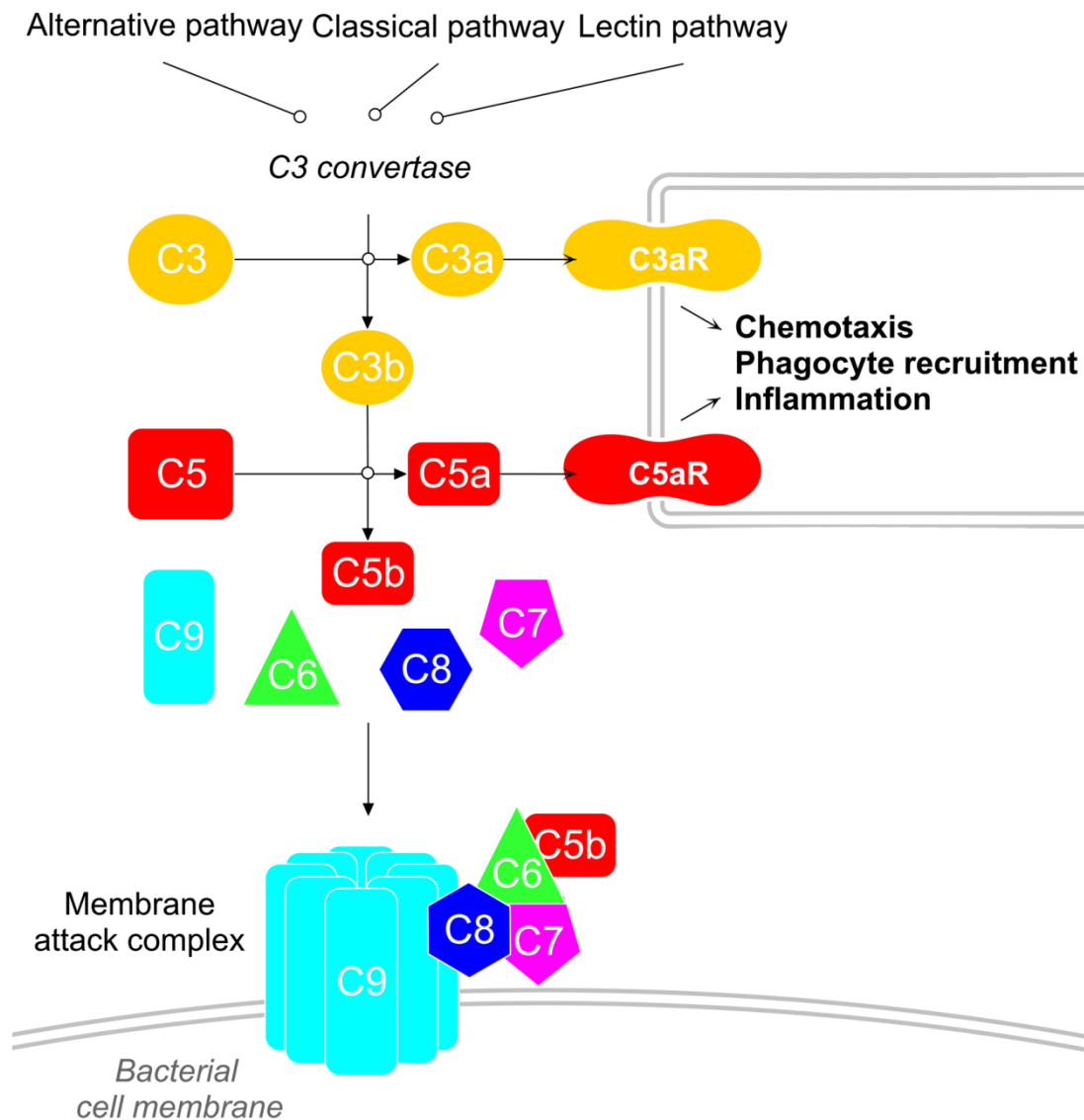
Fig. 2: The complement cascade of the immune system, simplified after bovine complement cascade pathway on KEGG database (Kanehisa et al., 2004). In all three pathways (alternative, classical, lectin) C3 convertase is activated and cleaves C3 to C3a and C3b. The latter cleaves C5 to C5a and C5b. C3a and C5a bind to their respective receptors C3aR and C5aR for immune cell activation. C5b together with C6, C7, C8 and C9 form the membrane attack complex to perforate bacterial cell membranes. C3, C5, C6, C7, C8, C9 = complement components 3, 5, 6, 7, 8, 9; C3aR = C3a receptor; C5aR = C5a receptor. Created with PathVisio 2.0.11 (van Iersel et al., 2008).
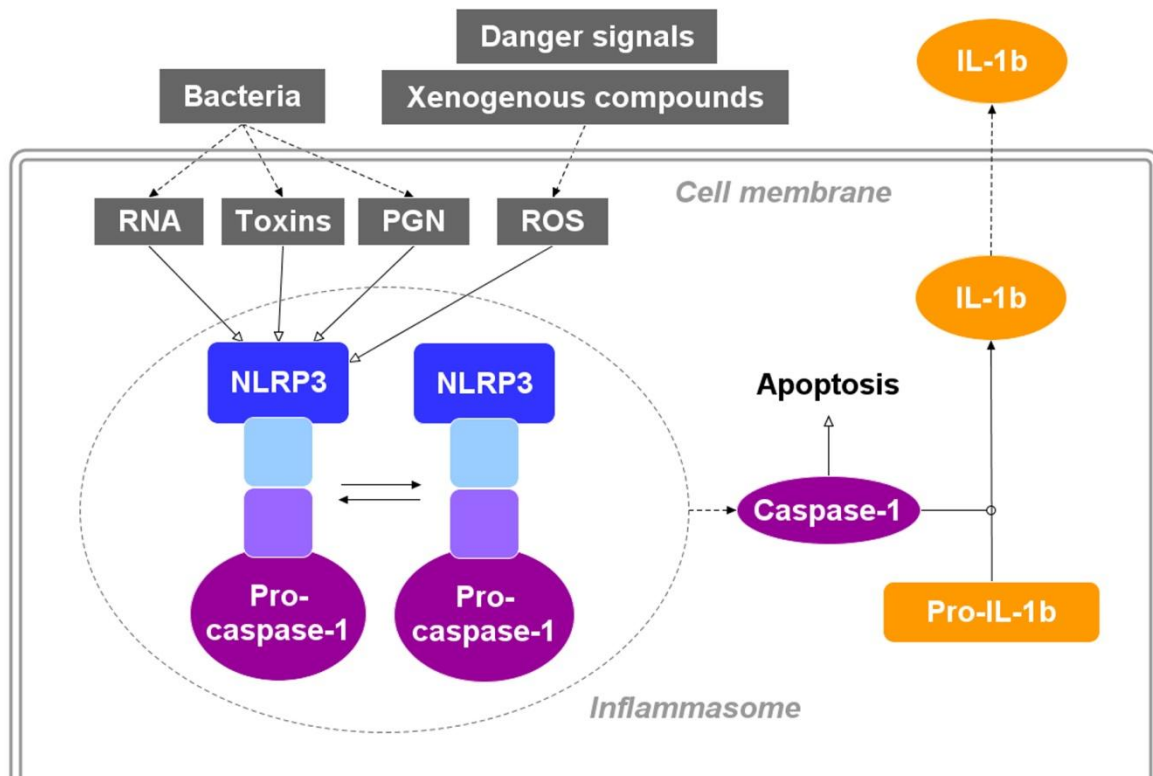
Fig. 3: The inflammasome NRPL3, simplified after bovine inflammasome pathway on KEGG database (Kanehisa et al., 2004). Upon recognition of pathogenic compounds and other danger signals the NLRP3 complex dimerizes and cleaves pro-caspase-1 to active caspase-1. The latter matures the inflammatory cytokine pro-interleukin-1, beta (pro-IL-1b) to active interleukin-1, beta (IL-1b) and induces apoptosis. NLRP3 = NACHT, LRR and PYD domains-containing protein 3; PGN = peptidoglycans; ROS = reactive oxygen species. Created with PathVisio 2.0.11 (van Iersel et al., 2008).

1.3. Genetic resistance to mastitis

It is well known that susceptibility to mastitis is determined by a combination of many factors: bacterial virulence, environmental conditions (housing, management, feeding, milking technique) and cow factors (milk yield, genetics). These are interdependent from each other and their impact depends on the type of pathogen (Burvenich *et al.*, 2003). The reason for the high prevalence of mastitis in modern dairy breeds seems to be a positive genetic correlation of milk yield and mastitis (Strandberg and Shook, 1989, Heringstad *et al.*, 2003). After this fact had been neglected for decades of intensive selection on milk yield, there are now many approaches of breeding for mastitis resistant cows. New phenotypic breeding goals have been defined (Boichard and Brochard, 2012). Intensive research is going on to

identify genomic markers for mastitis resistance in the Holstein breed (Griesbeck-Zilch *et al.*, 2009, Liu *et al.*, 2012, Russell *et al.*, 2012). Even a database of cattle candidate genes and genetic markers for milk production and mastitis is now available (Ogorevc *et al.*, 2009). However, prediction of a phenotypic trait by genetic markers is only accurate if this trait is influenced by few large loci on the genome, not by many small loci (Hayes *et al.*, 2010). The latter seems to be the case for mastitis resistance. Furthermore, there is only low heritability of conventionally estimated breeding values for mastitis resistance (Heringstad *et al.*, 2003). So it remains difficult to link phenotypic resistance to certain genetic factors when only the genomic architecture but not the physiological effect of it is studied.

1.4. Cattle breeds

1.4.1. White Park cattle

The British White Park cattle (WP) (Figure 4) have been very well described by Alderson (1997) in his book "A breed of distinction". In a few words, he states that it is the oldest European cattle breed. Its first descriptions have been made by pre-Christian Irish epics. Being free-ranging feral animals before, defined herds were established and emparked in the 13th, 14th and 15th century and five of them are still existent in the UK today. They have up or downward facing horns, sometimes referred to as "Auerochsen-like". The White Park cattle has a white coat colour and black muzzle, ears, eye-rims and feet - the dominant White Park pattern (Olson, 1999). This pattern is caused by a heterozygous duplication of the KIT gene on chromosome 6 and its aberrant insertion on chromosome 29 (Brenig *et al.,* 2013). It was depicted in the form of a white bull with black points in the cave of Lascaux as early as 17,000 years ago (Alderson, 1997). This distinct and unusual appearance might have been the reason for its use as a sacred, a decorative and a game animal in former times. Due to its hardiness it is kept in extensive low-input grazing systems or parks as suckler cows for beef production, but up to the 20th century it was also milked (Alderson, 1997). The Food and Agriculture Organization of the United Nations (FAO) considered the status of this breed as critical in Germany and as endangered-maintained in their home country UK (FAO, 2000). In the 2012 watch list of The British Rare Breeds Survival Trust they are listed as "minority" (RBST, 2012) and the British Cattle Movement Service (BCMS) gives their number with approximately 3,300 animals in the UK in 2012 (BCMS, 2012). Biedermann *et al.* (2009) published relationship data from 11 male and 33 female German White Park cattle. The zoological park Arche Warder (Zentrum für alte Haus- und Nutztierrassen e.V., Warder)

which is dedicated to the conservation and research of ancient domestic animal breeds keeps the largest herd in Germany (Biedermann *et al.*, 2009).

Fig. 4: White Park cow on pasture (Tierpark Arche Warder, Warder, Germany; photo: Diana Sorg).

1.4.2. Highland cattle

The Highland cattle (HLD) (Figure 5) have been imported to Germany from the UK. The origins of this breed are found in Scotland where they have been bred for hundreds of years (Dohner, 2001). Their alternative name "Kyloe" is derived from the fact that they were driven from islands to the mainland by swimming across the sea channels, called "Kyles". The coat colour is mostly red, black and white, but there are also dun (diluted black or red) and brindle (red and black striped) animals. This rather small animal has dense and long hair and extended horns which makes it especially adapted to a harsh climate and the threat by predators. As a very robust and frugal animal it can be kept outdoors throughout the year in extensive hill or mountain grazing systems. Although being a suckler cow for beef production

nowadays, it has been reported that it was additionally milked in former times (Dohner, 2001). Its herd book was established in 1885 and so it is one of the oldest registered cattle breeds (Mason, 2002). This ancient breed is not considered as a rare breed. In Germany there were 2,785 female and 385 male animals registered in 2010 (BLE, 2012) and approximately 38,000 Highland cattle in Great Britain (BCMS, 2012). Many farmers value this breed for landscape conservation and high-quality beef production and so there are folds, as Highland herds are called, in many countries around the world.

Fig. 5: Highland cow and calf on pasture (Rattenweiler, Germany; photo: Diana Sorg).

1.4.3. Brown Swiss

The Brown Swiss (BS) cow (Figure 6) is one of the most important dairy breeds in southern Germany and Switzerland. It is coloured from light grey to dark brown with white spots occurring occasionally. Recent numbers were 180,000 controlled cows in Germany with an average milk yield of 6,800 kg per year (European Brown Swiss Federation, 2012). It has

been bred in the USA at the end of the 19th century from 155 animals of the sturdy triple-purpose (dairy, meat and draught) Swiss Brown Mountain cow to produce a high-yielding dairy breed that can still be used as a dual-purpose animal for additional meat production (Mason, 2002). One of its main characteristics is the high percentage of BB genotypes for κ-casein, which is favourable for a high cheese yield (European Brown Swiss Federation, 2012).

Fig. 6: Brown Swiss cow at a breeding exhibition (photo: European Brown Swiss Federation, Bussolengo, Italy).

1.4.4. Red Holstein

The Red Holstein (RH) cow (Figure 7), being the red-allele carrying variant of the Holstein breed, exhibits a black-white or red-white pied coat colour. It was created as a particular dairy breed in the 19th century in the USA and Canada from the dual-purpose (dairy and milk) breed Dutch Black Pied (Mason, 2002). Due to the over 30-year long history of breeding for high production traits its performance is superior to most other dairy breeds and so it has

become the most important dairy breed in Germany (Blottner *et al.*, 2011). In 2010 there were 240,000 milk recorded Red Holstein and 2 million milk recorded Holstein cows (German Holstein Association, 2010) with average milk yields of 8,245 and 9,008 kg respectively. Holstein cows as a model breed have been largely studied in terms of genetic markers for production, fertility and health traits (Rupp *et al.*, 2007, Griesbeck-Zilch *et al.*, 2009, Beecher *et al.*, 2010).

Fig. 7: Red Holstein cow at a breeding exhibition (photo: Deutscher Holsteinverband e. V., Bonn, Germany).

2.   Aim of the study

The aim of this study was to compare the innate immune response of pbMEC from ancient and modern cattle breeds to a mastitis challenge with *E. coli* and *S. aureus in vitro*. Functional differences in the innate immune response should be described and possible reasons thereof discussed.

For that pbMEC cultures out of the milk from two ancient breeds and two modern dairy breeds were to be established. Heat-inactivated *E. coli* 1303 and *S. aureus* 1027 should be used to elicit the innate immune response of these cells *in vitro*. This response was to be measured in terms of mRNA expression of 39 target genes of the innate immune system via reverse transcription quantitative polymerase chain reaction (RT-qPCR). The target genes included representatives of the complement system, chemokines, inflammatory cytokines, antimicrobial peptides, acute phase proteins, TLR-signaling, scavenger receptors and others. In addition to this, the antimicrobial peptide LF, the anti-inflammatory cytokine IL-10 and the acute phase protein SAA were to be quantified as protein in the cell protein extract using enzyme linked immunosorbent assay (ELISA).

By studying the intra-mammary immune system of ancient breeds that have never been selected for high milk yield there is the hope to better understand the underlying genetic mechanisms of their frequently observed higher phenotypic resistance to mastitis and to identify possible candidate genes that could later on be used for genomic selection and the breeding of healthier animals.

3.   Material and methods

3.1.   Animals

To compare the cultivation of pbMEC from udder tissue and milk (pbMEC I), four healthy lactating RH cows on the research station Veitshof (Technische Universität München, Freising, Germany) were milked around the 150th day in milk. After slaughtering around the 150th day in milk, udder tissue from the same animals was obtained. Additionally one healthy BS cow was used to obtain a tendon sample after slaughtering. To compare the immune response of milk-derived pbMEC from different cattle breeds (pbMEC II), milk samples from four different cattle breeds were taken. Six healthy mid-to-late-lactation BS cows from research station Veitshof (Technische Universität München, Freising, Germany) and six healthy mid-to-late-lactation RH cows from research station Hirschau (Technische Universität München, Freising, Germany) were taken as examples for modern dairy breeds. The ancient breeds were represented by five healthy mid-to-late-lactation WP cows and three healthy mid-to-late-lactation HLD cows from the zoological park Tierpark Arche Warder (Zentrum für alte Nutz- und Haustierrassen e. V., Warder, Germany) as well as by two healthy mid-to-late lactation HLD cows from a private farm (Franz Sorg, Rattenweiler, Germany).

3.2.   Bacteria

Samples of frozen *E. coli* and *S. aureus* (Petzl *et al.*, 2008) were used to establish bacterial cultures as described in Danowski *et al.* (2012a). *E. coli* was cultivated at 37°C in LB-medium containing 10 g/L yeast extract (Sigma-Aldrich, Munich, Germany), 10 g/L NaCl and 5 g/L trypton (Sigma-Aldrich). *S. aureus* was cultivated in CASO-broth (Sigma-Aldrich) at 37°C to the log-phase of growth. Bacterial density was determined photometrically at 600 nm. At several densities a dilution series of *E. coli* was plated on LB-agar (Roth, Karlsruhe, Germany) and a dilution series of *S. aureus* was plated on blood agar (Oxoid, Wesel, Germany, with sheep blood from Fiebig, Idstein-Niederauroff, Germany), cultivated over night at 37°C and counted. Under the hypothesis that from one bacterium one colony had arisen, the desired cell density and corresponding optical density (OD) was determined and the cultivation was repeated up to the desired OD. Cultivation was stopped by placing the solutions on ice and a centrifugation step of 10 min at 1,850 x g. The bacteria were washed with sterile PBS, centrifuged again and inactivated for 30 min at 63°C in a water bath. A diluted sample of both harvested batches was cultivated on a plate at 37°C over night to verify inactivation.

3.3. Cell culture

Cell isolation from milk

The BS and RH cows were automatically milked into an autoclaved steel can in the milking parlour after cleaning and disinfecting the teat surface. After clipping, cleaning and disinfecting the udder surface, the WP and HLD cows from Tierpark Arche Warder were automatically milked into an autoclaved steel can with a portable milking machine (Figure 8a). The cows on the private farm were hand milked directly into autoclaved glass bottles (Figure 8b). Up to 2 l of fresh milk was centrifuged for 10 min at 1,850 x g and room temperature. Then the supernatant was decanted and the remaining cell pellet was washed with washing solution Hank's Balanced Salt Solution (HBSS, Sigma-Aldrich, Munich, Germany) containing 176.7 IU/ml penicillin, 0.176 mg/ml streptomycin, 0.088 mg/ml gentamicin and 8.3 µg/ml amphotericin B (Sigma-Aldrich). The cells were centrifuged for 5 min at 600 x g at room temperature and the supernatant discarded. The cell pellet was suspended in fresh washing solution and filtered through a 100 µm pore size nylon cell strainer (BD Biosciences Europe, Erembodegem, Belgium). After centrifugation for 5 min at 600 x g at room temperature the washed cell pellet was resuspended in pre-warmed (37°C) Dulbecco's Modified Eagle's Medium (DMEM) with nutrient mixture F-12 HAM (Sigma-Aldrich) containing 10% Fetal Bovine Serum (FBS; Life Technologies, Darmstadt, Germany), 5 ml ITS liquid media supplement, 176.7 IU/ml penicillin, 0.176 mg/ml streptomycin, 0.088 mg/ml gentamicin and 4.4 µg/ml amphotericin B (Sigma-Aldrich). The cell solution was seeded in a 25 cm^2 tissue culture flask (Greiner, Frickenhausen, Germany) and cultivated at 37°C and 5% CO_2.

Cell isolation from udder tissue

A deep sagittal cut into the udder was made to excise a piece of approximately 1.5 x 1.5 x 1.5 cm. The tissue was immediately immersed in washing solution as described in section *Cell isolation from milk*. Under aseptic conditions the tissue was minced manually using sterile scalpel blades and put into washing solution additionally containing 0.5 mg/ml collagenase IV, 0.4 mg/ml DNase type I, 0.5 mg/ml hyaluronidase 1-S (Sigma-Aldrich). The tissue was incubated at 37°C on a shaker for 3 h. Following this digestion step the solution was filtered through a 1 mm pore size metal sieve to remove large clumps of tissue and centrifuged for 5 min at 1,400 x g and room temperature. The supernatant was discarded and

the pellet resuspended in washing solution. The next filtration step used a 0.5 mm pore size metal sieve followed by 5 min centrifugation at 1,400 x *g* and room temperature. After removing the supernatant the pellet was again suspended in washing solution and filtered through a 100 μm nylon cell strainer (BD Biosciences Europe, Erembodegem, Belgium). Following the last centrifugation of 5 min at 600 x *g* and room temperature, the cell pellet was resuspended in medium and cultivated as mentioned above in section.
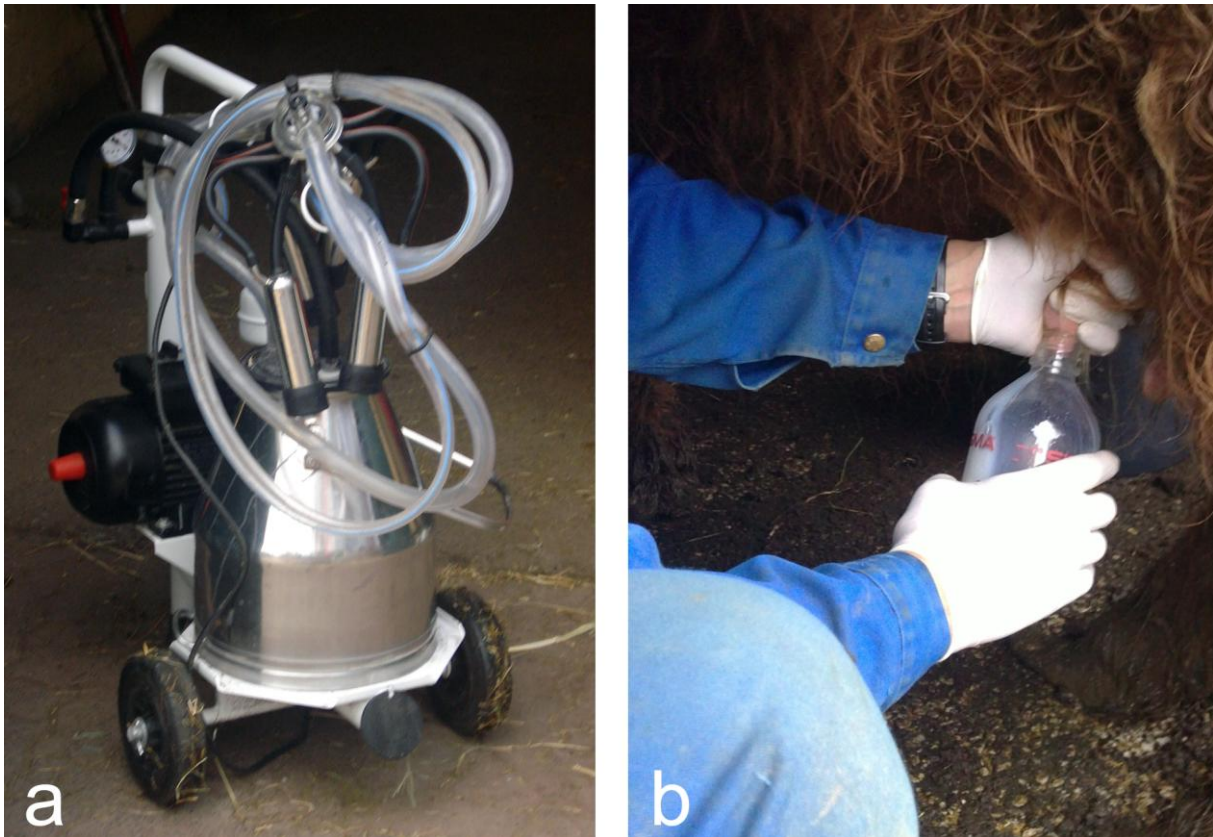

Fig. 8: (a) Portable milking machine (Tierpark Arche Warder, Warder, Germany; photo: Diana Sorg) (b) hand milking of a Highland cow into an autoclaved glass bottle (Rattenweiler, Germany; photo: Diana Sorg).

Isolation of fibroblasts

With the same protocol as for the udder tissue, a piece of 1 cm length of tendon was taken aseptically after slaughtering and extracted to establish a reference fibroblast culture for the fibroblast contamination check on the epithelial cultures.

Cell cultivation

The pbMEC I from four RH cows from the research station Veitshof (Technische Universität München, Freising, Germany) were used to compare pbMEC from udder tissue and milk. The pbMEC II from the ancient and moderns breeds were used for the stimulation experiment with mastitis pathogens. Growth and morphology of all pbMEC and fibroblasts were monitored daily by light microscopy. Medium was changed twice a week. When reaching about 70 to 80% confluence cells were detached using accutase (PAA, Pasching, Austria) and centrifuged for 5 min at 600 x *g* and room temperature. In the pbMEC I cultures, two wells of a six-well plate (Greiner, Frickenhausen, Germany) were seeded with 100,000 cells after the first, second and third passage. The rest was reseeded in tissue culture flasks for further cultivation. To compare frozen and non-frozen third passage cells an aliquot of the second passage pbMEC I was additionally stored in freezing medium consisting of 70 % DMEM/F-12 HAM, 20 % FBS and 10 % DMSO in liquid nitrogen. These cells were also reseeded at 100,000 cells per well in a six-well plate after 3 weeks storage. Fibroblasts were cultivated until second passage and cryopreserved in liquid nitrogen before reseeding at 100,000 cells per well in a six-well plate. PbMEC II were cultivated until third passage and stored in liquid nitrogen for further experiments. Additionally, a 16-well chamber slide (Nunc, Langenselbold, Germany) or a flexiPerm reusable cell culture chamber on a glass slide (Greiner) was seeded with 10,000 cells per well of each pbMEC and the fibroblast culture for immunocytochemistry.

Cell stimulation

PbMEC II from each of the animals in the breed comparison experiment were seeded at 30,000 cells per well in 12-well plates (Greiner). After reaching 70-80 % confluence the medium was removed and replaced with medium without FBS and antibiotics. On the following day three wells were detached and counted manually to determine an estimate of the cell count in the other wells. The other wells were washed with PBS and given fresh medium without FBS and antibiotics. Heat-inactivated *E. coli* 1303 and *S. aureus* 1027 (Petzl

et al., 2008) were added in a multiplicity of infection (MOI) of 30 colony forming units per cell to ensure the same bacterial load in every well. This MOI was chosen as a typical bacterial load used in other experiments with pbMEC (Gunther *et al.*, 2009, Danowski *et al.*, 2012a). Control wells were left untreated. Two wells of *E. coli* treated cells were each sampled after 6 and 30 h, two wells of *S. aureus* treated cells were each sampled after 30 and 78 h and two wells of control cells were each sampled at 6, 30 and 78 h.

3.4.   Immunocytochemistry

To check for contamination by non-epithelial cells, a staining of the epithelial-specific keratins, also called cytokeratins, was performed in all pbMEC cultures and a fibroblast sample as described in Danowski *et al* (2012a). In pbMEC I also a staining of the milk protein casein was performed to show their ongoing functionality. The cells that had been cultivated on chamber slides were washed with phosphate buffered saline (PBS) and fixed in ice cold methanol/acetone (1:1) for 10 min. After air drying at room temperature endogenous peroxidases were blocked in PBS with 1 % H_2O_2 for 30 min at room temperature and protected from light. Then the slides were washed in PBS with 0.05 % Tween20 (Sigma-Aldrich) (PBST) three times for 5 min each. Background staining by unspecific binding of the antibody was reduced by adding 30 µl goat serum (DAKO, Glostrup, Denmark) 1:10 diluted in PBST per well and incubating at room temperature for 30 min. Afterwards the liquid was decanted and fresh diluted goat serum was added to the negative control wells. The other wells were covered with the primary antibody and incubated over night at 4°C in moist atmosphere protected from light. For cytokeratin staining we used the monoclonal mouse anti-cytokeratin pan antibody clone C-11 (diluted 1:400 in PBST) (Sigma-Aldrich) and for casein staining we used the polyclonal rabbit anti-casein antibody (1:50 in PBST) (Genetex, Irvine, CA, USA). After washing as described above, the secondary antibody, horseradish peroxidase (HRP) labelled goat anti-mouse-immunoglobulin (DAKO) diluted 1:400 in PBST, was added and incubated for 1 h at room temperature protected from light. After washing, HRP was visualized by immersing the slides in PBS with 0.01 % 3,3'-diaminobenzidine (DAB; Sigma-Aldrich) and 0.01 % H_2O_2 for 15 min at room temperature protected from light. Subsequently the slides were washed and nuclei were stained with Haemalaun after Mayer (Roth, Karlsruhe, Germany) for 15 sec, following rinsing with tap water for 45 s. For 2 min each, the slides were dehydrated in 50 % ethanol, 100 % ethanol and Rotihistol (Roth). For storage they were covered with a cover slip and Eukitt (Roth).

3.5. Total RNA extraction and reverse transcription

PbMEC I and fibroblasts in the six-well plates were sampled after 5 days. Their total RNA was extracted with the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions. The pbMEC II in 12-well plates were harvested with the Allprep RNA/Protein kit (Qiagen, Hilden, Germany). Their total RNA and protein was extracted with an additional DNA digestion using the RNAse-free DNAse set (Qiagen) as indicated in the manual. RNA and protein were stored at -80°C. RNA concentration and purity was measured with the NanoDrop ND-1000 photometer (Peqlab, Erlangen, Germany) at 260 nm. RNA integrity was analysed with the Bioanalyzer 2100 and the RNA 6000 Nano Assay kit (Agilent, Waldbronn, Germany). 500 ng of pbMEC I and fibroblast RNA was transcribed to complementary DNA (cDNA) with 200 IU M-MLV (H-) Point Mutant Reverse Transcriptase (Promega, Mannheim, Germany), 5x buffer (Promega), 50 µM random hexamer primers (Invitrogen, Darmstadt, Germany) and 10 mM dNTPs (Fermentas, St. Leon-Roth, Germany) in a total volume of 60 µl. 100 ng of pbMEC II RNA was transcribed with 100 IU M-MLV (H-) Point Mutant Reverse Transcriptase (Promega), 5x buffer (Promega), 2.5 µM random hexamer primers (Invitrogen), 0.5 mM dNTPs (Fermentas) and 0.5 µM oligo-d(T) primer (Fermentas) in a total volume of 30 µl. A negative control containing pooled RNA from each RNA extraction run and no reverse transcriptase was created to detect contamination by genomic DNA. The temperature profile was 10 min at 21°C for annealing, 50 min at 48°C for transcription and 2 min at 90°C for degradation. CDNA was stored at -20°C.

3.6. Gene expression with reverse transcription quantitative PCR (RT-qPCR)

Comparison of milk and tissue-derived pbMEC I and fibroblasts

To avoid measuring gene expression of eventually contaminating fibroblasts only mammary epithelial cell-specific genes were chosen. Those were keratin 8 (KRT8), which is an intermediate filament protein of the cytoskeleton. It is generally used as a marker for epithelial cells (Karantza, 2011). Kappa casein (CSN3) is a major milk protein that is secreted by pbMEC (Threadgill and Womack, 1990). LAP, LF and LYZ1 encode for the antimicrobial peptides lingual antimicrobial peptide, lactoferrin and lysozyme which are produced by pbMEC to attack pathogens in the innate immune response (Carlsson *et al.*, 1989, Irwin, 2004, Huang *et al.*, 2012). The lactogenic hormone prolactin binds to the prolactin receptor PRLR (Bole-Feysot *et al.*, 1998, Viitala *et al.*, 2006). Vimentin (VIM), another filament protein of the cytoskeleton, is commonly used as a marker of mesenchymal cells, such as fibroblasts

(Herrmann and Aebi, 2004). Its expression was used to validate the reference fibroblast culture. Primer pairs were designed applying the HUSAR software (German Cancer research Center, Heidelberg, Germany) and PrimerBLAST (National Center of Biotechnology Information (NCBI), National Library of Medicine, National Institutes of Health, Bethesda, MA, USA). Primer details are given in table 1.

Table 1: Names, symbols, NCBI reference sequence number, sequences, amplicon lengths (L) and annealing temperatures (T) of the primers used to compare gene expression of tissue and milk-derived pbMEC (pbMEC I).

gene name (symbol)	NCBI reference sequence number	primer sequences (5'→ 3') forward reverse	L[1] (bp)	T[2] (°C)
keratin 8 (KRT8)	NM_001033610.1	ACTGGCTACGCAGGTGGACT CCGCAAGAGCCTTTCACTTG	181	62
kappa casein (CSN3)	NM_174294.1	TGCAATGATGAAGAGTTTTTTCCTAG GATTGGGATATATTTGGCTATTTTGT	151	54
lingual antimicrobial peptide (LAP)	NM_203435.3	AGAAATTCTCAAAGCTGCCG CAGCATTTTACTTGGGCTCC	107	62
lactoferrin (LF)	NM_180998.2	CGAAGTGTGGATGGCAAGGAA TTCAAGGTGGTCAAGTAGCGG	215	60
lysozyme 1 (LYZ1)	NM_001077829.1	AAGAAACTTGGATTGGATGGC ACTGCTTTTGGGGTTTTGC	185	60
prolactin receptor (PRLR)	NM_001039726.1	TGATGTTCATCTGCTGGAGAAGGGC TCCAGGTGCATGGGCTTCACG	195	64
vimentin (VIM)	NM_173969.3	TGGAGCGTAAAGTGGAATCC GACATGCTGTTCTTGAATCTGG	104	60

[1]Amplicon length

[2]Annealing and elongation temperature in qPCR

Real-time quantitative PCR (RT-qPCR) was done on the Rotorgene Q cycler (Qiagen) using 1 µl cDNA template, 5 µl SsoFast EvaGreen Supermix (Bio-Rad, Munich, Germany), water and a final concentration of 400 nM forward and reverse primers (Metabion, Martinsried, Germany) in a total volume of 10 µl. The temperature profile was 30 sec at 98°C, followed by

40 cycles with the two phases of 5 sec at 95°C and 20 sec at the primer-specific annealing temperature.

Comparison of cattle breeds with pbMEC II

39 target genes of the innate immune response and 6 putative reference genes were measured. Primer details are given in table 2. Primer specifity was checked as described for the pbMEC I. A 5-point dilution series of 3 representative cDNA samples from the experiment and untreated bovine spleen tissue cDNA was used to determine PCR efficiencies as described in the MIQE Guidelines for RT-qPCR (Bustin *et al.*, 2009) using one BioMark™ HD Gene Expression (GE) 48.48 Dynamic Array chip (Fluidigm, San Francisco, CA, USA).

Table 2: Names, symbols, NCBI reference sequence number, sequences and amplicon lengths of the primers used to compare gene expression of pbMEC from different cattle breeds (pbMEC II).

gene name (symbol)	NCBI reference sequence number	primer sequence (5' → 3') forward reverse	L^1 (bp)
reference genes			
actin, gamma 1 (ACTG1)	NM_001033618	AACTCCATCATGAAGTGTGAC GATCCACATCTGCTGGAAGG	234
keratin 8 (KRT8)	NM_001033610	TGGTGGAGGACTTCAAGACC CGTGTCAGAAATCTGAGACTGC	215
glyceraldehyd-3-phosphate dehydrogenase (GAPDH)	NM_001034034.1	GTCTTCACTACCATGGAGAAGG TCATGGATGACCTTGGCCAG	197
H3 histone, family 3A (H3F3A)	NM_001014389.2	ACTCGCTACAAAAGCCGCT ACTTGCCTCCTGCAAAGC	232
ubiquitine B (UBB)	NM_174133.2	AGATCCAGGATAAGGAAGGCAT GCTCCACCTCCAGGGTGAT	426
tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein, zeta polypeptide (YWHAZ)	NM_174814.2	CAGGCTGAGCGATATGATGA GACCCTCCAAGATGACCTAC	141
complement system			
complement component 1, q subcomponent, A chain (C1QA)	NM_001014945.1	CGTTGGACCGAATTCTGTCTC TGCTGTTGAAGTCACAGAAGCC	224

complement component 3 (C3)	NM_001040469	AAGTTCATCACCCACATCAAG	191
		CACTGTTTCTGGTTCTCCTC	
complement component 3a receptor (C3aR1)	NM_001083752.1	CCCTCCATCATCATCCTCAAC	167
		CACATTACCAAAGCCACCACC	
complement component 5a receptor (C5aR1)	NM_001007810	ATACCGTCCTTTGTGTTCCG	158
		ATTGTAAGCGTGACCAGCG	

C-C and C-X-C motif ligand chemokines

chemokine (C-C motif) ligand 2 (CCL2)	NM_174006.2	CTCACAGTAGCTGCCTTCAGC	205
		GCTTGGGGTCTGCACATAAC	
chemokine (C-C motif) ligand 5 (CCL5)	NM_175827.2	CCTCCCCATATGCCTCG	157
		TTGGCGCACACCTGG	
chemokine (C-C motif) ligand 20 (CCL20)	NM_174263.2	CTTGTGGGCTTCACACAGC	115
		GTTTCACCCACTTCTTCTTTGG	
chemokine (C-X-C motif) ligand 5 (CXCL5)	NM_174300.2	TTGTGAGAGAGCTGCGTTGT	150
		CCAGACAGACTTCCCTTCCA	
chemokine (C-X-C motif) ligand 8 (CXCL8)	NM_173925.2	AAGAATGAGTACAGAACTTCGATGC	160
		GTTTAGGCAGACCTCGTTTCC	

inflammatory cytokines

interleukin 1, beta (IL1B)	NM_174093.1	CAGTGCCTACGCACATGTCT	209
		AGAGGAGGTGGAGAGCCTTC	
interleukin 6 (IL6)	NM_173923.2	TGGTGATGACTTCTGCTTTCC	109
		AGAGCTTCGGTTTTCTCTGG	
interleukin 10 (IL10)	NM_174088.1	AGCTGTATCCACTTGCCAACC	119
		TGGGTCAACAGTAAGCTGTGC	
transforming growth factor beta 1 (TGFB1)	NM_001166068.1	CCTGAGCCAGAGGCGGACTAC	130
		GCTCGGACGTGTTGAAGAAC	
tumor necrosis factor (TNF)	NM_173966.2	CCACGTTGTAGCCGACATC	108
		ACCACCAGCTGGTTGTCTTC	

antimicrobial peptides

lingual antimicrobial peptide (LAP)	NM_203435.3	AGAAATTCTCAAAGCTGCCG	107
		CAGCATTTTACTTGGGCTCC	
lactoferrin (LF)	NM_180998.2	CGAAGTGTGGATGGCAAGGAA	215
		TTCAAGGTGGTCAAGTAGCGG	

lactoperoxidase (LPO)	NM_173933.2	TGGCTGTCAACCAAGAAGC	134
		TGAGGCTCGAAAATCTCCC	
lysozyme 1 K (LYZ1)	NM_001077829.1	AAGAAACTTGGATTGGATGGC	185
		ACTGCTTTTGGGGTTTTGC	
tracheal antimicrobial peptide (TAP)	NM_174776.1	AGGAGTAGGAAATCCTGTAAGCTGTGT	113
		AGCATTTTACTGCCCGCCCGA	
acute phase proteins			
haptoglobin (HP)	NM_001040470.1	AATGAACGATGGCTCCTCAC	176
		TTGATGAGCCCAATGTCTACC	
serum amyloid A3 (SAA3)	NM_181016.3	CCAACTACAGGGGTGCAGAC	103
		GCGTTACTGATCACTTTAGCAGC	
inflammasome			
NOD-like receptor family, pyrin domain containing 1 (NLRP1)	XM_003587406.1	ACCATATTTCCAGAGGCATCC	190
		TTGATTCAACCACGCTAAAGG	
NOD-like receptor family, pyrin domain containing 3 (NLRP3)	NM_001102219.1	AAACACTCCAACAACCTGGC	214
		AACCAGAGCTTCTTCAGATTGC	
caspase 1 (CASP1)	XM_002692921	ACGTCTTGCCCTTATTATCTGC	204
		GTACTGTCAGAGGTCCGATGC	
toll-like-receptor-pathway			
caspase 8 (CASP8)	NM_001045970.2	TAGCATAGCACGGAAGCAGG	295
		GCCAGTGAAGTAAGAGGTCAG	
monocyte differentiation antigen CD14 (CD14)	NM_174008.1	GCAGCCTGGAACAGTTTCTC	124
		ACCAGAAGCTGAGCAGGAAC	
interferon regulatory factor 3 (IRF3)	NM_001029845.2	GGCTTGTGATGGTCAAGGTT	100
		TGCAGGTCGACAGTGTTCTC	
lipopolysaccharide binding protein (LBP)	NM_001038674.1	CTTGGAGAGCAAGATTTGCG	174
		TCACCCTTGAACATCACATCC	
lymphocyte antigen 96 (LY96)	NM_001046517.1	TGTTTCAATACGTTCTGAGCCC	300
		TCAGTGTTCCCCTCGATGG	
myeloid differentiation primary response gene (88) k (MYD88)	NM_001014382.2	CTGCAAAGCAAGGAATGTGA	122
		AGGATGCTGGGGAACTCTTT	
toll-like receptor 2 (TLR2)	NM_174197.2	CATTCCTGGCAAGTGGATTATC	201
		GGAATGGCCTTCTTGTCAATGG	

toll-like receptor 4 (TLR4)	NM_174198.6	TGCTGGCTGCAAAAAGTATG	213
		TTACGGCTTTTGTGGAAACC	
scavenger receptors			
CD 68 molecule (CD68)	NM_001045902.1	GGCTCCAAGGAGGCAATAG	201
		GAATGAGAGGAGCAAGTGGG	
CD 163 molecule (CD163)	NM_001163413.1	CGAGTCCCATCTTTCACTCTG	185
		AGTGAGAGTTGCAGAGAGGTCC	
others			
myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse) (MX1)	NM_173940.2	AAGGCCACTATCCCCTGC	277
		CTCGTACTTTGGTAAACAGTCGG	
myxovirus (influenza virus) resistance 2 (mouse) (MX2)	NM_173941.2	CTTCAGAGACGCCTCAGTCG	232
		TGAAGCAGCCAGGAATAGTG	
S100 calcium binding protein A9 (S100A9)	NM_001046328.1	CTGGTGCAAAAAGAGCTGC	128
		AGCATAATGAACTCCTCGAAGC	
S100 calcium binding protein A9 (S100A12)	NM_174651.2	TGGGGAGGCGCTGCTCTAGAC	135
		TCGAAATGCCCCACCCGAACG	
v-rel reticuloendotheliosis viral oncogene homolog A (avian) (NF-kappa-B p65 subunit) (RELA (NFKB))	NM_001080242.2	GCCTGTCCTCTCTCACCCCATCTTTG	152
		ACACCTCGATGTCCTCTTTCTGCACC	

[1]L = amplicon length

To adjust Cq values to the measuring range a primer-specific preamplification was performed. For this 4 µl cDNA was amplified in a total volume of 20 µl with the iQ Supermix (Bio-Rad) and a final concentration of 25 nM of each primer pair. The thermal protocol was 95 °C for 3 min followed by 18 cycles of 95°C for 20 sec, 55°C for 3 min and 72°C for 20 sec. For RT-qPCR the preamplified cDNA was diluted 1:9 with water. QPCR was performed on the high-throughput BioMark™ HD system using GE Dynamic Array chips (Fluidigm). Spurgeon *et al.* (2008) describe this system in detail. Briefly, it applies microfluidic technology. PCR reactions take place on a micro-chip in the centre of a chip containing 48 x 48 or 96 x 96 reaction fields. Lines and valves leading from the 48 or 96 assay and 48 or 96 sample wells to the chip are controlled by pressure to manipulate nanolitre scales of samples and reagents in an automated manner. Thus, 48 x 48 = 2,304 or 96 x 96 = 9,216 PCR reactions can be carried out simultaneously in one single run. The samples of the stimulation experiment were measured on four 96.96 chips. One 6 h *E. coli* treated pbMEC II sample

was measured repeatedly on all chips as between-chip calibrator. It was chosen as a representative and stable sample that expressed all genes of interest to provide similar reaction conditions and expression levels as in the other samples. The sample mix used 1.25 µl 1:9 diluted cDNA in a total volume of 5 µl consisting of 2.5 µl SsoFast EvaGreen Supermix (Bio-Rad), 0.25 µl sample loading reagent (Fluidigm), 0.1 µl ROX diluted 1:3 (Life Technologies, Darmstadt, Germany) and water. The assay mix contained 2.5 µl assay loading reagent (Fluidigm), a final concentration of 4 µM of forward and reverse primer and water in a total volume of 5 µl. The final primer concentration in the PCR reaction was 400 nM. Assay and sample mix were transferred to the primed GE Dynamic Array 48.48 or 96.96. The samples and assays were mixed inside the chip by the Nanoflex IFC controller (Fluidigm). The PCR temperature profile was 40 sec at 98°C, followed by 40 cycles of 10 sec at 95°C and 40 sec at 60°C. Afterwards a melting curve of the amplified products was determined. Data was collected using BioMark Data Collection Software 2.1.1. built 20090519.0926 (Fluidigm) as the cycle of quantification (Cq) where the fluorescence signal of the amplified DNA intersected with the background noise.

3.7. Data analysis of the RT-qPCR

Comparison of milk and tissue-derived pbMEC I and fibroblasts

Primer specifity was checked by inspecting the melting curve of the PCR product for the existence of one single peak, indicating that only one product was present, and by running a 1.5 % agarose gel of the product to ensure that the fragment length was correct. Gene expression was measured in terms of cycle of quantification (Cq), the PCR cycle where the fluorescence signal intersected with the threshold. This was automatically detected by the Rotorgene Q PCR software version 1.7 (Build 94) (Qiagen). KRT8 was used as a reference gene for normalization of the target genes. It belongs to the cytoskeleton and was therefore suspected and confirmed to be stably expressed. Cq was subtracted from the target genes' Cq to obtain the dCq, the relative level of gene expression. Statistics and charts were produced with SigmaPlot 11.0 (Systat, Chicago, IL, USA) or SPSS Statistics Standard 19.0 (IBM, Armonk, NY, USA).

Comparison of cattle breeds with pbMEC II

The valid qPCR reactions were detected with Fluidigm Melting Curve Analysis Software 1.1.0. built 20100514.1234 and Real-time PCR Analysis Software 2.1.1. built 20090521.1135

(Fluidigm). Invalid reactions were not used and treated as missing data. Raw Cq values were processed with Genex 5.3.2 (MultID Analyses AB, Gothenburg, Sweden) applying between-chip calibration and reference gene normalization. Six putative reference genes were chosen upon literature review. With the Normfinder tool in Genex 5.3.2 (MultID), actin, gamma 1 (ACTG1), H3 histone, family 3A (H3F3A) and KRT8 were identified as stably expressed and afterwards used for target gene normalization. Cq values higher than 30 were regarded as invalid and treated as missing data. For the gene regulation analysis, but not for the replicate deviations, Cq values higher than 25 were regarded as invalid and replaced by 25 due to loss of measurement precision as suggested by the manufacturer. The raw Cq value was subtracted from the average of the reference genes' Cq to calculate the dCq value. The ddCq value was obtained by subtracting the dCq of the treated from the dCq of the control sample. Principal component analysis (PCA) on autoscaled dCq and autoscaled ddCq values was done with Genex 5.3.2 (MultID). Distribution of the qPCR and cell culture replicate deviations, termed as "within-chip variation", was calculated with gnuplot 4.4.0 (Sourceforge.net, Geeknet Inc., Fairfax, VA, USA). Between-chip deviations of the calibrator sample and within-chip deviations were calculated separately for high expression genes with an overall mean Cq below 20 and low expression genes with an overall mean Cq above 20, respectively. Statistics and charts were produced with SigmaPlot 11 (Systat) or SPSS Statistics Standard 19.0 (IBM). Genes were regarded as differentially expressed for $P<0.05$ in a paired t-test or signed rank test on dCq values between treatment and corresponding control. Fold changes of gene expression were calculated as 2^{-ddCq} according to Livak and Schmittgen (2001) for each sample and then expressed as a mean of all these fold changes to show the amount of regulation in each gene. Expression fold changes were compared between breeds with independent t-tests in SPSS (IBM).

3.8. Protein quantification with ELISA

Total protein content

Total protein content in the cell protein samples of pbMEC II was measured with the BCA assay. In this reaction Cu^{2+} is reduced to Cu^+ by the peptide bonds in the protein in a proportional manner. It then forms a chelate complex with two BCA molecules. This complex exhibits a purple colour and its OD is measured photometrically at 562 nm (Smith *et al.*, 1985). Briefly, $CuSO_4$ and bicin choninic acid (BCA) solution (Sigma-Aldrich) mixed 1:50 were incubated together with the protein samples (1:1 diluted in PBST) or a standard curve

of diluted bovine serum albumin (BSA) for 40 min at 37°C and measured with the Sunrise photometer (Tecan, Männedorf, Switzerland).

Lactoferrin

Lactoferrin (LF) was quantified using a competitive ELISA (Figure 9) as described in Danowski *et al.* (2012b). A 96-well microtiter plate (Nunc) was coated with 1 µg/well of a polyclonal goat anti-rabbit IgG antibody as primary antibody (immunised with rabbit IgG, Sigma-Aldrich) and stored at -20°C until use. After thawing at room temperature and washing four times with PBST 20 µl of cell protein sample were incubated at 4°C on a shaker overnight together with a polyclonal rabbit anti-bovine LF antibody as secondary antibody, diluted 1:400,000 in PBST. A standard curve of LF (Sigma-Aldrich) diluted in PBST was run on each plate. On the next day biotinylated LF (Sigma-Aldrich) diluted 1:400,000 in PBST was added and incubated at room temperature on a shaker and protected from light for 2 h. After washing streptavidin labelled horseradish peroxidase (SHRP) diluted 1:20,000 in PBST was added and incubated at room temperature on a shaker and protected from light for 15 min.
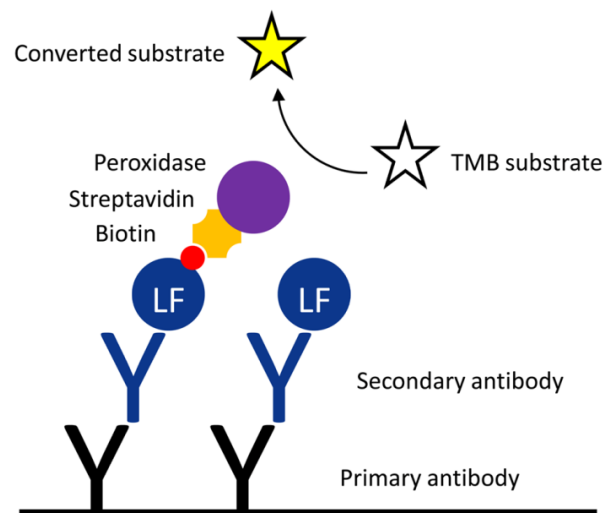

Fig. 9: Scheme of the competitive lactoferrin (LF) ELISA used for LF quantification in cell protein.

The plate was washed again before the chromogenic substrate 3,3,5,5-tetramethylbenzidine (TMB) was added and incubated at 37°C on a shaker protected from light for 40 min. Then the reaction was stopped with H_2SO_4 and the OD measured with the Sunrise photometer (Tecan) at 450 nm.

Interleukin-10

Interleukin-10 (IL-10) was measured with a sandwich ELISA (Figure 10) as described in Groebner *et al.* (2011). Briefly, 96-well microtiter plates (Nunc) were coated with monoclonal mouse anti-bovine IL-10 antibody clone CC318 (AbD Serotec, Düsseldorf, Germany) as capture antibody at 5 µg/ml in carbonate-bicarbonate buffer at 4°C on a shaker over night. Cell protein diluted 1:50 in PBST was incubated at room temperature on a shaker protected from light for 1 h. Biotinylated monoclonal mouse anti-bovine IL-10 antibody clone CC320 (AbD Serotec) was added as detection antibody at 1 µg/ml in PBST and incubated at room temperature on a shaker protected from light for 2 h. No standard curve was performed as there was no commercially available bovine IL-10 standard.
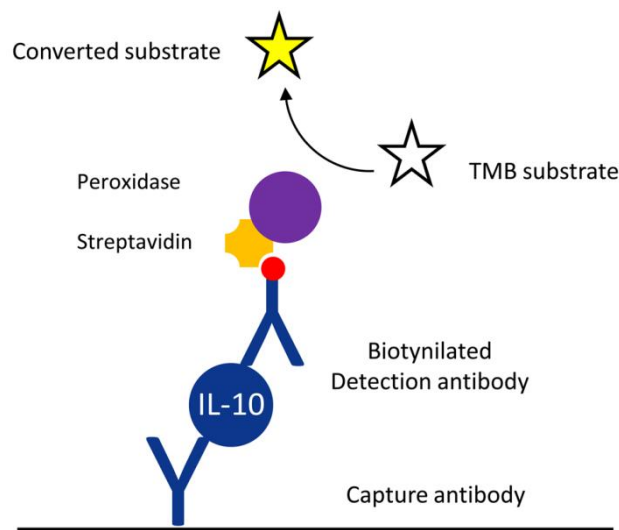

Fig 10: Scheme of the sandwich ELISA used for relative IL-10 quantification in cell protein.

Serum Amyloid A

Serum Amyloid A (SAA) was measured in one replicate each of the 30 h *E. coli* and control samples. For that the PHASE range multispecies Serum Amyloid A ELISA kit (Tridelta Development, Maynooth, Ireland) was applied (Figure 11) following manufacturer's instructions. Briefly, cell protein 1:67 diluted in PBST and standard was incubated together with SHRP labelled anti-SAA antibody (= detection antibody) on anti-SAA antibody (= capture antibody) coated 96-well strips at room temperature protected from light for 1 h. The colour reaction of the TMB substrate was measured on the Sunrise photometer (Tecan) at 450 nm.
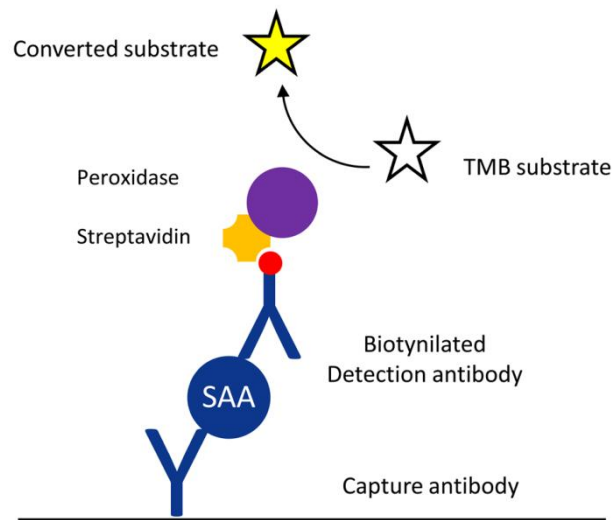

Fig. 11: Scheme of the sandwich ELISA used for SAA quantification in cell protein

3.9.   Data analysis of the ELISA

Total protein content was automatically quantified as µg/ml by Magellan data analysis software (Tecan) with a linear regression on the standard curve. LF contents were automatically quantified as ng/µl by Magellan data analysis software (Tecan) using the four parameter marquardt curve fit algorithm on the standard curve. LF was normalized to the total protein content and given as LF/cell protein in ng/µg. Due to the lack of a commercially available standard of bovine IL-10 it was determined relatively. OD was normalized to total cell protein. The normalized OD of a treated sample was divided by the normalized OD of the corresponding control sample and multiplied by 100 to obtain the change in IL-10 production in % of control. SAA contents were automatically quantified as ng/µl by the Magellan data analysis software (Tecan) using a linear regression on the standard curve. SAA contents where normalized to the total protein content and given as SAA/cell protein in ng/µg. Treated

and control samples were compared with SigmaPlot 11 (Systat) using a paired t-test or signed rank test. Breeds were compared with a one-way-ANOVA or independent t-tests in SPSS (IBM). It is important to remark that in all statistical tests no correction of P-values for multiple testing was used. As this study is of descriptive and exploratory character only and not of a diagnostic one, the goal was merely to show apparent differences. Many of these would have been masked by this stringent condition for significance. We are, however, aware of the fact that this increases the risk of false positive significances, so the results have been interpreted cautiously.

4. Results and discussion

4.1. Cell culture and immunocytochemistry

Cell culture can be defined as "a culture derived from dispersed cells taken from original tissue, from a primary culture, or from a cell line or cell strain by enzymatic, mechanical, or chemical disaggregation" (Freshney, 2005). The culture of animal cells had first been established as a means to multiply viruses to produce vaccines at the beginning of the 20th century. As early as that, scientists already studied the interactions of pathogenic bacteria with cultured chick embryo cells (Smyth, 1915). The first cultivation of primary bovine mammary epithelial cells (pbMEC) from udder tissue was published in 1961 (Ebner *et al.*, 1961). Tissue is the most common source for primary cell culture. It can be obtained from biopsies or after slaughtering of the animal and is then processed using mechanical rupturing, enzymatic digest or both. The idea to grow mammary epithelial cells not from tissue but from milk was first realized using human (Buehring, 1972) and baboon's milk (Rie *et al.*, 1976). Later, this protocol was adapted also for cow's milk to culture pbMEC (Buehring, 1990). This method has many advantages over the tissue-derived culture: it is non-invasive and therefore especially suited when valuable animals should not be harmed or killed, or when repeated sampling is desired. Also there is almost no contamination by fibroblasts (Buehring, 1990). This is why the milk-derived culture was chosen and evaluated against the tissue-derived culture (Sorg *et al.*, 2012). In both tissue (Figure 12a) and milk-derived pbMEC I (Figure 12b), the predominant cell type was of epithelial origin as proved by specific staining against the epithelial marker cytokeratin, and showed the typical cobblestone-like monolayer with slightly varying cell sizes. Only few fibroblasts were found in the tissue cultures. The fibroblast culture (Figure 12c) and negative control (Figure 12a, 12b insert) showed no cytokeratin staining. PbMEC I from milk also stained positively for casein (Figure 12d, insert: negative control). The casein staining demonstrated that the cultured pbMEC were still able to synthesize the milk protein casein. The pbMEC II cultures exhibited the typical cobblestone-like morphology as checked by light microscopy (Figure 13a) (Sorg *et al.*, 2013a). An average of 5.98 x 10^6 cells per animal in a range of 1 to 19 x 10^6 cells was harvested for cryopreservation. By visual inspection with light microscopy there were nearly 0% unstained nucleated cells detected, so the purity of all cultures was estimated at nearly 100% (Figure 13b). The negative controls showed approximately 0% visible stained cells (Figure 13b (insert)).
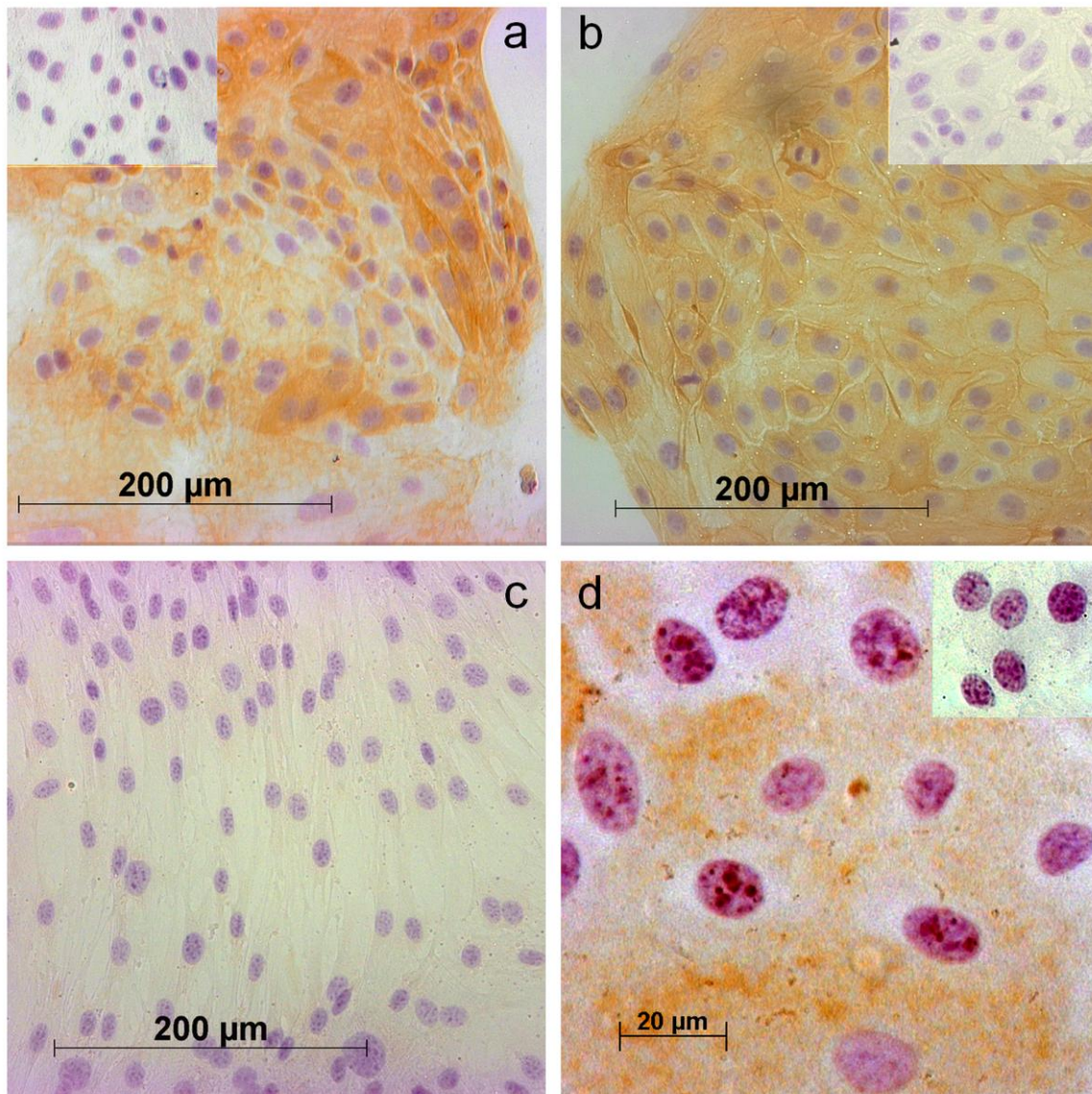
Fig. 12: (a) primary bovine mammary epithelial cells I (pbMEC I) cultivated from udder tissue, immunostained against the epithelial marker cytokeratin (insert: negative control), magnification 200x (b) pbMEC I cultivated from milk, immunostained against cytokeratin (insert: negative control), magnification 200x (c) primary bovine fibroblasts, immunostained against cytokeratin, magnification 200x (d) pbMEC I from milk, immunostained against the milk protein casein (insert: negative control), magnification 400x (Sorg et al., 2012).
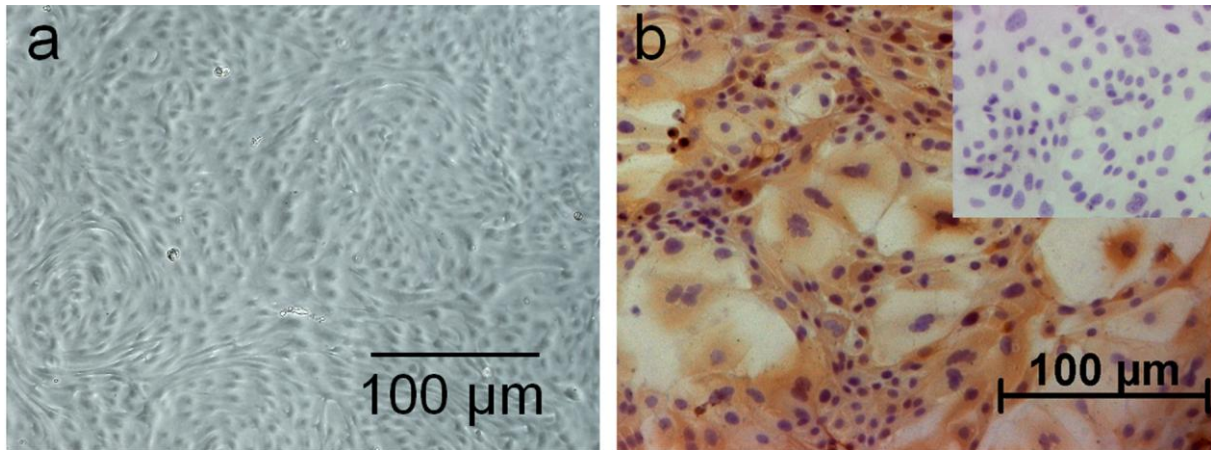
Fig. 13: (a) the typical cobblestone-like monolayer of primary bovine mammary epithelial cells (pbMEC) in culture, magnification 100x (b) pbMEC immunostained against the epithelial marker cytokeratin (insert: negative control), magnification 100x (Sorg et al., 2013a).

4.2. Gene expression of the cell culture comparison

Fibroblasts

The fibroblast marker VIM was highly expressed in the fibroblast sample (Cq value 13.5). The epithelial cell specificity of CSN3, KRT8, LAP, LF, LYZ1 or PRLR was confirmed as there was no expression detected in the fibroblast sample by checking the melting curves and gel electrophoresis of the PCR products (data not shown) (Sorg *et al.*, 2012).

Origin and cryopreservation comparison with pbMEC I

The initial cell count after extraction of primary cells is normally rather low. Hence, to obtain a sufficient cell count for an experiment it is necessary to let them proliferate. For successful proliferation cells should be grown at a certain minimal density to enable cell-cell communication. So they have to be passaged several times, subsequently providing more space in each culture vessel. It is understandable that the passage number in which primary cells are used for an experiment will always be a compromise between *in vivo* comparability and repeatability through cell culture replicates. The decision whether to store the primary cell cultures in liquid nitrogen is another such compromise. Often the primary cultures are sampled over a longer period of time, especially in trials with large animals. Unless they are cycle synchronized, dairy cows often need to be sampled at different times to ensure sampling in the same stage of their long lactation. To guarantee that in cell culture

experiments the same conditions apply for all the cultures, they are often cryopreserved before they are used. To validate the chosen experimental setup in our study we compared tissue- and milk-derived pbMEC over three passages and after cryopreservation (Sorg *et al.*, 2012).

The expression of the five epithelial-specific target genes of the tissue- and milk-derived cultures over three passages without frozen third passage samples is shown in Figure 14. The dCq values were subtracted from the value 20 to represent higher expression levels with higher lines and bars and lower expression levels with lower lines and bars, respectively.
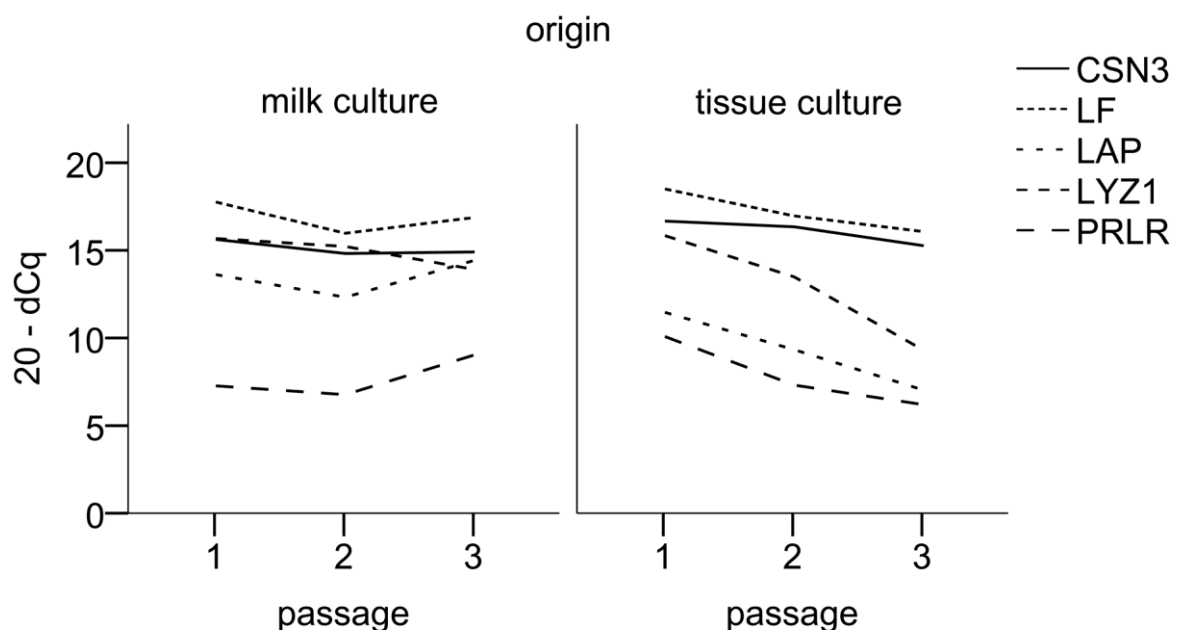
Fig. 14: Normalized relative gene expression of pbMEC I from milk (left) and udder tissue (right) over three passages without cryopreserved third passage samples. CSN3 = kappa casein, LF = lactoferrin, LAP = lingual antimicrobial peptide, LYZ1 = lysozyme 1, PRLR = prolactin receptor (Sorg et al., 2012).

The expression of the five epithelial-specific target genes in frozen and non-frozen third passage milk- and tissue-derived cultures is shown in Figure 15. Because of the low sample number no statistical calculations were performed. The results were interpreted qualitatively. Generally, RNA expression levels in the tissue-derived cultures decreased during cultivation, while this was not true for the milk-derived cells. This is a fact that can be explained by the different environment conditions of cell culture and living organs. In the udder the cells are embedded in surrounding tissue and supported by a basal membrane.

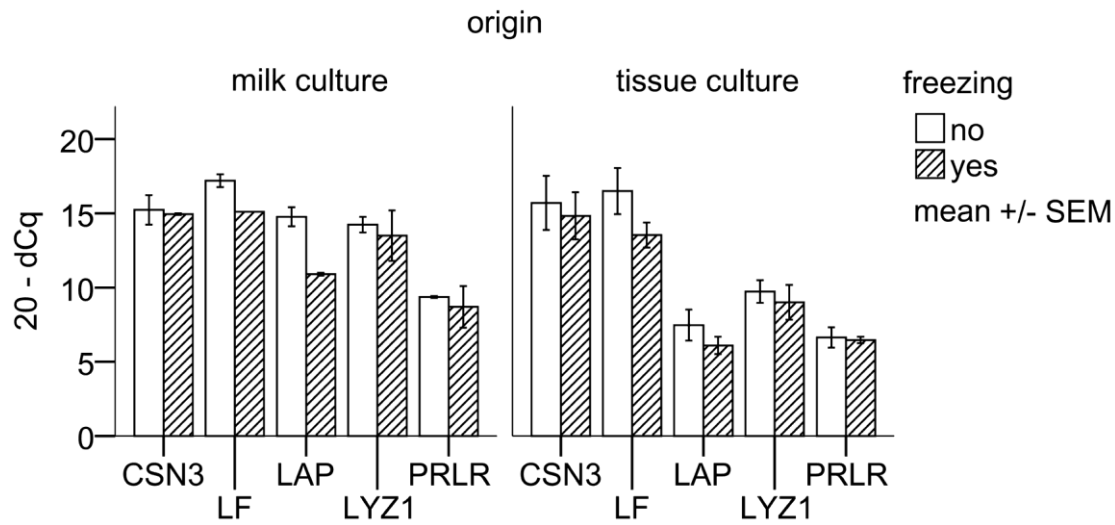*Fig. 15: Normalized relative gene expression of frozen and non-frozen third passage pbMEC I from milk (left) and udder tissue (right.) CSN3 = kappa casein, LF = lactoferrin, LAP = lingual antimicrobial peptide, LYZ1 = lysozyme 1, PRLR = prolactin receptor (Sorg et al., 2012).*

Cells are able to sense chemical and physical properties of their environment (Prasad Chennazhy and Krishnan, 2005). They receive signals from the ECM through adhesion receptors on the cell surface and this signalling influences cell proliferation, differentiation and apoptosis (Katz and Streuli, 2007). The loss of this signal is called anoikis (from ancient Greek 'αν' = 'without' and 'οικοσ' = 'house'), a state in which the cells sense the lack of a surrounding ECM (Bertrand, 2011). We assume that the tissue-derived cells which had been suddenly disrupted from their ECM by mechanical and enzymatic digestion underwent this process during the first three passages of cultivation, while the naturally exfoliated milk-derived cells had already adapted to their new environment. Although anoikis often leads to apoptosis, which is meant as a protection from reattaching in an inappropriate location in the body, Bertrand (2011) also mentions that epithelial cells can flexibly leave and re-enter an epithelium, and that anti-apoptotic signals can delay the onset of anoikis. There is not much knowledge about the comparability of tissue and milk-derived pbMEC. Krappmann *et al.* (2012) found a significant correlation of CSN3 and another milk protein gene expression in pbMEC isolated from milk with magnetic beads and udder tissue. In their pbMEC the CSN3 expression was much lower while in our study, the normalized expression levels were comparable. However, they had not cultivated them after extraction so the cells had had no opportunity to recover from the exfoliation. Interestingly our cells did not lose the ability to express the antimicrobial peptides LF, LAP and LYZ1, while in a study of Gunther *et al.*

(2009) pbMEC from two animals showed massively decreased LAP expression after three passages. Furthermore, we were able to show the continuous casein synthesis by immunocytochemistry in our cells.

The expression levels of CSN3, LYZ1 and PRLR were only slightly lower in frozen than in non-frozen third passage cultures. The effect was greater in LF and LAP. Overall expression levels and differences between frozen and non-frozen cultures were very similar in both origins. Other researchers found that viability (Cifrian *et al.*, 1994) and secretion ability (Talhouk *et al.*, 1990 and 1993) of pbMEC were not considerably influenced. Wellnitz and Kerr (2004) concluded that their cryopreserved pbMEC from udder tissue were a good model to study innate immunity *in vitro* even if the experiments took place weeks apart from each other**.**

We showed that milk-derived cells had similar or even higher expression levels than tissue-derived cells and had thus similar or lower loss of function in cell culture. The milk sampling has the additional advantage of non-invasiveness and repeatability. So we concluded it to be a suitable method for our *in vitro* experiments. Also the cryopreservation had acceptable effects on gene expression levels.

4.3. Technical evaluation of the Fluidigm BioMark HD™ measurements

Before interpreting the gene expression results from the mastitis challenge experiment in a physiological manner the precision and sensitivity of the qPCR platform was analysed with the obtained data set (Sorg *et al.*, 2013a). First of all the variability of raw Cq values on the different chips was compared. For that a quantile-quantile plot (Q-Q plot) was produced. Chip 1 versus chip 2 and 3 is shown exemplarily (Figure 16). The Cq values from two chips were each ranked in ascending order. Values with the same rank were paired as x- and y-coordinate and plotted in a diagram. Up to a Cq of 25 the dots formed the ideal diagonal straight line, which implies that the distribution of Cq values was the same on the chips. Above 25 the lines deviate and form a curve, meaning that above 25 the Cq values were not evenly distributed any more. This is a confirmation of the need to set a cut off at 25 when interpreting the gene expression results. The evaluation of between-chip variance was done using the calibration sample that was repeatedly measured on all chips.
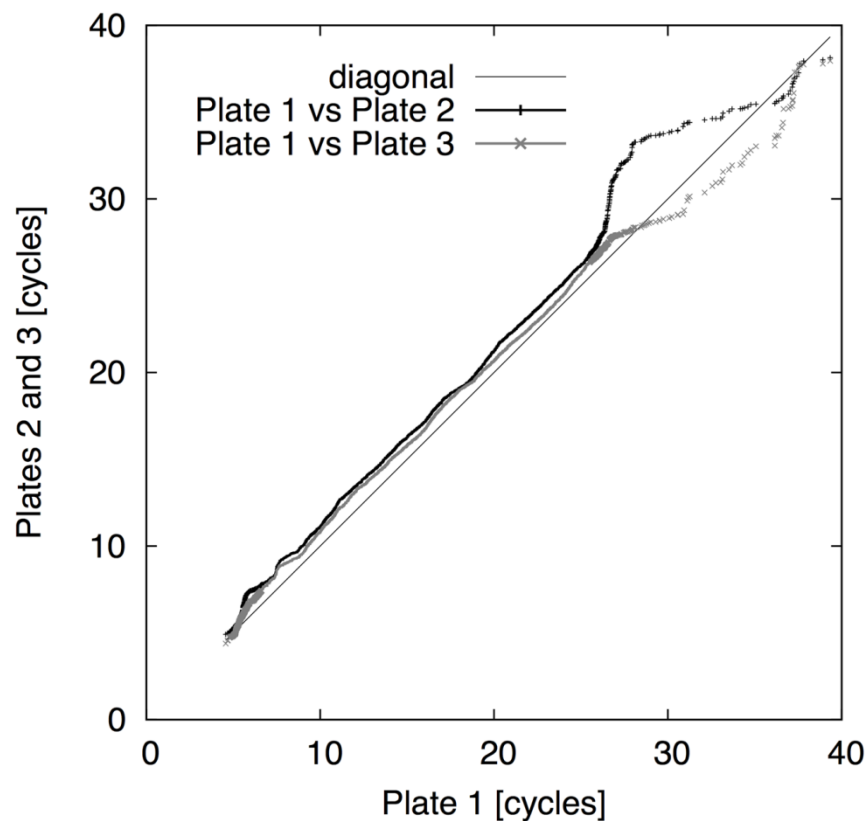
Fig. 16: quantile-quantile plot of the raw Cq values from three chips of the RT-qPCR measurements of the infection study (Sorg et al., 2013a).

To describe the between-chip variance we calculated the coefficient of variance (CV) of the Cq values of the repeatedly measured calibration sample for each gene across the four chips. We set a threshold between high and low expression genes for a mean Cq of 20 over all chips and samples. The mean CV (± SEM) was 4.3 ± 0.4 % for high expression genes and 3.3 ± 0.4 % for low expression genes. Surprisingly the CV was lower for the low expression genes and its SEM was the same in high and low expression genes. Although the between-chip variance was acceptably low, the calibration sample was used to normalize the raw Cq values between the chips to avoid introducing bias into the data when samples from different chips were compared.

The sensitivity of the platform was checked by comparing the Cq value differences of qPCR replicates (which were regarded as technical replicates) and the mean Cq values of cell culture replicates (which were regarded as biological replicates) over all four chips.
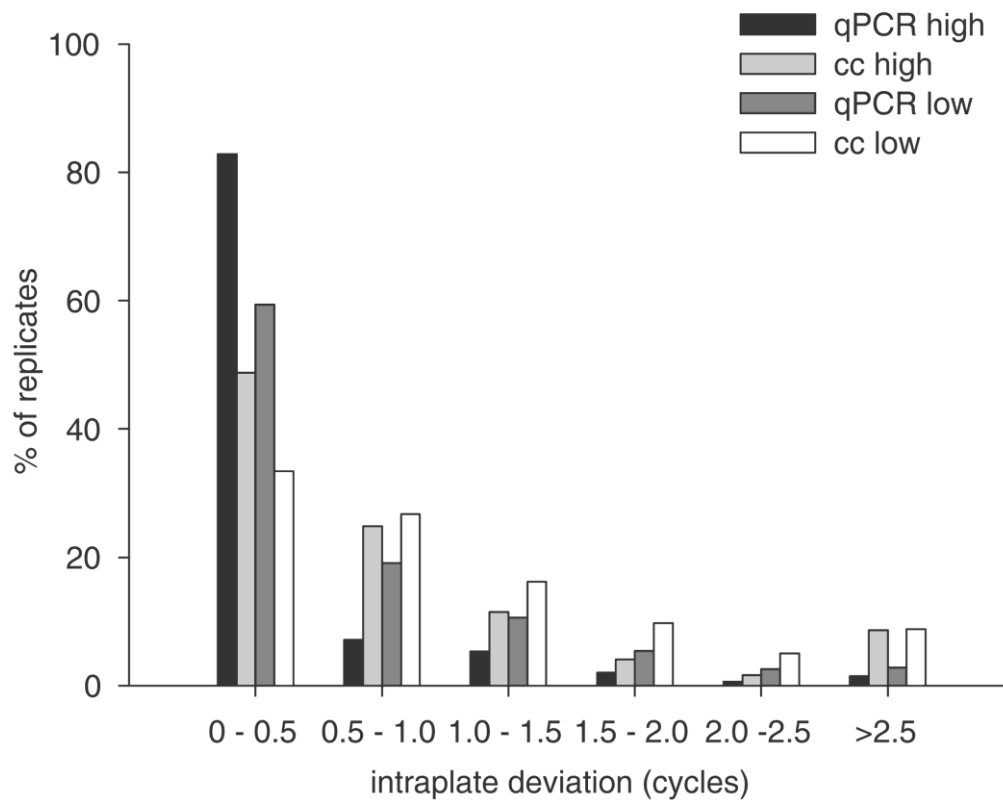
Fig. 17: Differences between the Cq values of qPCR (qPCR) replicates and between the mean Cq values of the cell culture (cc) replicates separated for high expression (high) and low expression (low) genes. The threshold of high and low expression was a mean Cq value of 20 over all samples and chips (Sorg et al., 2013a).

They were again separated for high and low expression genes (Figure 17). For high expression genes 83% of the qPCR replicate pairs and 59% of the cell culture replicate pairs were in the lowest deviation range between 0 and 0.5 cycles, respectively. For low expression genes it was 49% and 33%, respectively. It is no surprise that the low expression gene values showed higher differences than the high expression gene values. It is a natural effect that the Poisson distribution appears in aliquots from a sample with a low target concentration, as it is the case in diluted nucleic acid samples. The Poisson distribution predicts large variations in measured target quantities (Rutledge and Stewart, 2010). So it is advantageous to perform more technical replicates for an assay when it is known that the gene is little expressed. This should better cover this variation and increase the precision of the measurement. The mean between-chip CV was acceptable. The CV of the low expression genes was surprisingly lower than the CV of the high expression genes. But it must be kept in mind that the low expression gene CV does not reflect the true variability of

the data. The cut-off at Cq 30 in the pre-processing step must have lowered this variation. The smaller differences in the qPCR replicates than in cell culture replicates showed that the system was able to detect biological differences out of the background noise of measurement variability, which is necessary when comparing cultures from different animals and different treatments. The technical evaluation of our measurements was satisfactory and we moved on to the physiological interpretation of the results.

4.4. Gene expression and protein production of the innate immune response

Table 3 shows the normalized basal expression of 16 innate immune genes that were differentially expressed between breeds at one time point at least. At all three time points, CXCL8, LPO, CD68, CASP8, TLR2, TLR4 and MX2 were differentially expressed. All six evaluated genes of the TLR pathway were differentially expressed at 6 h. There was no consistent ranking in expression between the breeds over all genes, but notably in CASP8, CXCL8, TLR2 and TLR4 the ancient breeds had lower Cq values and therefore higher expression levels than the modern breeds. WP had higher expression levels of IL10, MX1 and MX2 than the other breeds. It also had a higher CCL20, CCL5, CD68, LF, LPO expression than RH.

Table 3: Basal mRNA expression (mean dCq and SEM) of innate immune genes in primary bovine mammary epithelial cells from four cattle breeds, unstimulated control after 6, 30 and 78 h.

	time											
	6 h				**30 h**				**78 h**			
	breed[1]				**breed**				**breed**			
	BS	**RH**	**WP**	**HLD**	**BS**	**RH**	**WP**	**HLD**	**BS**	**RH**	**WP**	**HLD**
	mean[2]				**mean**				**mean**			
gene	**SEM**				**SEM**				**SEM**			
chemokines												
CCL20	12.21	15.40	11.94	13.96	13.01	15.05	12.79	13.83	13.71_{ab}	15.83_a	12.47_b	14.64_{ab}
	0.63	1.24	1.63	1.14	0.60	0.95	0.93	0.73	0.78_a	0.90	1.43	0.90
CCL5	15.12_a	15.19_a	12.44_b	15.60_a	15.01	14.79	13.48	15.29	14.49_{ab}	14.74_{ab}	13.15_a	15.37_b
	0.67	0.73	0.63	0.80	0.72	0.66	0.72	0.83	0.47	0.46	0.46	0.83

	time											
	6 h				**30 h**				**78 h**			
	breed				**breed**				**breed**			
	BS	**RH**	**WP**	**HLD**	**BS**	**RH**	**WP**	**HLD**	**BS**	**RH**	**WP**	**HLD**
gene	**mean**				**mean**				**mean**			
	SEM				**SEM**				**SEM**			
CXCL8	10.36_{ab}	11.51_a	9.22_b	9.25_b	10.96_a	11.37_a	9.45_b	10.07_{ab}	11.07_{ab}	12.36_a	9.76_b	10.60_b
	0.50	0.29	0.74	0.48	0.57	0.29	0.44	0.38	0.42	0.22	0.75	0.55
cytokines												
IL6	7.22	6.88	8.92	8.03	7.66_a	7.52_a	9.56_b	7.92_{ab}	8.39_a	8.59_a	11.06_b	9.20_{ab}
	0.35	0.45	0.94	0.88	0.26_a	0.52	0.89	0.62	0.18	0.66	0.87	0.88
IL10	15.12_a	15.22_a	12.05_b	15.09_a	14.86_a	14.77_a	13.05_a	14.86_a	14.37_a	14.77_a	12.46_b	14.87_a
	0.68	0.68	0.76	0.77	0.67	0.66	0.67	0.87	0.44	0.44	0.38	0.98
antimicrobial peptides												
LF	9.44	10.45	9.70	9.12	8.06	9.38	8.92	8.66	5.56_a	7.93_b	6.21_{ab}	7.11_b
	0.66	0.24	0.55	0.38	0.67	0.56	0.59	0.49	0.51	0.84	0.85	0.33
LPO	15.15_{ab}	15.97_a	14.20_b	15.37_{ab}	15.09_{ab}	15.87_a	14.34_b	15.26_{ab}	15.30_{ab}	15.62_a	13.96_b	15.06_{ab}
	0.63	0.28	0.50	0.18	0.53	0.36	0.63	0.16	0.42	0.50	0.67	0.27
scavenger receptor												
CD68	13.28_{ab}	13.66_a	12.49_b	12.42_b	13.56_a	13.73_a	12.66_b	13.07_{ab}	14.16_{ab}	14.46_a	13.24_b	13.58_{ab}
	0.25	0.01	0.25	0.50	0.19	0.19	0.33	0.43	0.23	0.27	0.39	0.40
TLR pathway												
CASP8	7.61_a	7.82_a	6.62_b	6.64_b	7.90_a	7.90_a	6.85_b	7.01_b	7.76_{ab}	8.24_a	7.04_c	7.12_{bc}
	0.17	0.16	0.15	0.21	0.31	0.25	0.09	0.20	0.13	0.28	0.21	0.22
LBP	16.99_a	16.45_{ab}	16.60_{ab}	15.56_b	15.91	15.93	15.38	15.62	14.72	15.16	14.69	14.74
	0.44	0.35	0.21	0.56	0.55	0.26	0.34	0.51	0.40	0.58	0.91	0.43
LY96	4.92_a	5.53_b	4.43_{ac}	4.10_c	4.91	5.29	4.48	4.30	5.45	5.51	4.56	4.44
	0.08	0.29	0.18	0.21	0.13	0.63	0.20	0.28	0.34	0.52	0.27	0.27
MYD88	7.40_a	7.38_{ab}	7.18_{ab}	6.88_b	6.89	7.24	6.96	6.82	6.38	7.05	6.73	6.73
	0.18	0.15	0.19	0.17	0.20	0.39	0.19	0.21	0.18	0.38	0.08	0.29

	time											
	6 h				**30 h**				**78 h**			
	breed				**breed**				**breed**			
	BS	**RH**	**WP**	**HLD**	**BS**	**RH**	**WP**	**HLD**	**BS**	**RH**	**WP**	**HLD**
gene	**mean**				**mean**				**mean**			
	SEM				**SEM**				**SEM**			
TLR2	14.54_a	15.21_a	14.44_{ab}	13.47_b	14.04_{ab}	14.73_a	13.75_b	13.25_{bc}	13.68_{ab}	14.57_a	13.34_b	13.02_b
	0.17	0.36	0.48	0.33	0.37	0.32	0.23	0.27	0.36	0.33	0.19	0.38
TLR4	8.87_{ab}	9.37_a	8.10_c	8.20_{bc}	8.76_{ab}	9.25_a	7.87_c	8.09_{bc}	7.91_a	9.14_b	7.58_a	7.97_a
	0.20	0.16	0.30	0.29	0.30	0.17	0.25	0.21	0.32	0.28	0.31	0.18
others												
MX1	6.14_{ab}	6.54_{ab}	4.37_a	7.03_b	6.52	7.47	4.69	7.49	7.15	7.42	5.20	7.16
	0.34	0.88	0.54	1.27	0.41	0.89	0.87	1.48	0.71	0.68	0.27	1.56
MX2	11.59_a	11.42_a	8.22_b	11.61_a	11.29_{ab}	12.21_a	8.84_b	12.38_a	11.86_{ab}	12.59_a	9.20_b	12.05_{ab}
	0.72	0.97	0.63	1.24	0.57	1.06	1.08	1.28	0.84	0.96	0.45	1.33

[1]BS = Brown Swiss, RH = Red Holstein, WP = White Park, HLD = Highland

[2]means with different subscript letters within one time point are significantly different between the breeds (P<0.05)

Tables 4 and 5 show the relative fold changes of expression between control and treated cells. Only genes that were differentially expressed in one breed (P<0.05) or were at least 1.5-fold up regulated are presented. Table 4 shows the fold changes in gene expression after 6 h and 30 h exposure to *E. coli*. The most regulated gene was SAA3 with nearly 290-fold and 1900-fold after 6 h and 30 h *E. coli* in RH. After 6 h, fold changes of C3 and CASP8 were lower in HLD than in BS, CCL2 and LY96 were lower than in RH, and LPO was lower than in WP. C3, chemokines and cytokines were generally stronger up regulated. Antimicrobial peptides were only up regulated in the modern breeds. More of the S100 and MX genes were differentially expressed in the modern breeds. After 30 h exposure to *E. coli*, only BS differed from RH in CD14 fold change. C3, chemokines, cytokines and antimicrobial peptides were strongly up regulated. The S100 and MX genes were only significantly up regulated in the modern breeds. Table 5 shows the fold changes in gene expression after 30 h and 78 h exposure to *S. aureus*. There were no breed differences after 30 h *S. aureus*. The few differentially expressed genes were the antimicrobial peptides LPO and LYZ1 in WP and TLR4 in BS. After 30 h *S. aureus* LYZ1 had the highest significant fold change with 1.6 in WP.

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After 78 h incubation with *S. aureus*, HLD differed from BS in TGFB1. They were both down regulated and different from RH which was up regulated. LY96 was slightly elevated in HLD compared to WP and RH. The highest significant fold change was found in LF in RH with 1.6. SEM was generally very high. In general, the modern breeds had a higher number of regulated genes than the ancient breeds (Tables 4 and 5).

Table 4: Mean fold changes of the normalized relative gene expression of innate immune genes in pbMEC from four cattle breeds after 6 h and 30 h stimulation with *E. coli.*

gene		*E. coli* 6 h				*E. coli* 30 h			
		breed[1]				breed			
		BS	RH	WP	HLD	BS	RH	WP	HLD
complement system									
C3	fold change[2]	4.4 a**	4.2 ab**	6.1 ab*	1.9 b	8.6***	11.1**	6.8*	11.9*
	SEM	0.9	1.1	2.1	0.5	1.8	3.7	2.3	4.0
chemokines									
CCL2	fold change	28.8 ab**	10.8 a***	27.5 ab*	4.4 b	43.9***	27.3**	26.8*	18.2**
	SEM	12.3	1.9	11.8	1.0	13.1	9.0	12.1	9.2
CCL5	fold change	24.1	4.0*	4.8*	2.0*	51.6**	26.6*	55.4*	3.9*
	SEM	18.3	1.7	1.5	0.5	33.3	14.1	37.7	1.3
CCL20	fold change	39.4**	36.8**	110.8*	17.7*	50.2***	119.6*	74.5*	37.9*
	SEM	17.1	13.8	65.5	7.6	11.1	75.8	42.2	20.5
CXCL5	fold change	8.1**	8.0***	11.7*	3.9*	7.8***	9.0**	7.2*	6.4*
	SEM	2.1	1.6	4.0	1.0	1.3	2.6	2.3	2.0
CXCL8	fold change	20.1*	21.2***	33.8*	7.0*	21.9***	26.7**	16.0*	11.2*
	SEM	7.7	8.3	13.4	3.1	7.5	9.5	6.1	4.2
inflammatory cytokines									
IL6	fold change	3.6*	3.4**	7.7*	2.1*	5.3***	5.1*	11.2*	3.2*
	SEM	1.1	0.6	3.00	0.4	1.2	1.7	5.7	0.8
IL10	fold change	18.8*	3.3*	4.1	1.7	53.1**	25.2*	57.5	3.5
	SEM change	11.0	1.1	1.8	0.4	35.1	13.3	33.8	1.2
TGFB1	fold change[3]					1.7	0.9	0.9	1.4
	SEM					0.7	0.0	0.1	0.4

		treatment							
		E. coli 6 h				*E. coli* 30 h			
		breed				breed			
		BS	**RH**	**WP**	**HLD**	**BS**	**RH**	**WP**	**HLD**
TNF	fold change	21.9**	21.6	54.0*	6.0*	21.5***	40.6**	65.1	13.1**
	SEM	7.0	9.0	32.7	2.0	4.1	17.2	35.7	6.5
antimicrobial peptides									
LAP	fold change	2.8**	1.5	6.8	2.1	19.5**	4.6	84	25.2
	SEM	0.6	0.5	2.7	0.8	8.0	1.0	53.5	14.3
LF	fold change	3.2**	3.1*	2.8	1.7	8.6**	12.0**	9.3*	7.3*
	SEM	0.6	1.0	0.9	0.4	2.3	4.2	4.0	2.7
LPO	fold change	1.9$_{ab}$	0.9$_{ab}$	2.9$_a$	0.9$_b$	4.2*	1.7	9.2*	2.2
	SEM	0.4	0.1	0.8	0.2	1.2	0.2	4.1	0.7
LYZ1	fold change	2.1	2	3.7	1.9	40.2**	12.7*	32.1*	13.4*
	SEM	0.5	0.6	1.0	0.5	27.6	6.5	14.2	4.6
TAP	fold change	54.8*	1.3	33.5	8.5	105.7**	11.8*	234.8	47.8
	SEM	32.8	0.1	12.9	6.1	57.9	1.7	117.8	41.0
acute phase proteins									
SAA3	fold change	98.7	289.4*	418.2	10.8	618.2**	1912.3**	1769.1*	69.9*
	SEM	90.1	263.4	172.5	3.7	272.4	1445.1	1076.6	39.1
TLR signalling									
CASP8	fold change	1.3 $_a$ *	1.1 $_{ab}$	1.2 $_{ab}$	1.0 $_b$	1.4*	1.3*	1.2	1.4
	SEM	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2
CD14	fold change	1.3	1.1	1.2	1.8	1.8$_a$*	1.0$_b$	1.1$_{ab}$	1.8$_{ab}$
	SEM	0.1	0.1	0.1	0.4	0.3	0.1	0.2	0.4
LBP	fold change	2.5	2.2	1.4	1.2	1.2	1.4	1.2	2.7
	SEM	0.7	0.7	0.4	0.3	0.1	0.4	0.5	0.2
LY96	fold change	1.0$_{ab}$	1.1$_a$*	0.9$_{ab}$	1.0$_b$				
	SEM	0.0	0.0	0.1	0.0				
TLR2	fold change	5.7	1.7***	6.9*	1.5	5.0*	2.0*	2.8	2.2*
	SEM	2.1	0.1	3.3	0.2	2.1	0.3	0.9	0.4
others									
MX1	fold change	2.8	1.6*	1.2	1.5	4.4*	3.5**	3.1	2.9
	SEM	1.4	0.2	0.3	0.4	1.4	0.8	1.1	1.3

		E. coli 6 h				E. coli 30 h			
		breed				**breed**			
		BS	**RH**	**WP**	**HLD**	**BS**	**RH**	**WP**	**HLD**
MX2	fold change	11.6*	2.0*	1.5	1.8	8.6***	8.3*	6.5	3.9
	SEM	7.6	0.5	0.4	0.6	2.3	2.8	3.3	1.6
S100A9	fold change	3.9**	2.4	8.6*	2.1	12.0***	12.6*	14.2	20.4
	SEM	0.8	0.9	4.0	0.6	4.1	4.1	6.0	15.9
S100A12	fold change	2	N/A[4]	6.5	2.6	4.2**	2.1	5.1	1.7
	SEM	0.5	N/A	3.1	1.0	0.9	1.2	1.4	0.5
regulated genes[5]		14	14	10	6	22	18	11	12

treatment

[1]BS = Brown Swiss (n=6), RH = Red Holstein (n=6), WP = White Park (n=5), HLD = Highland (n=5)

[2]stars indicate significant differences between treated and control dCq: * P<0.05, ** P<0.01, *** P<0.001, fold change means with different subscript letters within one time point differ between breeds (P<0.05)

[3]empty genes: no significant breed differences in expression fold changes and no fold changes > 1.5 at this time point

[4]missing data

[5]where P<0.05 for dCq difference between treated and control samples

Table 5: Mean fold changes of the normalized relative gene expression of innate immune genes in pbMEC from four cattle breeds after 30 h and 78 h stimulation with *S. aureus*.

gene		S. aureus 30 h				S. aureus 78 h			
		breed[1]				**breed**			
		BS	**RH**	**WP**	**HLD**	**BS**	**RH**	**WP**	**HLD**
complement system									
C3[2]	fold change[3]					1.1	1.6	1.1	1.2
	SEM					0.1	0.4	0.1	0.1
chemokines									
CCL2	fold change					1.0	1.3	1.3	1.6
	SEM					0.1	0.1	0.2	0.3
CCL5	fold change					1.5*	1.6	1.3	1.2
	SEM					0.2	0.4	0.6	0.3

treatment

gene		S. aureus 30 h				S. aureus 78 h			
		breed				breed			
		BS	RH	WP	HLD	BS	RH	WP	HLD
CCL20	fold change					1.7	0.9	3.3	1.5
	SEM					0.6	0.2	1.3	0.4
CXCL8	fold change					1.3	1.2	1.9	1.1
	SEM					0.2	0.1	0.6	0.2
inflammatory cytokines									
IL10	fold change					1.5*	1.5	1.0	1.1
	SEM					0.2	0.5	0.4	0.3
TGFB1	fold change					0.9_b*	1.2_c*	0.9_{abc}	0.8_a
	SEM					0.0	0.1	0.1	0.0
TNF	fold change					1.2	1.2	4.5	1.8
	SEM					0.3	0.1	2.9	1.1
antimicrobial peptides									
LAP	fold change					1.2	N/A[3]	5.1	1.9
	SEM					0.2	N/A	3.4	0.8
LF	fold change					1.0	1.6*	1.3	1.1
	SEM					0.1	0.2	0.4	0.1
LPO	fold change	1.0	1.2	1.2*	1.0	1.2	0.9	1.7	1.5
	SEM	0.3	0.2	0.2	0.1	0.2	0.1	0.7	0.4
LYZ1	fold change	1.1	0.9	1.6*	1.9	1.6	1.2	2.9	1.2
	SEM	0.1	0.2	0.3	0.5	0.5	0.2	1.2	0.1
TAP	fold change	0.9	1.1	1.5	0.9	5.2	0.7*	6.9	1.5
	SEM	0.3	0.4	0.3	0.2	4.3	0.2	3.7	0.8
acute phase proteins									
SAA3	fold change	1.7	2.5	3	1.2	5.1	3.1	5.4	1.2
	SEM	0.5	1	1.7	0.3	3.8	1.5	4.3	0.2
TLR signalling									
LBP	fold change					1.0	1.4	0.9	1.8
	SEM					0.2	0.4	0.2	1.0
LY96	fold change					1.1_{ab}	1.0_b	1.0_b	1.1_a*
	SEM					0.0	0.1	0.0	0.0

gene		S. aureus 30 h				S. aureus 78 h			
		breed				breed			
		BS	RH	WP	HLD	BS	RH	WP	HLD
TLR2	fold change					1.0	1.0	1.3	1.9
	SEM					0.1	0.1	0.2	1.1
TLR4	fold change	1.3*	1.0	1.0	1.1	1.0	1.2*	1.0	1.0
	SEM	0.2	0.1	0.1	0.0	0.1	0.1	0.1	0.0
scavenger receptor									
CD68	fold change					1.1	1.3	1.2	1.7
	SEM					0.2	0.2	0.3	0.5
others									
MX1	fold change	2.1	1.2	1.0	1.7	1.7	1.2	1.1	0.8
	SEM	0.7	0.4	0.3	0.3	0.4	0.2	0.3	0.2
MX2	fold change	1.9	1.9	1.1	1.9	2.9	1.3	1.4	1.0
	SEM	0.7	1.3	0.4	0.4	1.3	0.2	0.5	0.2
S100A9	fold change					1.3	1.3	2.2	1.3
	SEM					0.2	0.2	0.6	0.2
S100A12	fold change					1.3_a*	0.8_b	1.2_{ab}	1.6_{ab}
	SEM					0.1	0.2	0.2	0.5
regulated genes[4]		1	0	2	0	4	4	0	1

[1]BS = Brown Swiss (n=6), RH = Red Holstein (n=6), WP = White Park (n=5), HLD = Highland (n=5)

[2] empty genes: no significant breed differences in expression fold changes and no fold changes > 1.5 at this time point

[3]stars indicate significant differences between treated and control dCq: * $P<0.05$, ** $P<0.01$, *** $P<0.001$, fold change means with different subscript letters differ between breeds ($P<0.05$)

[4]where $P<0.05$ for dCq difference between treated and control samples

Figure 18 shows the PCA on the dCq values of the basal expression (untreated control samples) (Figure 18a) and the ddCq values, the differences between control and treated dCqs (Figure 18b) (Sorg *et al.*, 2013b). Each symbol represents all data of all respective samples from one animal. A clustering is visible in the basal expression (Figure 18a): RH and

BS form two subgroups and are separated from the cluster of WP and HLD. The PCA on the ddCqs shows no such separation (Figure 18b).
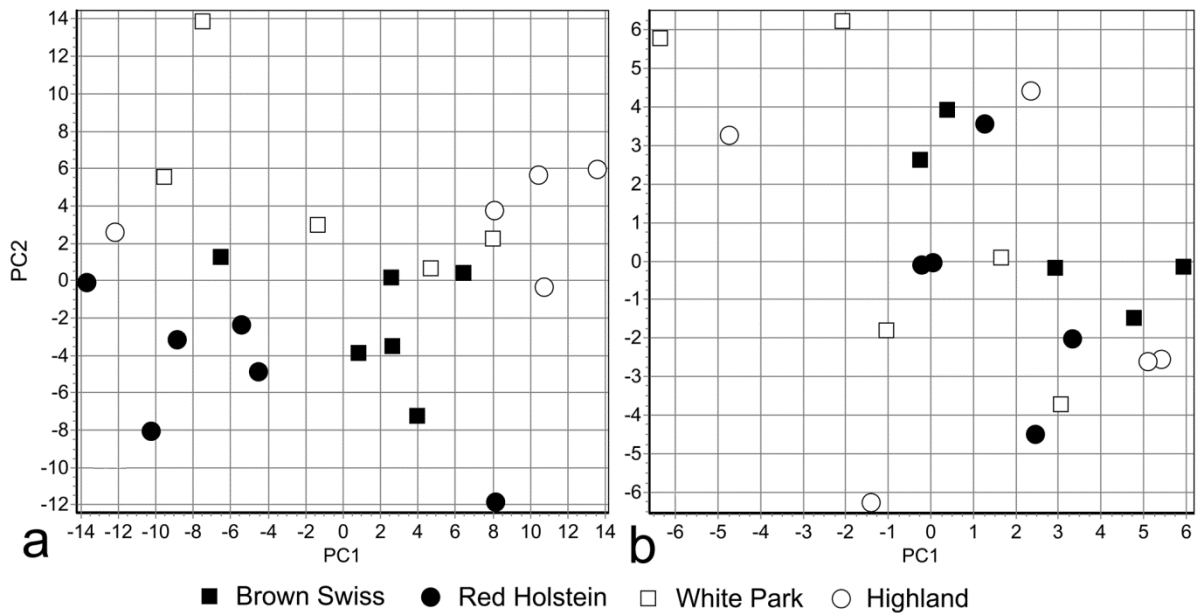
Fig. 18: Principal component analysis of (a) dCq values (basal expression of unstimulated control) and (b) ddCq values (difference between treated and control dCq) of 28 target genes in primary bovine mammary epithelial cells from four cattle breeds after stimulation with E. coli and S. aureus. Each symbol represents all respective samples from one animal (Sorg et al., 2013b).

The protein production of LF, IL-10 and SAA is shown in Figures 19, 20 and 21 respectively (Sorg *et al.*, 2013b). It is shown together with the particular corresponding mRNA expression. LF mRNA up regulation could be observed in most *E. coli* treatments and after 78 h *S. aureus* in RH, the LF protein was only increased after 30 h *E. coli* in RH and WP (Figure 19). BS even showed a decrease under this treatment. In the untreated cells at 78 h BS had higher gene expression levels than RH and HLD. In the untreated and the *S. aureus* treated cells after 30 h HLD had higher protein levels than WP.
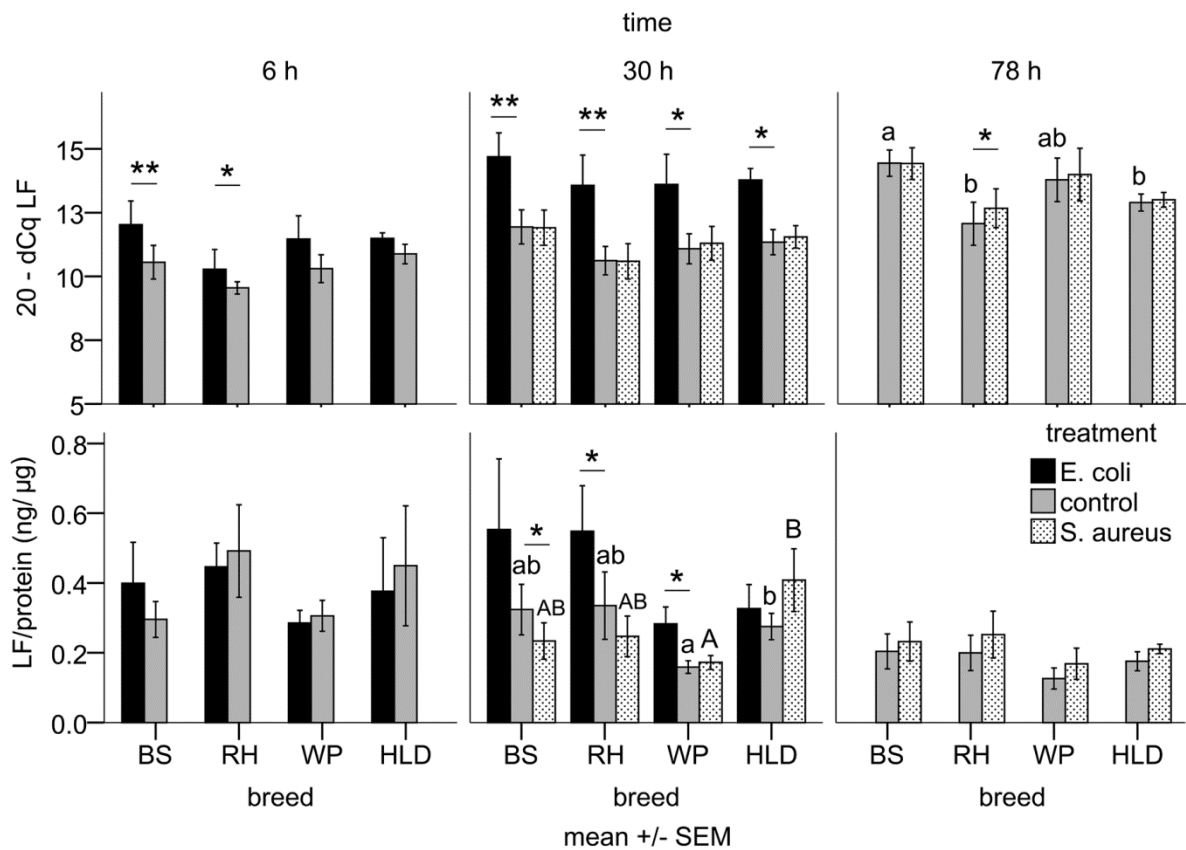
Fig. 19: Lactoferrin (LF) gene expression as 20 – dCq and LF protein production per total cell protein as ng/µl in pbMEC II from Brown Swiss (BS, n=6), Red Holstein (RH, n=6), White Park (WP, n=5) and Highland (HLD, n=5) cattle after a bacterial challenge. Stars indicate significant differences between treatment and control (*=P<0.05, **=P<0.01), lower and upper case letters indicate significant differences (P<0.05) between breeds in control and S. aureus treated samples, respectively (Sorg et al., 2013b).

In IL10 gene expression fold changes and IL-10 protein in % of control there were no significant breed differences (Figure 20). In contrast to the often significant up regulation in IL10 mRNA expression (Tables 4 and 5) there was no consistent trend in IL-10 protein increase over the breeds and treatments. BS had a qualitative increase of about 50 and 25 % after 30 h and 78 h *S. aureus* respectively. In RH there was a qualitative increase of about 60 % after 30 h *E. coli*. WP showed no distinct increase or decrease compared to controls. HLD had an about 50 % increase with 6 h *E. coli* and 78 h *S. aureus* and an approximate 100 % increase with 30 h *S. aureus*. Qualitatively, HLD was the most reactive in IL-10 protein regulation, but had the least reactive IL10 gene expression pattern of the breeds. In contrast to this, WP had a considerable, however not significant reaction to the treatments in mRNA expression, but showed almost no protein regulation.
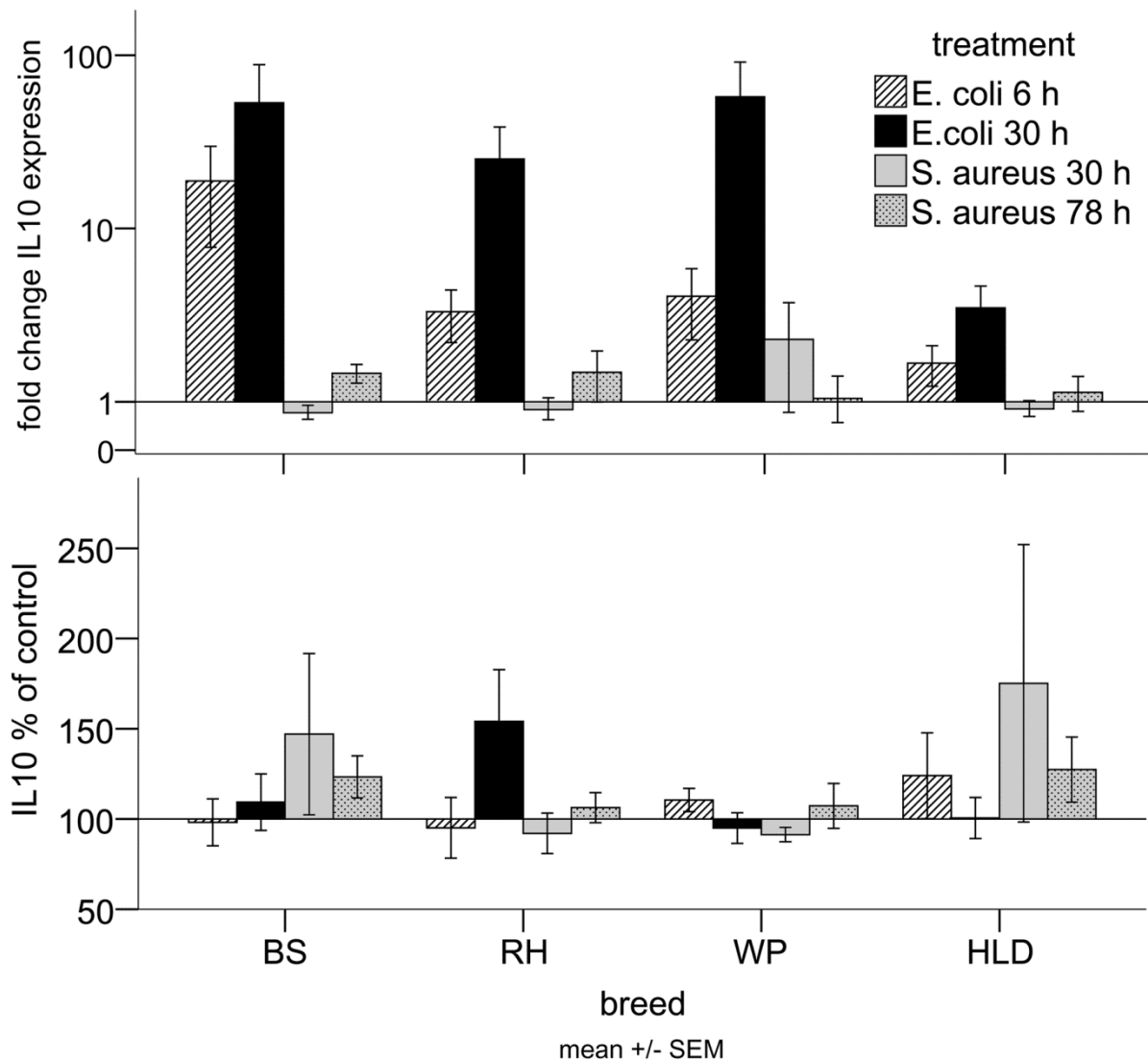
Fig. 20: Interleukin 10 (IL10) fold changes in gene expression and interleukin-10 (IL-10) protein production in % of control in pbMEC II from Brown Swiss (BS, n=6), Red Holstein (RH, n=6), White Park (WP, n=5) and Highland (HLD, n=5) cattle after a bacterial challenge (Sorg et al., 2013b).

SEM was extraordinarily high in IL-10 protein, indicating a large between-cow variation. The significant up regulation of SAA3 in 30 h *E. coli* treated samples in all breeds was only reflected by a significant SAA protein increase in BS (Figure 21). While in gene expression there were no breed differences, the basal (control) levels of SAA protein were lower in BS and RH than in HLD and the levels of the treated samples were lower in BS than in HLD. A qualitative breed gradient was visible for both control and treated protein levels in the order of BS<RH<WP<HLD.
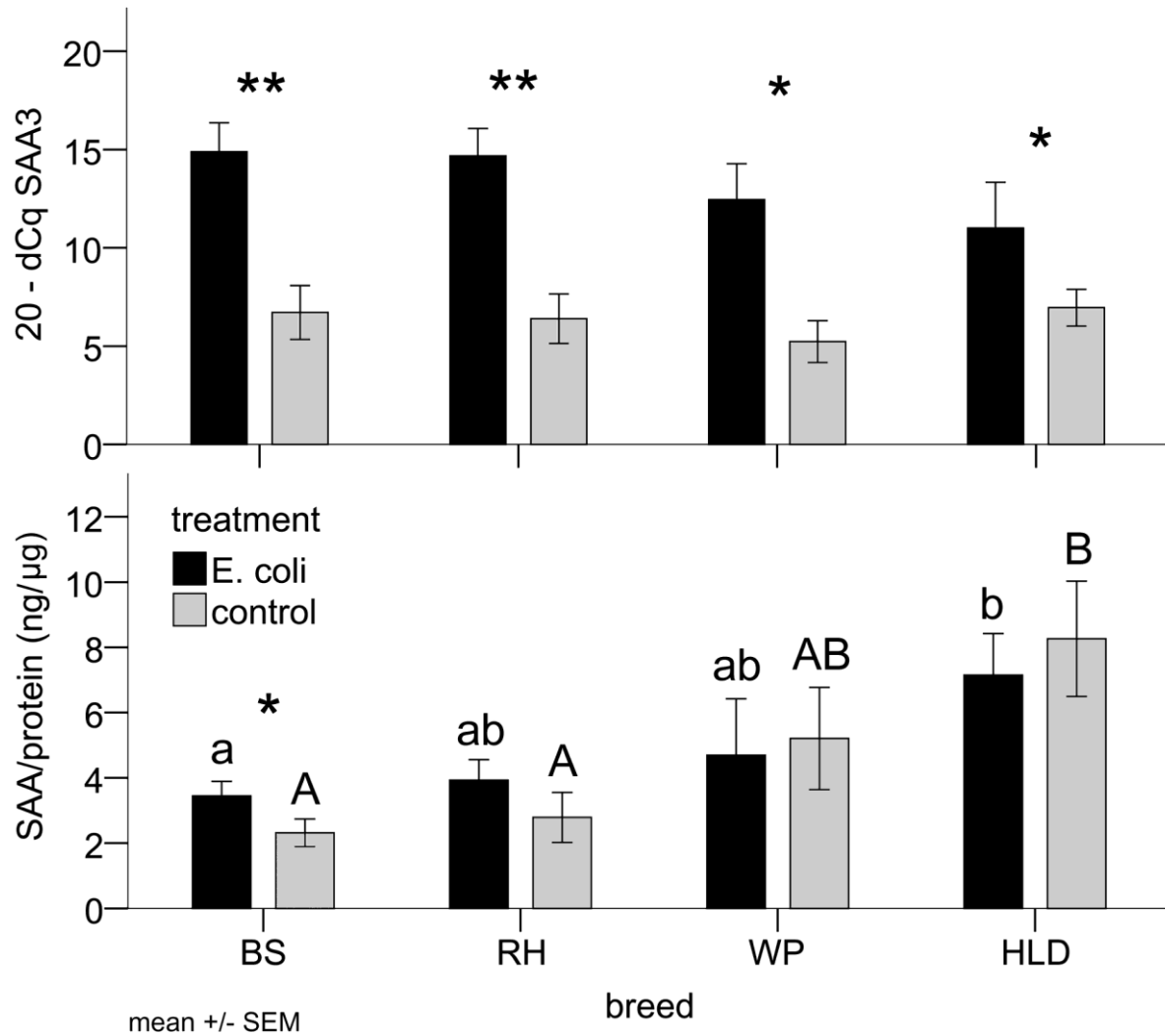
Fig. 21: Serum amyloid A3 (SAA3) gene expression as 20-dCq and serum amyloid A (SAA) protein production per total cell protein as ng/µg in pbMEC II from Brown Swiss (BS, n=6), Red Holstein (RH, n=6), White Park (WP, n=5) and Highland (HLD, n=5) cattle after a bacterial challenge. Stars indicate significant differences between treatment and control (*=P<0.05, **=P<0.01), lower and upper case letters indicate significant differences (P<0.05) between breeds in E. coli treated and control samples, respectively (Sorg et al., 2013b).

Breed differences are visible in the basal expression and to a lesser extent in the response to the stimulation

On the level of basal expression in the PCA, there was a visible separation of ancient from modern breeds and within the two modern breeds. This is corresponding with a study of Blott *et al.* (1998) where the allele frequencies of 37 cattle breeds were studied to compare breed relationships (Figure 22). In that study, Holstein-Friesian (HO) was clearly distant from Brown Swiss (BS), while White Park (WP) and Highland (HL) were closer together. In our PCA on ddCq levels, however, this separation was lost and the points were widely spread (Figure 19b). The ancient breed animals were further apart from each other than the modern breed animals, indicating a greater variation in immune response while modern breed animals were more similar to each other.
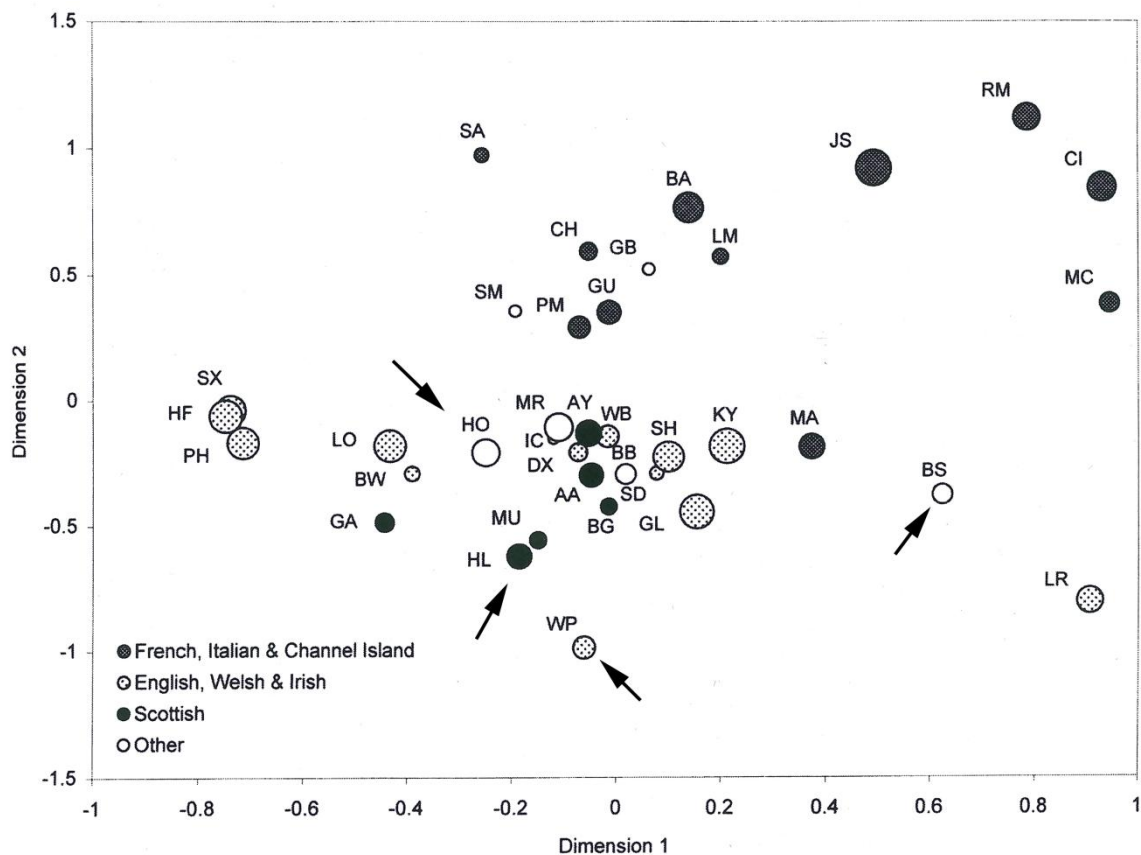
Fig. 22: Principal component analysis of allele frequencies from 37 cattle breeds in a study of Blott et al. (1998), slightly adapted: inserted arrows indicate Holstein-Friesian (HO), Brown Swiss (BS), Highland (HL) and White Park (WP) for better visualization. Three components are plotted, with the third represented by the diameter of the points, so that points that are distant from the viewer are smaller than those that are closer and clusters of breeds can be formed within the three-dimensional space.

It could have been expected that the WP animals would be lying much closer together, as the exceedingly small German WP cattle population has been shown to be more inbred (average coefficient of inbreeding = 16%) than many other cattle breeds worldwide (average coefficient of inbreeding = 0.3 – 5%) (Biedermann *et al.*, 2009). It is likely that the rigorous selection on conformal traits in the modern dairy breeds led to a high resemblance and small variations.

Although the ranking of the breeds in gene expression levels was not consistent over all genes, there was a consistency in TLR pathway expression: the higher basal expression of the components of the TLR pathway in the ancient breeds could have an effect on the rapidity of pathogen recognition and lead to a more efficient triggering of the immune response. The higher basal levels of SAA protein in the ancient breeds could have a protective effect against pathogens, as SAA is an opsonising agent (Shah *et al.*, 2006). Interestingly, basal LF protein levels were lower in the ancient breeds, but differed significantly only between WP and HLD. WP and HLD also differed in basal expression of MX1 and CCL5. This suggests that the breeds are all different from each other and cannot simply be grouped together in 'modern' and 'ancient'. It is difficult to interpret the fold changes of gene expression. Due to the considerably high SEM some visibly high fold changes happened to be not significant. The PCA on ddCq did not reveal any clustering. But apart from this, in the modern breeds there were more genes significantly regulated, especially for the antimicrobial peptides, the TLR pathway and the MX genes. HLD had the lowest fold changes in SAA3 expression, but the highest basal levels of SAA protein after 30 h. The whole picture of breed differences is very heterogeneous and inconsistent. But it looks like in those parts of the immune system where we found a difference between the breeds, a higher basal expression led to a lower response. Kandasamy *et al.* (2012) measured the immune response of cows that had before been classified as 'high-' and 'low-responder' animals to an intramammary *E. coli* infusion. The low-responder animals had a weaker immune response, which was, however, more effective and led to a shorter resolution phase of the inflammation. Hence, a strong immune response is not in all cases beneficial for the animal. Another example for this phenomenon is the tolerance of the *Bos indicus* Sahiwal cattle to the indigenous protozoan parasite *Theileria annulata.* After an experimental infection, they showed fewer clinical symptoms, recovered from a higher dose of pathogen and had lower acute phase protein levels compared to Holstein calves (Glass *et al.*, 2005). In another

experiment, macrophages from Holstein cattle showed higher up regulation of inflammatory and immune response genes than those from Sahiwal cattle (Glass *et al.*, 2012).

To our knowledge there are no studies on the intra-mammary immune system of ancient cattle breeds like WP and HLD. It has been found that the immune system of modern dairy breeds shows differences in details, but is generally conserved (Bannerman *et al.*, 2008a; Bannerman *et al.*, 2008b), which is in accordance with our results. In one of those studies, the *in vivo* response of Holstein and Jersey cows to *E. coli* differed only in the time point of milk cytokine and SCC increase, not in overall levels (Bannerman *et al.*, 2008a). After an *S. aureus* challenge, Holstein and Jersey animals also responded with similar levels of milk SCC and cytokines and varied only in neutrophil and NAGase activity (Bannerman *et al.*, 2008b). Different LF contents in milk have already been measured in Holstein, Jersey and Simmental cows (Krol *et al.*, 2010) and in dairy and beef cattle (Tsuji *et al.*, 1990), which adds to our findings of different LF contents in pbMEC. Several polymorphisms have been found in the LF gene in different cattle breeds that could cause a differential LF expression and production (O'Halloran *et al.*, 2009). To compare SAA levels in different breeds, we only found one study: SAA in blood serum increased more rapidly in Angus than in Romosinuano steers (an indigenous Colombian breed) after an LPS challenge and remained at higher levels for 8 h (Carroll *et al.*, 2011). Although there was no significant rise in SAA protein after pathogen stimulation in our ancient breed pbMEC, their absolute levels were higher than in the modern breeds. Cattle breed differences in gene expression and protein production of the immune system have not been systematically studied so far, but our findings and the above mentioned studies show that there is evidence for such diversity.

The animal differences within each breed, revealed by the high SEMs and by the wide spread of the symbols representing animals in the PCAs, could also reflect the existence of a substantial between-cow variation in the immune response which has already been shown for Holstein cattle *in vitro* and *in vivo* (Kandasamy *et al.*, 2012). The underlying genetic polymorphisms could be linked to a certain breed, but they could be spread all over the cattle population as well. Furthermore, it has been implied that a part of so far unexplained phenotypic variation in the dairy cow is due to epigenetic regulation (Singh *et al.*, 2010).

E. coli induces a much stronger immune response than S. aureus

In all the breeds there was a much stronger reaction to *E. coli* than to *S. aureus.* This remarkable pathogen difference has already been noted by other researchers *in vitro* (Griesbeck-Zilch *et al.*, 2008, Gunther *et al.*, 2011, Danowski *et al.*, 2012a) and *in vivo* (Bannerman *et al.*, 2004, Lee *et al.*, 2006, Petzl *et al.*, 2008). It was first thought that a lack of *S. aureus* recognition by TLRs was the reason for the weak immune response, but Yang *et al.* (2008) found that TLR2 and TLR4 were both properly activated by the two exact strains of pathogens that we had used, too. *S. aureus* however, failed to activate NFKB, a transcription factor that initiates the transcription of many proinflammatory genes. After experimental blocking of the NFKB activation, their pbMEC still responded weakly to *S. aureus*. This leads to the conclusion that *S. aureus* recognition by TLRs triggers the immune response via a completely different pathway and may even dispose of a means to block or interfere with the TLR signalling cascade. The fact that we measured such an extraordinarily weak immune response compared to other studies could be found in the experimental design. Maybe we missed the proper time frame for the peak in immune response which had been at 3 h in the study of Yang *et al.* (2008) and at 2 – 4 h in a study of Strandberg *et al.* (2005) with gram-positive bacterial lipoteichoic acid stimulation (LTA). It could also have been a wrong dose of inoculum. Too low a dose would not trigger the immune response sufficiently. But Wellnitz and Kerr (2004) showed that also with a too elevated MOI there was no more significant immune response. Furthermore, it could be a strain-dependent effect, as other researchers obtained a more pronounced reaction to other *S. aureus* strains like *S. aureus* M60 *in vitro* (Griesbeck-Zilch *et al.*, 2008) or *S. aureus* 305 *in vivo* (Bannerman *et al.*, 2004). The often chronic outcome of *S. aureus* mastitis could be based on this failure of a proper immune response to clear out the colonization of the udder by the pathogen.

Signalling and defence molecules are strongly activated, in contrast to the TLR pathway

By far the most influenced gene was SAA3, which was up to 1900-fold up regulated in RH after 30 h *E. coli.* It is known to have antibacterial effects and the reaction of our cells is a confirmation of its suggested use as an inflammation marker (Molenaar *et al.*, 2009). A strong induction by *E. coli* was also observed in the chemokines and inflammatory cytokines on the one hand as well as in C3, in the antimicrobial peptides, the S100A genes and the MX genes on the other. This shows the excellent bidirectional functionality of our pbMEC: while acting as sentinels by attracting and activating immune cells upon pathogen recognition, at the same time they exerted their function in actively combating the pathogen with defence

proteins. The TLR-pathway was not as markedly regulated. It seems that a small regulation in this starting point in the signalling cascade builds up and results in a strong regulation of the resulting end product. This is understandable when realizing that a single activated molecule can itself activate many molecules in a row and this happens in each step of the cascade. Strandberg *et al.* (2005) found no effect of LPS or LTA on TLR4 and TLR2 expression in pbMEC and the bovine mammary epithelial cell lines MAC-T, although there was a pronounced inflammatory response. Thus, we agree with what those authors concluded, which is that pbMEC have a fully functional, constituently active and immediately responsive set of TLRs that does not need to be up regulated upon pathogen recognition.

Protein levels do not correspond well with mRNA expression

The weak correspondence of mRNA and protein levels could have several possible reasons. First of all, the dynamics of gene expression and protein production over time are not necessarily the same. It is possible that a fast response to the pathogens in 6 h LF gene expression is translated into a measurable protein reaction only after 30 h and later. Furthermore, LF is secreted into the cell culture medium. Unfortunately it was below the measuring range of the ELISA protocol in most of the medium samples (data not shown), so that no evaluation was possible. But this proportion of LF could account for the gap between mRNA expression and cellular LF protein. In addition to this, in the human LF gene there is an alternative transcript variant from a different LF gene promoter that leads to a shorter isoform of the LF mRNA and protein, called delta LF (Mariller *et al.*, 2012). It exerts intracellular functions as a transcription factor in cell cycle regulating genes. The mRNAs differ in exon 1 (Hoedt *et al.*, 2010). Our primer pair had been designed to bind in exon 7 and 8 before this fact was known to us. If the same transcript variant exists in the bovine transcriptome - which is possible as the LF gene is highly conserved among mammal species - we could not discriminate between the expressions of these two. However, it is possible that the antibody in the ELISA did only bind to LF and not to delta LF because of the different amino acid sequence and structure. Another potential posttranscriptional regulation is the microRNA miR-214 which has been shown to regulate LF mRNA expression and function in mammary epithelial cells (Liao *et al.*, 2010). Also for IL10 and for another SAA-encoding gene, SAA2, microRNAs have been found that could lead to a posttranscriptional regulation and a massive variation in protein production (Longley *et al.*, 1999, Sharma *et al.*, 2009).

5. Conclusions

For the first time the intra-mammary immune system of the ancient HLD and WP cattle has been characterized *in vitro.* It has often been suspected that the immune system of the ancient breeds must be different from the modern breeds due to a very different selection process and an adaptation to a different natural environment. At least in the innate immune system of pbMEC this seems not to be the case. The differences between BS, RH, WP and HLD were more pronounced in the basal mRNA and protein expression than in the response to the pathogen stimulation. The breeds differed in parts of the innate immune system, but activated mainly the same pathways at similar expression levels. Those were the TLR-mediated triggering of the innate immune response which comprised the expression of inflammatory cytokines and chemokines on the one hand, and the expression of antimicrobial peptides, the acute phase gene and protein SAA3, complement component C3 and other defence molecules on the other hand. It could be suspected that the often observed smaller susceptibility to mastitis of the ancient breeds is caused by higher basal innate immune levels which do not need to be as highly up regulated after pathogen recognition as in the modern breeds. There also was a large between-cow variation which seemed to be independent of the breed. Breed-independent genetic polymorphisms, epigenetic regulation and posttranscriptional regulation by microRNAs could be responsible for that. These mechanisms remain subject for further research. In addition to that, we studied only one part - one cellular component - of the complex immune network that operates in the udder. It is still possible that there are breed differences in the reactivity of neutrophils and macrophages or even in the adaptive immune response. Thus, our findings from pbMEC need to be compared to the reaction of other cell types or to the *in vivo* response.

The culture of pbMEC from milk has been found to be a suitable method for our purposes that could easily be applied to study other ancient and rare breeds to obtain a broader picture of breed differences, and the same holds true for the successful application of a high-throughput qPCR system.

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Sorg D, Potzel A, Beck M, Meyer HHD, Viturro E, Kliem H 2012. Effects of cell culture techniques on gene expression and cholesterol efflux in primary bovine mammary epithelial cells derived from tissue and milk. *In Vitro Cellular & Developmental Biology - Animal* 48, 550-553.

Danowski K, Sorg D, Gross J, Meyer HHD, Kliem H 2012. Innate defense capability of challenged primary bovine mammary epithelial cells after an induced negative energy balance in vivo. *Czech Journal of Animal Science* 57, 207-220

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Appendix

Appendix I

Sorg D, Potzel A, Beck M, Meyer HHD, Viturro E, Kliem H 2012a. Effects of cell culture techniques on gene expression and cholesterol efflux in primary bovine mammary epithelial cells derived from tissue and milk. *In Vitro Cellular & Developmental Biology - Animal* 48, 550-553 (Reprinted with kind permission from Springer Science and Business media)

Appendix II

Danowski K, Sorg D, Gross J, Meyer HHD, Kliem H 2012. Innate defense capability of challenged primary bovine mammary epithelial cells after an induced negative energy balance in vivo. *Czech Journal of Animal Science* 57, 207-220 (Reprinted with permission)

Appendix III

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Appendix III

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REPORT

Effects of cell culture techniques on gene expression and cholesterol efflux in primary bovine mammary epithelial cells derived from milk and tissue

D. Sorg · A. Potzel · M. Beck · H. H. D. Meyer · E. Viturro · H. Kliem

Abstract Primary bovine mammary epithelial cells (pbMEC) are often used in cell culture to study metabolic and inflammatory processes in the udder of dairy cows. The most common source is udder tissue from biopsy or after slaughter. However, it is also possible to culture them from milk, which is non-invasive, repeatable and yields less contamination with fibroblasts. Generally, not much is known about the influence of cell origin and cell culture techniques such as cryopreservation on pbMEC functionality. Cells were extracted from milk and udder tissue to evaluate if milk-derived pbMEC are a suitable alternative to tissue-derived pbMEC and to test what influence cryopreservation has. The cells were cultivated for three passages and stored in liquid nitrogen. The relative gene expression of the five target genes kappa-casein, lingual antimicrobial peptide (LAP), lactoferrin, lysozyme (LYZ1) and the prolactin receptor normalised with keratin 8 showed a tendency to decrease in the tissue cultures, but not in the milk-derived cultures, suggesting a greater influence of the cultivation process on tissue-derived cells, freezing lowered expression levels in both cultures. Overall expression of LAP and LYZ1 tended to be higher in milk cells. Cholesterol efflux was measured to compare passages one to seven in milk-derived cells. Passage number did not alter the efflux rate ($p \leq 0.05$). We showed for

the first time that the extraction of pbMEC from milk can be a suitable alternative to tissue extraction.

Keywords Primary bovine mammary epithelial cells · Gene expression · Passage · Cryopreservation · Cholesterol

Mammary gland biology is often studied in vitro. In dairy cows, most often, primary bovine mammary epithelial cells (pbMEC) are used for experiments in different fields. Examples are lactation and milk constituent biosynthesis (Groves and Larson 1965), cell-to-cell-interaction studies (Close et al. 1997), cell-to-extracellular matrix (ECM) interaction studies (Katz and Streuli 2007), plasma membrane transporter studies (Paye et al. 2007) and investigations on the innate immune system (Griesbeck-Zilch et al. 2008). Usually pbMEC are obtained by tissue culture from slaughtered animals. The extraction and cultivation from cow's milk has been known for over 20 yr (Buehring 1990) but never found considerable attention, although it has many advantages like non-invasiveness, repeatability and less contamination by fibroblasts. There still exists a need to demonstrate the suitability as a true alternative to the tissue culture in terms of gene expression and cell functionality. We performed an explorative study comparing gene expression levels from pbMEC cultivated from milk and from udder tissue and testing the influence of passage number and cryopreservation in liquid nitrogen. In an additional trial with milk-derived pbMEC, the efflux of cholesterol in cells from different passages was studied to show the sustained functionality of these cells.

Milk samples from four healthy lactating dairy cows (Red Holstein) were taken 1 wk before slaughter shortly before the 150th day of lactation. Cows were selected upon inspection of SCC. The mean SCC was 108,000±41,000 cells/ml SEM. An SCC below 200,000 cells/ml was regarded as healthy. PbMEC were extracted from 2 l fresh whole milk of each cow and cultivated with the method described in Danowski et al.

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Table 1. Primer sequences of the genes measured in RT-qPCR

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)
CSN3	TGCAATGATGAAGAGTTTTTTCCTAG	GATTGGGATATATTTGGCTATTTTGT
KRT8	ACTGGCTACGCAGGTGGACT	CCGCAAGAGCCTTTCACTTG
LAP	AGAAATTCTCAAAGCTGCCG	CAGCATTTTACTTGGGCTCC
LF	CGAAGTGTGGATGGCAAGGAA	TTCAAGGTGGTCAAGTAGCGG
LYZ1	AAGAAACTTGGATTGGATGGC	ACTGCTTTTGGGGTTTTGC
PRLR	TGATGTTCATCTGCTGGAGAAGGGC	TCCAGGTGCATGGGCTTCACG
VIM	TGGAGCGTAAAGTGGAATCC	GACATGCTGTTCTTGAATCTGG

(2012) adapted from Buehring (1990). Additionally, udder tissue from the same four cows was taken aseptically immediately after slaughtering with the method described in Griesbeck-Zilch et al. (2008). After the first, second and third passage each, 100,000 cells were seeded in a six-well tissue-culture plate and grown for 5 d until harvest, the rest was reseeded in a 25-cm^2 tissue-culture flask for further proliferation. After the third passage, additionally, an aliquot was stored in liquid nitrogen for 3 wk before being reseeded at 100,000 cells in a six-well plate in the same way. Primary bovine fibroblasts were extracted from a healthy cow's tendon and cultivated with the same protocol as the mammary tissue. An immunocytochemical staining of the epithelial marker cytokeratin in pbMEC and fibroblasts was done using the protocol and antibodies described in Danowski et al. (2012). The polyclonal rabbit anti-casein antibody (1:50 in PBS-Tween) (Genetex, Irvine, CA) was used for the casein staining in milk-derived cells with the same protocol as the cytokeratin staining. After growing for 5 d, cells were washed with PBS and total RNA was extracted with the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. Reverse transcription was carried out using 500 ng of RNA in a reaction with the M-MLV (H-) Point Mutant Enzyme (Promega, Wisconsin) and random hexamer primers (Invitrogen GmbH, Darmstadt, Germany). Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was done on Rotor-Gene Q cycler (Qiagen GmbH, Hilden, Germany) and the SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Inc. Munich, Germany) according manufacturer's instructions and the primers listed in Table 1. To avoid measuring expression in eventually contaminating fibroblasts, only mammary epithelial cell-specific genes were

Figure 1. Light microscopy of (a) primary bovine mammary epithelial cells (pbMEC) from udder tissue, immunostained against cytokeratins (insert: negative control), magnification ×200 (b) pbMEC from milk immunostained against cytokeratins (insert: negative control), magnification ×200 (c) primary bovine fibroblasts immunostained against cytokeratins, magnification ×200 (d) pbMEC from milk immunostained against casein (insert: negative control), magnification ×400.
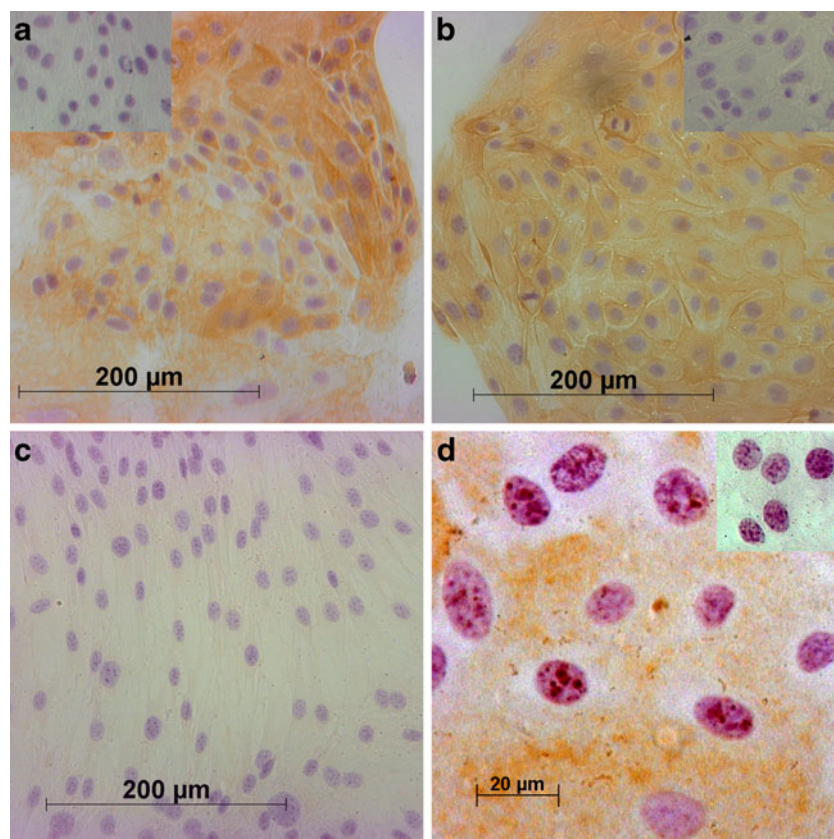
Figure 2. (*a*) Relative normalised gene expression of primary bovine mammary epithelial cells (pbMEC) cultured from milk and udder tissue over three passages (*b*) relative normalised gene expression from the same pbMEC cultures in third passage with and without prior freezing in liquid nitrogen. *CSN3* kappa-casein, *LF* lactoferrin, *LAP* lingual antimicrobial protein, *LYZ1* lysozyme1, *PRLR* prolactin receptor.
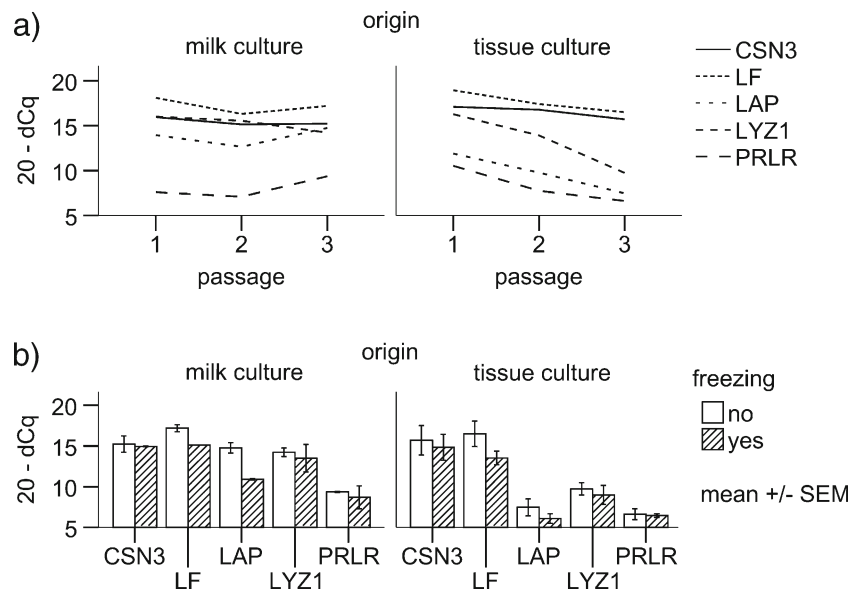
selected. The absence of expression of the candidate genes was tested in the fibroblast culture. Keratin 8 (KRT8) is an intermediate filament protein of the cytoskeleton and a marker for epithelial cells. Kappa-casein (CSN3) is a major milk protein. Lingual antimicrobial peptide (LAP), lactoferrin (LF) and lysozyme (LYZ1) are antimicrobial peptides. The prolactin receptor (PRLR) responds to the lactogenic hormone prolactin. Vimentin (VIM) is a filament protein of the cytoskeleton and used as a marker for fibroblasts. mRNA expression was determined relatively to the reference gene KRT8 by subtracting target gene Cq from KRT8 Cq to obtain the dCq value. Due to the low sample number, no calculation of significant differences between group means was conducted. The results are discussed qualitatively. Mammary epithelial cells secrete cholesterol into the milk in vivo. Therefore the cholesterol efflux in pbMEC has been chosen as an example of cell functionality after isolation and culture. For the cholesterol efflux trial pbMEC from the milk of five Brown Swiss cows were cultured over seven passages with the same protocol as described above without freezing. Cholesterol efflux assays were performed in duplicates using a method previously optimised and described by Gelissen et al. (2006) with minor modifications. Briefly, cells were incubated for 48 h with [3 H]-labelled cholesterol (Moravek Biochemicals, Brea, CA) and afterwards equilibrated in serum-free medium for 18 h. For induction of cholesterol efflux, 20 μg/ml apolipoprotein AI (ApoAI, Sigma Aldrich, Munich, Germany) was added as an acceptor. Negative controls received media without ApoAl. After 6-h incubation, media were removed and cells were washed and dissolved in 0.1 M NaOH solution. Radioactivity (dpm, disintegrations per minute) was measured in the cell extract and in the media, and the rate of cholesterol efflux (percent) calculated as dpm in medium/(dpm in medium+dpm in cell extract). Data were analysed by one-way ANOVA repeated measurement for the passage comparison.

The predominant cell type in both tissue (Fig. 1*a*) and milk culture (Fig. 1*b*) was of epithelial origin as proved by specific staining against cytokeratin and showed the typical cobble-stone shape. A few fibroblasts were found in the tissue-derived pbMEC culture. The fibroblast culture (Fig. 1*c*) and negative control (Fig. 1*a, b* insert) showed no cytokeratin staining. pbMEC from milk stained positively for casein (Fig. 1*d*, insert: negative control), confirming the sustained functionality of the cells. VIM as a fibroblast marker was highly expressed in the fibroblast sample (Cq value 13.5). No expression of CSN3, KRT8, LAP, LF, LYZ1 or PRLR could be detected (data not shown). The selected genes were regarded as valid to measure epithelial cell expression. Figure 2 shows the gene expression of the passage and freezing comparison as 20—dCq for a better visualisation: higher values represent higher gene expression. The gene expression of tissue cells decreased during cultivation in all five genes while in milk cells there was no distinct trend visible. CSN3
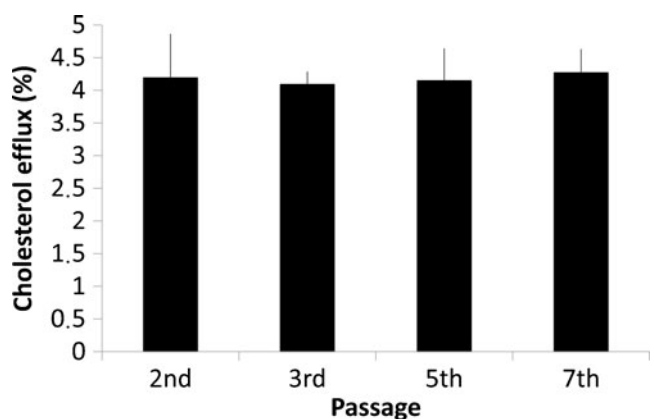

Figure 3. Effect of passage number on cholesterol efflux rate (percent) in five primary bovine mammary epithelial cell cultures isolated from milk, mean+standard deviation.

and LF gene expression was similar in both cultures, but LAP and LYZ1 were markedly higher expressed by the milk cells. The gene expression in milk-derived cells was similar or higher in the third compared to the first passage. In contrast to this, tissue-derived cells showed a decreased gene expression in the third passage. The passage number in which cells are taken for an experiment is always a compromise between a sufficient cell number and in vivo comparability. So it is understandable that gene expression is down regulated in the course of passages, as the in vivo conditions can never be mimicked perfectly and cells sense the chemical and physical surroundings via adhesion receptors (Katz and Streuli 2007). The question arises, how big is the extent of functionality loss and is it acceptable. Seeing that the milk-derived cells had similar starting levels of gene expression as the tissue cells, the origin did not seem to have much influence in a qualitative way. The sustained gene expression in the milk cells in contrast to the decreased expression in the tissue cells is a hint that the former culture is at least equally suited and might be even superior to the latter under certain circumstances. Interestingly, our cultured pbMEC cells did not lose immune defence capability in terms of expressing LAP, LYZ1 and LF, in contrast to a study of Gunther et al. (2009). In that study, pbMEC from udder tissue of two animals almost lost the ability to express LAP with or without stimulation with *E. coli* after three passages. This could be explained by the fact that LAP was qualitatively much lower expressed in our tissue-derived cells than in our milk-derived cells.

The same question of sustained functionality holds true for the decision whether to store the cells prior to use. Especially in trials with dairy cows, the samplings of animals are often at different times to ensure comparing animals at the same lactation stage. To avoid bias through different cell culture conditions, the cells are stored and reseeded together for treatment. Freezing had only a slight effect on the levels of CSN3, LYZ1 and PRLR in both cultures. This is in accordance to other studies that found that viability (Cifrian et al. 1994) and secretion ability (Talhouk et al. 1990, 1993) were not considerably influenced. LAP and LF, however, showed a greater difference in both cultures. Therefore, before setting up an experiment there is the need to test if the desired genes are still satisfactorily expressed after cryopreservation. But this need is the same for milk and tissue-derived cells.

The cholesterol efflux ability of second, third, fifth and seventh passage cultures remained intact during time, as it showed no significant variations ($p \leq 0.05$) (Fig. 3). The mean values ranged between 4.10 and 4.28% with a standard deviation between 0.19 and 0.67%. This is another confirmation for the sustained functionality of milk-derived pbMEC in culture and a hint that they could be a suitable model for studying cholesterol metabolism in vitro.

To our knowledge, this is the first time that gene expression and cholesterol efflux from cultured pbMEC was compared for tissue and milk origin. We demonstrated in this preliminary study that culturing pbMEC from milk seems to be a suitable alternative to isolating these cells from tissue. Expression levels of five target genes over three passages were similar or higher than in the cultures from tissue, indicating that the loss of function was similar or even lower in the milk cell culture. These findings are supported by the sustained cholesterol efflux over seven passages.

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

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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).

Czech J. Anim. Sci., 57, 2012 (5): 207–219

Original Paper

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, Denmark) 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
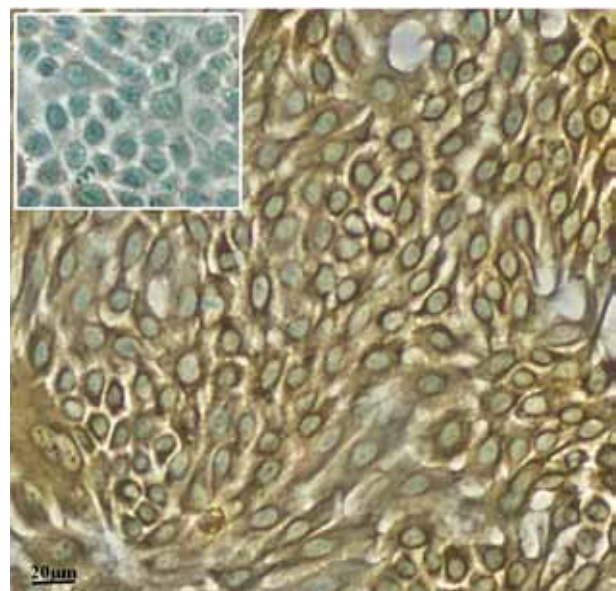

Figure 1. Immuno-histological identification of pbMEC by cytokeratine staining. Positive brown staining of cytokeratines 4, 5, 6, 8, 10, 13, and 18. The insert shows the negative control

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

Genes		Abbrevia-tion	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

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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
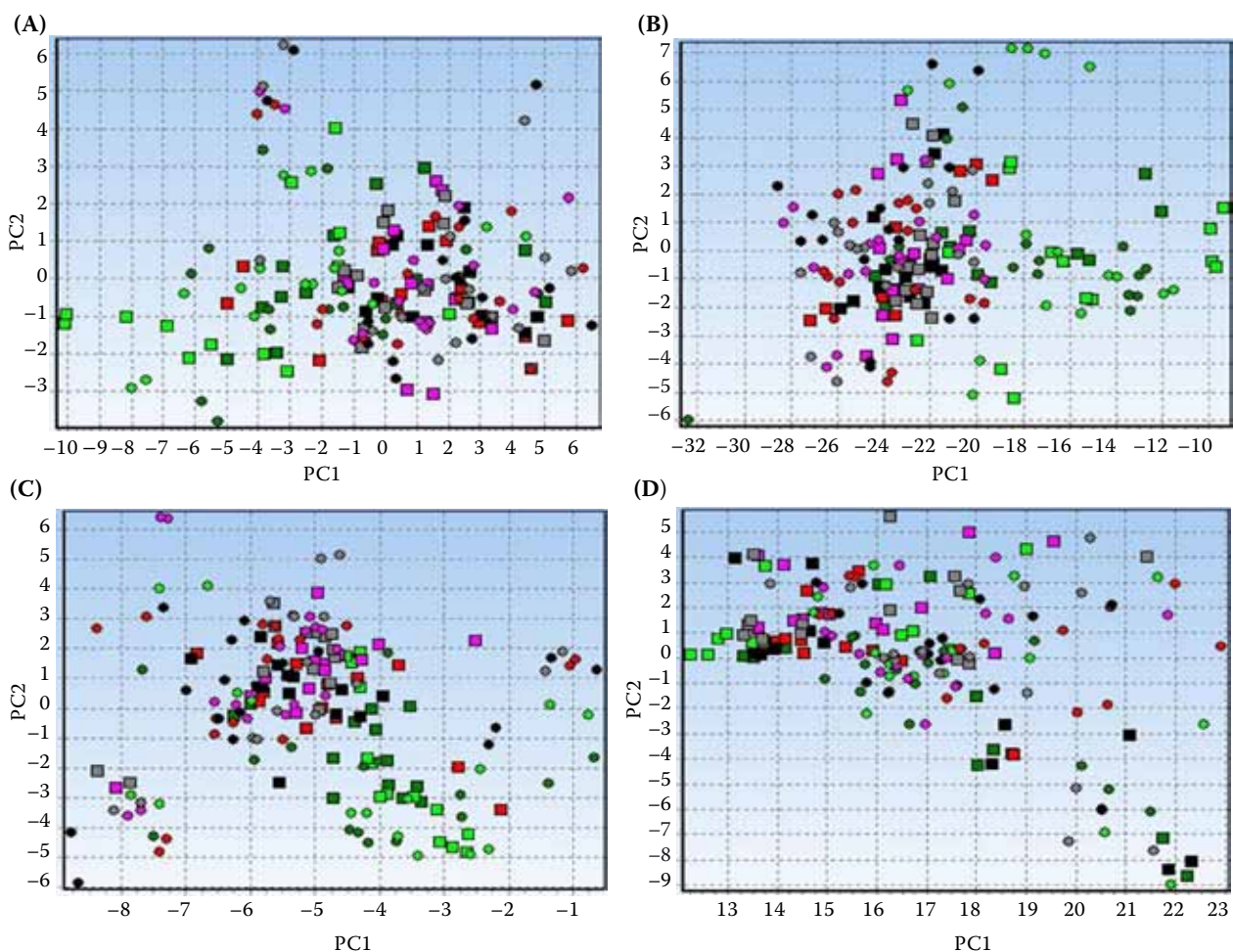
Figure 2. Principal component analysis (PCA) of four different immune functional gene groups presented on Δ*Cq* level: **A** = antimicrobial peptides and receptors (AMPR), **B** = cytokines, **C** = chemokines, **D** = apoptosis. Data sets are arranged according to feeding regime (control = square, restriction = 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)

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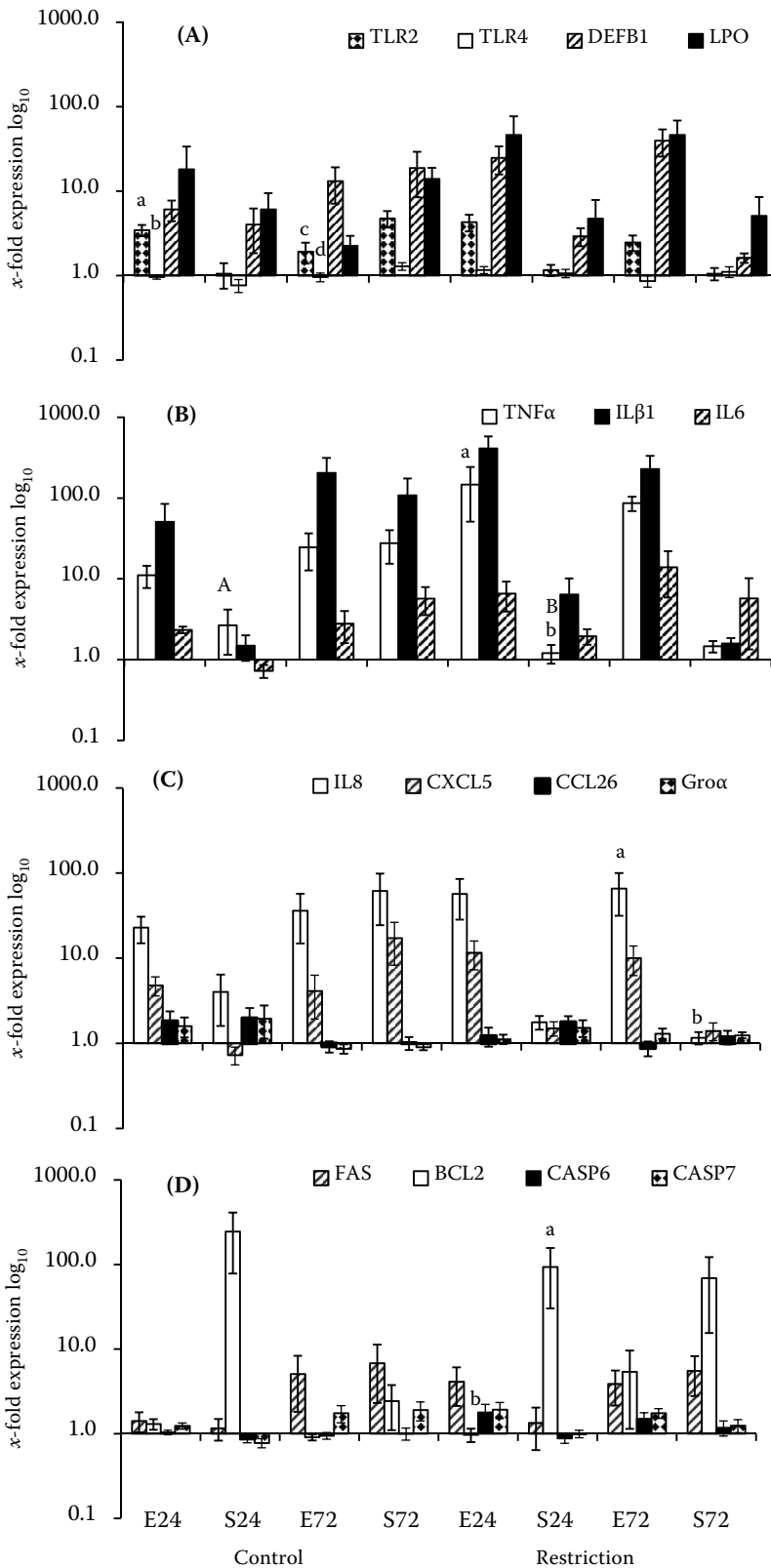
Figure 3. Relative gene expression of means presented as $2^{-\Delta\Delta Cq}$ in $\log_{10}$ scales ± SEM: **A** = antimicrobial peptides and receptors (AMPR), **B** = cytokines, **C** = chemokines, **D** = apoptosis related genes. S24, S72 = *S. aureus* infection for 24 and 72 h; E24, E72 = *E. coli* 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 ΔCq-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

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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 $51 \pm 2\%$ 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.

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animal

Microfluidic high-throughput RT-qPCR measurements of the immune response of primary bovine mammary epithelial cells cultured from milk to mastitis pathogens

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Bovine mastitis, the inflammation of the udder, is a major problem for the dairy industry and for the welfare of the animals. To better understand this disease, and to implement two special techniques for studying mammary gland immunity in vitro, *we measured the innate immune response of primary bovine mammary epithelial cells (pbMEC) from six Brown Swiss cows after stimulation with the heat-inactivated mastitis pathogens,* Escherichia coli *1303 and* Staphylococcus aureus *1027. The cells were extracted and cultivated from milk instead of udder tissue, which is usually done. The advantages of this technique are non-invasiveness and less contamination by fibroblasts. For the first time, pbMEC gene expression (GE) was measured with a microfluidic high-throughput real-time reverse transcription-quantitative PCR platform, the BioMark HD™ system from Fluidigm. In addition to the physiological analysis, the precision and suitability of this method was evaluated in a large data set. The mean coefficient of variance (± s.e.) between repeated chips was 4.3 ± 0.4% for highly expressed and 3.3 ± 0.4% for lowly expressed genes. Quantitative PCR (qPCR) replicate deviations were smaller than the cell culture replicate deviations, indicating that biological and cell culture differences could be distinguished from the background noise. Twenty-two genes (complement system, chemokines, inflammatory cytokines, antimicrobial peptides, acute phase response and toll-like receptor signalling) were differentially expressed (P < 0.05) with E. coli. The most upregulated gene was the acute phase protein serum amyloid A3 with 618-time fold.* S. aureus *slightly induced CCL5, IL10, TLR4 and S100A12 expression and failed to elicit a distinct overall innate immune response. We showed that, with this milk-derived pbMEC culture and the high-throughput qPCR technique, it is possible to obtain similar results in pbMEC expression as with conventional PCR and with satisfactory precision so that it can be applied in future GE studies in pbMEC.*

Keywords: bovine mastitis, gene expression profiling, microfluidic qPCR, primary bovine mammary epithelial cells, innate immune response

Implications

We show that a time- and cost-efficient high-throughput quantitative PCR (qPCR) system, applied on primary bovine mammary epithelial cells (pbMEC) cultured from milk, is a convenient alternative to the two major standard procedures in measuring gene expression. We obtained similar results as studies with pbMEC from udder tissue and measurements on DNA microarrays or conventional qPCR. We suggest that the milk-derived pbMEC culture and the microfluidic high-throughput qPCR system could be applied in future experiments with pbMEC.

Introduction

Bovine mastitis, the inflammation of udder tissue, is one of the most frequent and most costly diseases in dairy cows. Bacteria are by far the most common cause of mastitis. *Escherichia coli* induces predominantly acute and severe mastitis, whereas *Staphylococcus aureus* often leads to mild and chronic mastitis (Petzl *et al.*, 2008). In order to better understand this disease, primary bovine mammary epithelial cells (pbMEC) have been intensively studied *in vitro*. They synthesize

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and secrete milk, but they also have immune capacity: on recognition of pathogens via toll-like receptors (TLRs), they produce inflammatory cytokines and chemokines to attract immune cells. They also secrete antimicrobial peptides and acute phase proteins as a first defence (Rainard and Riollet, 2006). pbMECs are generally extracted from udder tissue of slaughtered cows via enzymatic digest; however, we used exfoliated cells isolated from milk. The advantages of this method are its non-invasiveness and repeatability and non-contamination by fibroblasts (Buehring, 1990). A high-throughput gene expression (GE) instrument, the BioMark HD™ real-time reverse transcription-quantitative PCR (RT-qPCR) platform (Fluidigm, San Francisco, CA, USA), was chosen to determine the relative expression of 45 genes of the innate immune response of milk-derived pbMEC after *E. coli* and *S. aureus* stimulation. Spurgeon *et al.* (2008) describe the function and the advantages of this novel technique in detail. Briefly, with the applied microfluidic technology to manipulate nanolitre scales of samples and reagents in an automated manner, it is possible to measure the expression of up to 96 genes in up to 96 samples in one run. The system has successfully been used by other researchers. Jang *et al.* (2011) measured the expression of microRNA and found that the sensitivity of the measurement increased compared with conventional singleplex RT-qPCR. They also measured higher fold changes than with an Affymetrix microarray. Furthermore, they reported that the sample and reagent consumption was 50 to 100 times lower and the throughput was 5 to 20 times higher than in conventional RT-qPCR. These attributes of the system make it especially attractive when only small amounts of sample, such as in primary cell culture, are available, and when whole pathways and functional groups of genes are screened.

Material and methods

Cell culture

Fresh milk from six healthy Brown Swiss cows in mid and late lactation was taken after cleaning and disinfecting the teat surface. Mammary epithelial cells (MECs) were extracted by centrifugation and washed with Hank's Balanced Salt Solution containing antibiotics as described in a study by Danowski *et al.* (2012). Briefly, the cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) with a nutrient mixture F-12 HAM, 10% FBS (Life Technologies, Darmstadt, Germany), ITS liquid media supplement and antibiotics (Sigma-Aldrich, Munich, Germany) at 37°C and 5% CO_2. At reaching confluence, they were split using accutase (PAA, Pasching, Austria). After the second passage, a sample was reseeded at 10 000 cells per well in a 16-well chamber slide (Nunc, Langenselbold, Germany) for immunocytochemistry (IC). The rest were resuspended in freezing medium consisting of 70% DMEM, 20% FBS and 10% DMSO, and stored in liquid nitrogen until all cultures were collected. Cells from every animal were reseeded at 30 000 cells per well in a 12-well tissue culture plate and cultivated until confluence. The mean value of three counted wells was

used to estimate the cell count in the other wells. Heat-inactivated *E. coli* 1303 and *S. aureus* 1027 (Petzl *et al.*, 2008) were added in a multiplicity of infection (MOI) of 30 colony forming units per cultured cell to ensure that every culture received the same bacterial load per cell. This MOI was chosen on review of the literature as a typical bacterial load often used in similar experiments (Gunther *et al.*, 2009; Danowski *et al.*, 2012). *E. coli* treated (6 and 30 h), *S. aureus* (30 and 78 h) and control wells (6, 30 and 78 h) were sampled in duplicates by washing the wells with phosphate buffered saline (PBS) and dissolving the cell layer in lysis buffer of the AllPrep RNA/Protein Kit (Qiagen, Hilden, Germany).

IC

IC was conducted with the method described in a study by Danowski *et al.* (2012). Briefly, after fixation of the chamber slides in methanol : acetone (1 : 1), washing, blocking of endogenous peroxidases in 1% H_2O_2 and reduction of background staining with goat serum (DAKO, Glostrup, Denmark), monoclonal mouse anti-cytokeratin pan antibody clone C-11 (1 : 400 in PBS-Tween, Sigma-Aldrich) was incubated over night at 4°C. After washing, horse radish peroxidase (HRP)-labelled goat anti-mouse-immunoglobulin (1 : 400 in PBS-Tween, DAKO) was incubated for 1 h at room temperature. HRP was visualized with diaminobenzidine and 0.01% H_2O_2. Nuclei were stained with Haemalaun after Mayer (Roth, Karlsruhe, Germany).

RNA extraction and reverse transcription

Total RNA and cell protein was extracted with the AllPrep RNA/Protein Kit (Qiagen) according to the manufacturer's protocol with an additional DNase treatment (RNAse-free DNase set, Qiagen). RNA concentration was measured with the Nanodrop ND-1000 spectrophotometer (Peqlab, Erlangen, Germany). RNA quality was analysed with RNA 6000 nano chips and kit on the 2100 Bioanalyzer (Agilent, Böblingen, Germany) and then stored at −80°C. For reverse transcription, 100 ng RNA and a master mix prepared from 5× buffer and 100 U M-MLV H(−) reverse transcriptase (Promega, Mannheim, Germany), 0.5 mM dNTPs and 0.5 μM Oligo-d(T) primer (Fermentas, St. Leon-Rot, Germany) and 2.5 μM random hexamer primers (Invitrogen Life Technologies, Darmstadt, Germany) were used in a total volume of 30 μl. A pooled RNA sample of all samples from each RNA extraction run was transcribed to cDNA with the same reaction mix without reverse transcriptase and included in the quantitative PCR (qPCR) measurements to check for contamination by genomic DNA. The incubation programme consisted of an annealing phase at 21°C for 10 min, transcription phase at 48°C for 50 min and degrading phase at 90°C for 2 min. cDNA was stored at −20°C.

PCR primer pairs

The mRNA sequences of the studied genes were taken from the National Center for Biotechnology Information (NCBI) Gene database (NCBI, National Library of Medicine, Bethesda, MD, USA). Primer pair oligos (Metabion, Martinsried, Germany)

were designed using HUSAR (DKFZ, German Cancer Research Center, Heidelberg, Germany) or PrimerBLAST (NCBI). Specificity of primer pairs was checked via melting curve analysis and gel electrophoresis of the amplified product (data not shown). PCR efficiencies of the assays were determined with a 5-point dilution series of two representative samples from the experiment and untreated bovine spleen tissue cDNA in qPCR triplicates with the calculation described in Bustin *et al.* (2009). Primer sequences and gene names are shown in Supplementary Table S1. The analysis was performed on a relative quantification of mRNA expression in treated samples *v.* control samples for each target gene separately.

RT-qPCR

4 µl cDNA was preamplified with the thermal protocol: 95°C for 3 min followed by 18 cycles of 95°C for 20 s, 55°C for 3 min and 72°C for 20 s. The reaction volume was 20 µl containing the iQ Supermix (Bio-Rad, Munich, Germany) and 25 nM of each primer pair. Preamplified cDNA was subsequently diluted with water 1 : 9. qPCR was conducted on the BioMark™ HD system. PCR efficiencies of the assays were measured on a gene expression (GE) Dynamic Array 48.48 chip (Fluidigm). The 84 preamplified cDNA samples from the stimulation experiment were measured together with 213 other preamplified cDNA samples, no reverse transcriptase (NoRT) control and no template control (NTC) from cultured pbMEC on four GE Dynamic Array 96.96 chips (Fluidigm). One 6 h *E. coli* treated pbMEC sample was measured repeatedly on all four 96.96 chips and used as between-chip calibrator. It was chosen as a representative and stable sample that expressed all genes of interest to provide similar reaction conditions and expression levels as in the other samples. 5 µl sample premix consisting of 2.5 µl SsoFast EvaGreen Supermix (Bio-Rad), 0.25 µl Sample loading reagent (Fluidigm), 0.1 µl ROX (diluted 1 : 3, Invitrogen), 1.25 µl preamplified and 1 : 9 diluted cDNA and water, as well as 5 µl assay premix consisting of 2 µl 10 µM primer pairs in the final concentration of 4 µM, 2.5 µl Assay loading reagent (Fluidigm) and water were prepared and transferred to the primed GE Dynamic Array 96.96. The samples and assays were mixed inside the chip using the Nanoflex IFC controller (Fluidigm). The final concentration of primers in the individual reaction was 400 nM. Thermocycling parameters included an initial phase of 98°C for 40 s followed by 40 cycles, consisting of 95°C for 10 s and 60°C for 40 s. After completion of the run, a melting curve of the amplified products was determined. Data were collected using Bio-Mark Data Collection Software 2.1.1. built 20090519.0926 (Fluidigm) as the cycle of quantification (Cq), where the fluorescence signal of the amplified DNA intersected with the background noise.

Data preprocessing and analysis

Fluidigm Melting Curve Analysis Software 1.1.0. (build 20100514.1234, Fluidigm) and Real-time PCR Analysis Software 2.1.1. (build 20090521.1135 (Fluidigm)) were used to determine the valid PCR reactions. Invalid reactions were not used for later analyses and treated as missing data. Raw Cq values were processed with Genex 5.3.2 (MultiD Analyses AB, Gothenburg, Sweden) using between-chip calibration and reference gene normalization. Six putative reference genes had been identified after review of the available literature. Stability of their expression was evaluated with the Normfinder tool in Genex 5.3.2 (MultiD Analyses AB). For the gene regulation analysis, the cut-off was set to 25 and higher values were replaced with 25. Cq values >30 were regarded as invalid and treated as missing data. The subtraction of reference gene Cq mean from target gene Cq value yielded the ΔCq value. Genex 5.3.2 was used for principal component analysis (PCA) on the auto-scaled ΔCq values. Distribution of within-chip deviation of the BioMark™ HD chips was calculated with gnuplot 4.4.0 (Sourceforge.net, Geeknet Inc., Fairfax, VA, USA). Genes with an overall Cq mean below 20 were termed as 'high expression', above 20 as 'low expression' genes for the within-chip and between-chip deviation analysis. Statistics and charts were produced with SigmaPlot 11 (SYSTAT, Chicago, IL, USA) or SPSS Statistics Standard 19.0 (IBM, Armonk, NY, USA). Genes were observed as differentially expressed for $P < 0.05$ in a paired *t*-test on ΔCq of control and treated samples at each time point. The fold change in expression was calculated with the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen, 2001) for every sample and then expressed as the mean of all these fold changes. It must be noted that no correction for multiple testing was imposed on the *P*-values, although we are aware that this increases the risk of false positive significances. This study is of explorative and descriptive character only, not of a diagnostic one. Such a correction would have been too stringent and masked many of the differences that we found between treatment and control. RT-qPCR was conducted following the MIQE (minimum information for publication of quantitative real-time PCR experiments) guidelines (Bustin *et al.*, 2009).

Results

Cell culture and IC

The extracted cells attached after 24 h and proliferated after a few days. A mean total cell count of 6.5 million cells per culture with a range between 1 and 19 million cells per culture was harvested after the second passage and stored in liquid nitrogen. The cells showed the typical cobblestone-like monolayer in cell culture with varying cell sizes (Supplementary Figure S2 (a)). The purity of all the cultures was estimated at nearly 100%. Thorough visual inspection with light microscopy detected 0% unstained nucleated cells after immunocytochemical staining against cytokeratins, whereas in the negative controls there were 0% stained cells visible (Supplementary Figure S2 (b)).

RT-qPCR

Supplementary Figure S3 shows the quantile–quantile (Q–Q) plot of the Cq values from chips one to three exemplarily to depict the correspondence of Cq variation between these chips.

The fourth chip contained only few samples from this experiment. The rest consisted of NTCs, negative RT controls and RT calibration samples. Those were not comparable with the samples of the other three chips in terms of Cq value range. With the remaining comparable samples, it was not possible to draw a valid Q–Q plot *v.* the other chips. The Cq values from two chips were each ranked according to their value in ascending order. Beginning with the lowest two values from both chips, they were paired to form coordinates of points. The resulting curve was a straight ascending diagonal line that showed that the ranked Cq values increased in the same rate up to cycle 25. From there the Cq values started to increase in inconsistent intervals, indicated by the bends in the curves. Supplementary Figure S4 shows the distributions of the Cq

differences of qPCR replicates (qPCR) and the differences of the mean Cq values of cell culture replicates over all chips (within-chip deviation). The qPCR replicates of 25 high and 20 low expression genes had 83% and 59% of the values in the lowest deviation range between 0 and 0.5 cycles, respectively. The cell culture replicates for high and low expression genes had 49% and 33% of the values in that range, respectively.

The mean coefficient of variation (CV; $\pm$ s.e.) of the calibration sample over the four chips was $4.3 \pm 0.4\%$ for the high expression genes and $3.3 \pm 0.4\%$ for the low expression genes after Cq values over 30 had been cut-off and Cq values over 25 had been set to 25. A visualization of the Cq values is shown in Supplementary Figure S5.

Immune response of the pbMEC
With the Normfinder tool within Genex 5.3.2 (MultID) *ACGT1*, *KRT8* and *H3F3A* were identified as stably expressed over all samples and all conditions and thus being suitable reference genes. They were used for normalization of the target gene Cq values, resulting in the ΔCq value. Of the 39 target genes, 28 were successfully quantified. *C1QA*, *C3aR1*, *C5aR1*, *CASP1*, *CD163*, *IL1B*, *HP*, *IRF3*, *NLRP1*, *NRLP3* and *RELA* were found to have too many invalid PCR reactions to be subjected to processing. Differentially expressed genes ($P < 0.05$) between treatment and control are shown in Tables 1 and 2. Twenty-two genes were differentially expressed with the *E. coli* stimulation, but only four with the *S. aureus* stimulation. *E. coli* strongly activated complement component 3 (*C3*), chemokines and inflammatory cytokines after 6 and 30 h, as well as antimicrobial peptides after 30 h. The two myxovirus resistance genes (myxovirus (influenza virus) resistance 1, interferon inducible protein p78 (mouse) (*MX1*), myxovirus (influenza virus) resistance 2 (mouse) (*MX2*)) and the two S100 calcium-binding genes (S100 calcium-binding protein A9 (*S100A9*), S100 calcium-binding protein A12 (*S100A12*)) were also similarly upregulated after 30 h *E. coli*. The most induced gene was serum amyloid A3

Table 1 *Differentially expressed genes (*P < 0.05*) between treatment and control in pbMECs from six Brown Swiss cows after 6 and 30 h stimulation with heat-inactivated* Escherichia coli *1303*

	x-time fold change[1]			
	E. coli 6 h		*E. coli* 30 h	
	Mean	s.e.	Mean	s.e.
Complement system				
C3	4.4	0.9	8.6	1.8
Chemokines				
CCL2	28.8	12.3	43.9	13.1
CCL5	ns[2]	ns	51.6	33.3
CCL20	39.4	17.1	50.2	11.1
CXCL5	8.1	2.1	7.8	1.3
CXCL8	20.1	7.7	21.9	7.5
Inflammatory cytokines				
IL6	3.6	1.1	5.3	1.2
IL10	18.8	11.0	53.1	35.1
TNF	21.9	7.0	21.5	4.1
Antimicrobial peptides				
LAP	2.8	0.6	19.5	8.0
LF	3.2	0.6	8.6	2.3
LPO	ns	ns	4.2	1.2
LYZ1	ns	ns	40.2	27.6
TAP	54.8	32.8	105.7	57.9
Acute phase response				
SAA3	ns	ns	618.2	272.4
TLR pathway				
CASP8	1.3	0.1	1.4	0.1
CD14	ns	ns	1.8	0.3
TLR2	ns	ns	5.0	2.1
Others				
MX1	ns	ns	4.4	1.4
MX2	11.6	7.6	8.6	2.3
S100A9	3.9	0.8	12.0	4.1
S100A12	ns	ns	4.2	0.9

pbMEC = primary bovine mammary epithelial cells; CCL = (C-C motif) ligand; CXCL = (C-X-C motif) ligand; IL = interleukin; TNF = tumour necrosis factor; LAP = lingual antimicrobial peptide; LF = lactoferrin; LPO = lactoperoxidase; LYZ = lysozyme; TAP = tracheal antimicrobial peptide; SAA3 = serum amyloid A3; TLR = toll-like receptor; CASP8 = caspase 8.
[1]Calculated with the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen, 2001).
[2]Not significant ($P > 0.05$).

Table 2 *Differentially expressed genes between treatment and control in pbMECs from six Brown Swiss cows after 30 and 78 h stimulation with heat-inactivated* Staphylococcus aureus *1027*

	x-time fold change[1]	
S. aureus	Mean	s.e.
30 h		
TLR pathway		
TLR4	1.3	0.2
78 h		
Chemokines		
CCL5	1.5	0.2
Inflammatory cytokines		
IL10	1.5	0.2
Others		
S100A12	1.3	0.1

TLR = Toll-like receptor; *CCL5* = (C-C motif) ligand 5; *IL10* = interleukin 10.
[1]Calculated with the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen, 2001).

(*SAA3*) with 618-time fold after 30 h exposure to *E. coli*. With *S. aureus*, the most induced genes were the chemokine (C-C motif) ligand 5 (*CCL5*) and the anti-inflammatory cytokine interleukin 10 (*IL10*) after 78 h.

The PCA on the relative expression of the target genes is shown in Supplementary Figure S6. *E. coli* samples form a distinct subgroup only slightly overlapping with the other samples. No separation between *S. aureus* and control samples is visible.

Discussion

Precision of the BioMark^TM HD system
The Q–Q plot (Supplementary Figure S3) shows that the correspondence of the distribution of Cq values between the chips was very good, as the points formed almost the ideal diagonal line. However, values over 25 were not evenly distributed and deviated from the line. This is a confirmation of the need to set a cut-off at 25 when processing the data from this system. The within-chip deviations of the qPCR replicates were found to be smaller than those of the cell culture replicates. This is very important as small biological differences could be masked by the noise in measurement and may not be detected with this method. It is understandable that the replicate deviations were higher in low expression genes. In diluted nucleic acid samples with low target concentrations, the Poisson distribution occurs as a natural effect. It predicts large variations in target quantities in aliquots from the same sample (Rutledge and Stewart, 2010). This should be kept in mind when deciding how many qPCR replicates of an assay are to be carried out. It is recommended to run more replicates for genes that are known to be less expressed to cover this variation and increase the precision of the measurement. The mean between-chip CV was acceptable. The CV of the low expression genes was surprisingly lower than the CV of the high expression genes. However, this must be interpreted with caution, as it is likely that the low expression gene CV does not reflect the true variability of the data because of the cut-off at Cq 30. It only reflects the variability of the remaining data after preprocessing and cut-off.

General considerations
So far the expression of the immune response in pbMEC has either been measured by conventional RT-qPCR or on DNA microarrays. To our knowledge, this is the first time that a high-throughput RT-qPCR technique was applied to study a large set of genes in pbMEC cultured from milk. So far large sets of GE data are only available from pbMEC extracted from udder tissue in microarray studies (Gunther *et al.*, 2009). Gunther *et al.* (2009) also reported that the immune response to *E. coli* was much faster and stronger than to *S. aureus*; however, the authors were still able to identify several significantly upregulated genes by *S. aureus*. Generally, they identified higher fold changes in the regulated genes, but this could be because of the microarray technique or to different cell culture conditions. In their study, *SAA3* was also

the most up regulated gene by *E. coli* (Gunther *et al.* (2009)), followed by the chemokine *CCL5*, lingual antimicrobial peptide (*LAP*) and *MX2* (Gunther *et al.*, 2009), which were also highly upregulated in our study. Our cells proved to be able to express a similar set of inflammatory cytokines (*IL6*, *IL10* and tumour necrosis factor (*TNF*)) and chemokines (chemokine *CCL2*, *CCL5*, chemokine *CCL20*, chemokine (C-X-C motif) ligand 5 (*CXCL5*), and chemokine *CXCL8*) compared with the study by Gunther *et al.* (2009). Lutzow *et al.* (2008) measured the intra-mammary immune response of dairy cows to *S. aureus in vivo* and found upregulated inflammatory cytokines and chemokines, as well as defence proteins. Both were measured on a DNA microarray and validated with RT-qPCR. However, two important innate immune genes, *TNFα* and *CD14*, were identified as differentially expressed by the RT-qPCR, but not on the microarray. Swanson *et al.* (2009) infected heifers with *Streptococcus uberis* and measured the transcriptional changes in the mammary tissue on a DNA microarray. Of the regulated genes, they validated 11 innate immune genes with RT-qPCR. Three of these showed a different direction of regulation or no regulation in the validation. These findings underline the need to carefully interpret microarray results and validate them with qPCR.

Pathogen differences
The remarkable pathogen differences in immune response have been noted before (Griesbeck-Zilch *et al.*, 2008; Petzl *et al.*, 2008). However, the total failure of *S. aureus* to stimulate the innate immune defence in our study is remarkable. A direct comparison can be made with the study by Danowski *et al.* (2012) where milk-derived pbMECs were stimulated with the same strains of pathogens as in our study. There the PCA showed no distinct separation of *S. aureus* samples from *E. coli* and control, similar to our PCA. Therefore, it can be assumed that the weak *S. aureus* effect in our study is a reproducible physiological effect. Possibly, the dose of inoculation was insufficient. It could have been too low, taking into account that there is a dose-dependent immune response of pbMEC to lipopolysaccharide (LPS) and *S. aureus* (Wellnitz and Kerr, 2004) and a study by Swanson *et al.* (2009) with pbMEC from tissue showed an upregulation in four of nine measured immune genes to *S. aureus* with a much higher MOI of 1000. Another possibility is that we missed the proper time frame of the immune response: one study showed an early immune response of MECs to *S. aureus* that decreased to resting levels after 8 to 16 h (Strandberg *et al.*, 2005). Our bacteria had been isolated from a clinical case of mastitis and were shown to have elicited weak but measurable symptoms of mastitis when administered *in vivo* intra-mammary (Petzl *et al.*, 2008); thus, the question remains whether this strain exhibits sufficient virulence only in a live, but not in a heat-inactivated form. The fact that udder infections with *S. aureus* often remain subclinical and become chronic could be explained by this lack of a strong immune response of the MECs.

Pathogen recognition

E. coli should be recognized by TLR4 and its cofactor CD14, which binds to conserved LPS patterns of gram-negative bacteria (Lu *et al.*, 2008). Of this complex, only *CD14* has been regulated by *E. coli* in our study. In contradiction to the statement that MECs do not express *CD14* (Rainard and Riollet, 2006), we were able to measure an expressed and weakly regulated *CD14*. Apoptosis inducer caspase 8 (*CASP8*) is activated by the gram-positive bacteria recognizing *TLR2* (Aliprantis *et al.*, 2000). *CASP8* and *TLR2* were upregulated by *E. coli*. It has been shown in human cells that *TLR2* is able to respond to gram-negative bacteria when expressed in combination with the cofactor lymphocyte antigen 96 (*LY96*, also called *MD2*; Dziarski *et al.*, 2001). This cofactor was expressed but not regulated by *E. coli* in our cells, as well as other members of the pathway (LPS-binding protein (*LBP*), *TLR4* and myeloid differentiation primary response gene 88 (*MYD88*)). *TLR4*, on the other hand, was weakly upregulated by *S. aureus*. Although based on mRNA expression only, nothing can be said about the actual interaction of the pathogen components with the TLRs; both *TLR2* and *TLR4* mRNAs were present in all control and treated samples, and changes in expressions of *TLR2* and *TLR4* were pathogen specific in our study. The whole TLR signalling pathway here seemed to be less influenced than in other studies. It seems that it is not necessary to strongly upregulate the TLR pathway components for an efficient immune response. Strandberg *et al.* (2005) found a similar weak TLR activation in bovine MECs upon LPS stimulation and still came to the conclusion that a functioning and locally effective immune system was present.

Inflammatory cytokines and chemokines

These signalling and modulating molecules were highly influenced by *E. coli*. This is consistent with most other studies mentioned already and confirming that pbMECs exert a major sentinel function to trigger the immune response. However, in contrast to another study (Lahouassa *et al.*, 2007), we detected a modulation of the immune response by upregulation of the anti-inflammatory cytokine *IL10*, suggesting an instant self-regulation to avoid damage to the tissue.

Antimicrobial peptides and acute phase response

LAP and tracheal antimicrobial peptide (*TAP*) belong to the β-defensins, and together with lactoperoxidase (*LPO*), lysozyme (*LYZ1*) and lactoferrin (*LF*) they are antimicrobial peptides, able to inhibit and damage bacteria directly. All five studied antimicrobial peptides were differentially expressed after *E. coli* stimulation, with *TAP* being by far the most influenced one. This is a confirmation of the findings of Lopez-Meza *et al.* (2009) that MECs are the source for *TAP* found in the udder and in milk. Not many data are available for antimicrobial peptide expression of cultured pbMEC. Although it has been reported that repeated subcultivation of pbMECs lowered their ability to express *LAP* with and without stimulation (Gunther *et al.*, 2009), all of our three passage cultures expressed *LAP* and responded to the stimulation.

The acute phase gene *SAA3* was the most upregulated one, same as in the study by Gunther *et al.* (2009). The antibacterial SAA protein is an opsonin for gram-negative bacteria (Shah *et al.*, 2006), and because of its massive increase during mastitis it has been suggested as a biomarker for this disease (Larsen *et al.*, 2010).

Complement system

The known lack of the classical pathway of the complement system in the mammary gland (Rainard and Riollet, 2006) was confirmed by the absence of complement component 1, q subcomponent, A chain (*C1QA*) expression. However, *C3* expression was induced showing that the alternative pathway was functional in our cells. C3 can opsonize bacteria and makes them available for phagocytosis, and it regulates the inflammatory response (Rainard and Riollet, 2006). In another study, it was also upregulated by *E. coli* and *S. aureus* in pbMEC (Griesbeck-Zilch *et al.*, 2008). These findings suggest that C3 in milk is at least partially synthesized by the epithelial cells and not just transported through the blood–udder barrier. Complement component 5a receptor 1 (*C5AR1*) and complement component 3a receptor 1 (*C3AR1*) were both found expressed in a part of the samples, and no statistical evaluation was done because of the missing data (data not shown). The expression of *C5AR1* in epithelial cells has been discussed controversially; however, one study found C5aR protein expression in the bovine MEC line MAC-T in a subpopulation of 10% to 12% of the cells (Nemali *et al.*, 2008). *C5AR1* encodes for the receptor of complement component 5a (C5a), which is mainly present on granulocytes, macrophages and some lymphocytes. C5a leads to cellular responses of the cells such as chemotaxis, phagocytosis and enzyme release (Rainard and Riollet, 2006). However, this author also mentions the stimulation of cytokine synthesis by C5a. This could be one possible function of pbMEC when recognizing C5a via the C5a receptor.

Others

S100A9 and *S100A12* encode for calgranulins, which are a group of mediator molecules with calcium-binding, pro-inflammatory, regulatory, anti-oxidant and protective properties. The S100-A12 protein has been shown to inhibit *E. coli* growth *in vitro* (Lutzow *et al.*, 2008). S100 genes are known to be upregulated in infected udder tissue and pbMEC (Gunther *et al.*, 2009). As in the pbMEC study by Gunther *et al.* (2009), *MX2* and *MX1* were induced by the *E. coli* treatment. MX proteins belong to the large GTPases family and have different antiviral capacities; their expression is stimulated by interferon and virus recognition (Lee and Vidal, 2002). Their potential effect on mastitis remains to be subject of further research.

Conclusions

For the first time, a high-throughput microfluidic RT-qPCR platform was applied to study a large set of genes in pbMEC cultured from milk. The sensitivity of the measurement was

found to be satisfactory for our purposes. We found it to be less time, sample, reagent and cost consuming than the conventional RT-qPCR, and unlike DNA microarrays, it does not require additional validation via conventional qPCR. With this technique and with cells cultivated from milk instead of tissue, we obtained similar results as other studies about the immune system in pbMEC. This confirms that our results are comparable with the results from conventional qPCR and tissue cultured pbMEC. With conventional qPCR, usually there are only few genes measured in each experiment. Therefore, it is necessary to assemble many different studies with different experimental conditions to achieve an overview of the immune response. We showed that, with microfluidic qPCR, it is now possible to do this in one experiment. The same holds true to other functions of these cells; cholesterol, fatty acid and milk protein metabolism are also important fields of study and could be screened in exactly the same way.

It is a subject of further research to analyse why *S. aureus* often fails to elicit a distinct immune response and what genes are exactly involved if there is a response. For that, microfluidic qPCR could be applied to screen a larger set of immune genes by omitting PCR replicates. The activation of antimicrobial peptides, the acute phase gene *SAA3*, *S100A12* and *S100A9* confirmed the diversified defence capability of pbMEC against *E. coli*. On the other hand, our pbMECs proved to be able to act as sentinel cells by expressing chemokines and inflammatory cytokines for the attraction and activation of immune cells. They were also able to express the anti-inflammatory gene *IL10* to modulate the immune response. However, many details and interactions of the immune response are still unclear and we suggest that this experimental set-up could be applied for further studies. Different pathogens and additional genes could be tested to broaden the picture as well as make it more detailed.

Acknowledgements

Supplementary materials

For supplementary materials referred to in this article, please visit http://dx.doi.org/10.1017/S1751731112002315

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Mammary immunity of White Park and Highland cattle compared with Brown Swiss and Red Holstein

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Summary

Mastitis is a frequent disease in modern dairy cows, but ancient cattle breeds seem to be naturally more resistant to it. Primary bovine mammary epithelial cells from the ancient Highland and White Park ($n = 5$) cattle and the modern dairy breeds Brown Swiss and Red Holstein ($n = 6$) were non-invasively isolated from milk, cultured, and stimulated with the heat-inactivated mastitis pathogens *Escherichia coli* and *Staphylococcus aureus* to compare the innate immune response *in vitro*. With reverse transcription quantitative polymerase chain reaction (RT-qPCR), the breeds differed in the basal expression of 16 genes. Notably CASP8, CXCL8, Toll-like receptors 2 and 4 (TLR2 and TLR4) expression were higher in the ancient breeds ($P < 0.05$). In the modern breeds, more genes were regulated after stimulation. Breed differences ($P < 0.05$) were detected in C3, CASP8, CCL2, CD14, LY96 and transforming growth factor β1 (TGFβ1) regulation. Principal component analysis separated the ancient from the modern breeds in their basal expression, but not after stimulation. ELISA of lactoferrin and serum amyloid A protein revealed breed differences in control and *S. aureus* treated levels. The immune reaction of ancient breeds seemed less intensive because of a higher basal expression, which has been shown before to be beneficial for the animal. For the first time, the innate immune response of these ancient breeds was studied. Previous evidence of breed and animal variation in innate immunity was confirmed.

Keywords: *breed comparison, primary bovine mammary epithelial cells, innate immune response, ancient and modern cattle breeds, mastitis*

Résumé

La mastite est une maladie fréquente chez les vaches laitières modernes. Or, les races bovines anciennes semblent être naturellement plus résistantes. Dans le présent travail, des cellules primaires bovines épithéliales mammaires des races anciennes *Highland* et *White Park* ($n = 5$), ainsi que des races laitières modernes *Brown Swiss* et *Red Holstein* ($n = 6$) ont été isolées du lait de façon non-invasive. Ensuite, elles ont été cultivées, puis stimulées avec les pathogènes de la mastite *Escherichia coli* et *Staphylocoque doré* – tous les deux préalablement inactivés par la chaleur – pour ainsi comparer la réponse immunitaire innée *in vitro*, utilisant la technique reverse transcription quantitative polymerase chain reaction (RT-qPCR). Il s'avère que les races diffèrent dans l'expression basale de 16 gènes. Notamment, les expressions de CASP8, CXCL8, TLR2 et TLR4 étaient élevées dans les races anciennes ($P < 0.05$). Dans les races modernes, c'est le nombre global des gènes régulés après stimulation qui était plus élevé. Des différences entre les races ($P < 0.05$) ont été détectées quant à la régulation de C3, CASP8, CCL2, CD14, LY96 et TGFβ1. L'analyse des composantes principales a permis de cloisonner les races anciennes des races modernes dans l'expression basale, mais pas après stimulation. Les mesures ELISA de lactoferrin et de sérum amyloïde A protéine ont dévoilé des différences interraciales entre le groupe du contrôle et du groupe *Staphylocoque doré*. Dans son ensemble, la réaction immunitaire de races anciennes apparaissait moins intensive en fonction d'une expression basale plus grande. Une telle atténuation avait préalablement été décrite comme étant bénéfique pour l'animal. Pour la première fois la réponse immunitaire innée de ces races anciennes a été étudiée ici. De précédentes preuves de la variation interraciale, ainsi qu'inter-animale, ont pu être confirmées par le présent travail.

Mots-clés: *comparaison de races, cellules primaires épithéliales mammaires bovines, réponse immunitaire innée, races bovines anciennes et modernes, mastite*

Resumen

La mastitis es una enfermedad de gran incidencia en ganado bovino moderno destinado a producción lechera. Sin embargo, razas más ancestrales y hoy en día casi en desuso parecen poseer una mayor resistencia natural a esta enfermedad. En el presente estudio se establecieron cultivos celulares de celulas mamarias provenientes de las razas ancestrales Highland y White Park ($n = 5$) y de las razas modernas Brown Swiss y Red Holstein ($n = 6$), para después ser infectados con los patógenos *Escherichia coli* y *Staphylococcus aureus*. Mediante reverse transcription quantitative polymerase chain reaction (RT-qPCR) se pudo determinar que la expresión basal de 16

genes era diferente en las distintas razas. Los genes CASP8, CXCL8, TLR2 y TLR4 demonstran una mayor expresión en las razas ancestrales ($P < 0.05$). Un mayor número de genes sufría una estimulación de su expresión tras la infección con los patógenos en las razas modernas. Asi mismo fueron encontradas diferencias significativas ($P < 0.05$) entre razas en la regulación de C3, CASP8, CCL2, CD14, LY96 y TGFβ1. La concentración de las proteínas lactoferrina y serum amyloid A también es diferente en las distintas razas en células control y tratadas con *Staphylococcus aureus*. La reacción inmune tras infección fue generalmente menos intensa en células provenientes de razas ancestrales, posiblemente debido a una mayor expresión basal en estas razas, un hecho que ha sido demostrado beneficioso para el animal en trabajos previos. En resumen, los datos de este trabajo confirman la hipótesis previa de una mayor inmunidad innata en razas bovinas ancestrales en comparación con las razas modernas empleadas hoy en día.

Introduction

Inflammation of the udder, or mastitis, causes major financial losses for farmers and diminishes the welfare of the animals. Gram-negative bacteria such as the environment-associated *Escherichia coli* mostly induce acute mastitis that can be mild or severe with grave systemic clinical symptoms (Burvenich *et al.*, 2003). In contrast with that the animal-associated Gram-positive *Staphylococcus aureus* often leads to subclinical and chronic infections with no or only mild symptoms (Riollet, Rainard and Poutrel, 2001). To better understand the disease process, primary bovine mammary epithelial cells (pbMEC) can be studied *in vitro*. Besides producing milk, these cells possess immune functions. Upon pathogen recognition via Toll-like receptors (TLRs) they secrete chemokines and inflammatory cytokines to attract immune cells and trigger the adaptive immune response. At the same time they also produce antimicrobial peptides and acute phase proteins to combat the pathogen directly (Rainard and Riollet, 2006). Modern dairy breeds are potentially more affected by mastitis than ancient breeds owing to intensive selection of milk production traits that have a negative genetic correlation with mastitis resistance (Strandberg and Shook, 1989). Observations from cattle farmers report that ancient cattle breeds that have never been selected for high milk yield seem to be naturally more resistant or tolerant to mastitis. This could be caused by different environmental and management conditions, but it could also be partly based on different genetics. However, prediction of traits by genetic values is only accurate if there are few large loci responsible for the trait rather than many small loci (Hayes *et al.*, 2010). Regarding the large number of so far identified candidate genes for mastitis traits (Ogorevc *et al.*, 2009) the latter can be assumed in the case of mastitis resistance. In addition, conventional estimation of breeding values showed that heritability of mastitis resistance is generally low (Heringstad, Klemetsdal and Steine, 2003). It is difficult to find genetic markers for phenotypic resistance when only the genomic architecture but not the resulting functional outcome is studied. That is why we looked at the functional phenotype of the innate immune system in pbMEC of two ancient and two modern cattle breeds. The Brown Swiss (BS) is one of the modern dairy breeds that are commonly used in Germany with 180 000 milk-controlled cows listed in Germany and an average milk yield of 6 800 kg/year (European Brown Swiss Federation, 2012). The Red Holstein (RH) cow is the red-allele carrying variant of the Holstein breed. It has been bred for high production traits for decades. Holstein is superior to most other dairy cattle breeds worldwide in terms of production and it is the most important dairy breed in Germany with 240 000 recorded RH and 2 million recorded Holstein cows that have an average milk yield of 8 245 and 9 008 kg/year (German Holstein Association, 2010). The British White Park (WP) cattle (Figure 1a) has been extensively described (Alderson, 1997) and is thought to be the oldest European cattle breed. Its descriptions as a sacred animal dates back to the pre-Christian Irish epics in the first century AD. It is hardy, robust and kept in extensive low-input grazing systems or parks for beef production (Alderson, 1997). Data from 11 male and 33 female WP cattle were available in Germany in 2009 (Biedermann *et al.*, 2009) and the breed has been considered as endangered-maintained in the UK, their country of origin (FAO, 2000). In Germany, the largest herd is kept in the Arche Warder, a zoological park for ancient domestic animal breeds (Biedermann *et al.*, 2009). The robust and hardy highland cattle (HLD) (Figure 1b) were originally bred in Scotland hundreds of years ago (Dohner, 2001). It was primarily used in extensive hill or mountain grazing systems for beef production, but was also used to some extent for dairy production (Dohner, 2001). With the herd book established in 1885, it is one of the oldest registered cattle breeds (Mason, 2002). Recent livestock numbers in Germany were 2 785 female and 385 male animals in 2010 (BLE, 2012). Our goal was to investigate possible phenotypic breed differences in the innate immune response against mastitis. Therefore, we cultivated pbMEC out of milk from these four breeds and stimulated them with the two major mastitis pathogens *E. coli* and
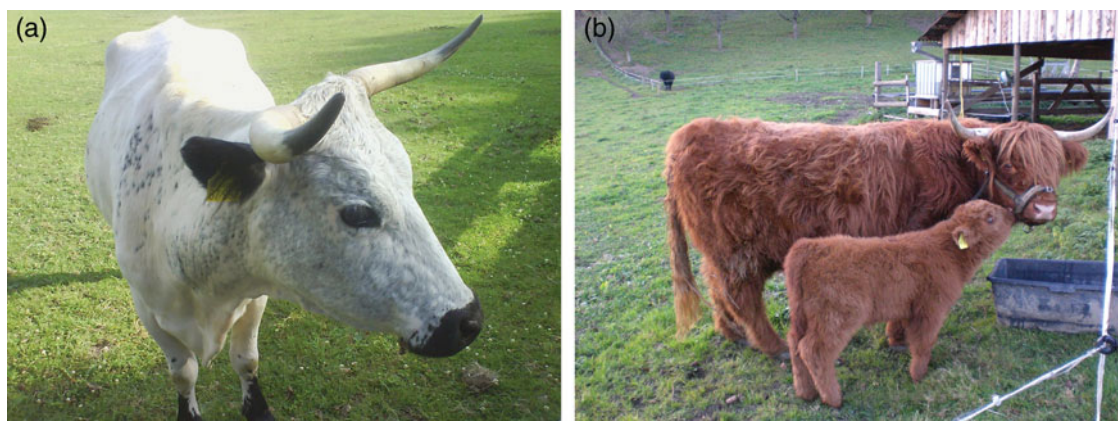
Figure 1. (a) White Park cow (Arche Warder, Zentrum für alte Haus- und Nutztierrassen e.V., Warder, Germany; photo: Diana Sorg). (b) Highland cow and calf (Rattenweiler, Germany; photo: Diana Sorg).

S. aureus. The breeds were compared in their mRNA expression of 39 target genes of the innate immune system via reverse transcription quantitative polymerase chain reaction (RT-qPCR) and in the synthesis of three antimicrobial proteins via enzyme-linked immunosorbent assay (ELISA).

Material and methods

Cell extraction from milk

Usually, pbMEC are cultivated from udder tissue after biopsy or slaughter. We chose to culture them from milk because it is a non-invasive method and therefore especially suited for rare and valuable animals. It yields less contamination by fibroblasts (Buehring, 1990) and has been shown to be an applicable alternative to tissue sampling (Sorg *et al.*, 2012). For the modern breeds, six healthy BS and six healthy RH cows (from research stations of Technische Universität München, Freising, Germany) in mid-to-late lactation were sampled in the milking parlour by machine milking into an autoclaved milk pail. For the ancient breeds, five healthy HLD (from Arche Warder and a private farm in Rattenweiler, Germany) and five healthy WP cattle (from Arche Warder) in mid-to-late lactation were automatically milked with a portable milking machine into an autoclaved milk pail or by hand milking into autoclaved glass bottles. The cells were extracted and cultivated with the method described in Danowski *et al.* (2012a) until third passage and stored in liquid nitrogen. Briefly, the milk was centrifuged at 1 850 *g* for 10 min to obtain the cell pellet. The pellet was washed twice with Hank's balanced salt solution (HBSS) containing 200 units/ml penicillin, 0.2 mg/ml streptomycin, 0.1 mg/ml gentamicin and 8.3 μg/ml amphotericin B (Sigma-Aldrich, Munich, Germany) and centrifuged at 600 *g* for 5 min. It was then resuspended in Dulbecco's modified Eagle's medium with nutrient mixture F12 Ham (DMEM/F12 Ham, Sigma-Aldrich)

containing 10 percent fetal bovine serum (FBS, Gibco Life Technologies, Darmstadt, Germany), $1 \times$ ITS supplement (Sigma-Aldrich), antibiotics as described above and 1.76 μg/ml amphotericin B (Sigma-Aldrich). The cells were cultivated in 25 cm^2 tissue culture flasks (Greiner, Frickenhausen, Germany) at 37 °C and 5 percent CO_2. For two subsequent passages, they were expanded into 75 cm^2 flasks (Greiner) by gently detaching them with accutase (PAA, Pasching, Austria). Growth and morphology was checked daily by light microscopy. After the third passage, they were resuspended in freezing medium (70 percent DMEM/F12 Ham, 10 percent FBS, 20 percent dimethyl sulfoxide (DMSO)) and stored in liquid nitrogen. Before freezing, a 16-well chamber slide (Nunc, Langenselbold, Germany) was cultivated for immunocytochemistry by seeding with 10 000 cells per well.

Bacteria

E. coli 1303 (Petzl *et al.*, 2008) and *S. aureus* 1027 (Petzl *et al.*, 2008) had been isolated from cows with clinical mastitis and shown to trigger the immune response *in vivo* (Petzl *et al.*, 2008) and *in vitro* (Gunther *et al.*, 2011). They were cultivated and harvested with the method used in Danowski *et al.* (2012a, b) and stored at −80 °C. Briefly, one colony of *E. coli* and of *S. aureus* was each cultured at 37 °C in Luria–Bertani (LB) medium containing 10 g/l yeast extract (Sigma-Aldrich), 10 g/l NaCl and 5 g/l trypton (Sigma-Aldrich) or in CASO-broth (Sigma-Aldrich), respectively, to the log-phase of growth. Bacterial density was determined photometrically at 600 nm. At several densities, a dilution series of *E. coli* and *S. aureus* was cultivated on LB agar (Roth, Karlsruhe, Germany) or on blood agar (Oxoid, Wesel, Germany, with sheep blood from Fiebig, Idstein-Niederauroff, Germany), respectively. The colonies were counted to determine the desired bacterial count and the corresponding optical density (OD). The cultivation was repeated up to the desired OD and stopped by placing

the solutions on ice. The bacteria were harvested by centrifugation for 10 min at 1 850 g and washed in PBS twice. They were inactivated for 30 min at 63 °C in a water bath. A diluted sample of both harvested cell pellets was cultivated on a plate at 37 °C overnight to verify inactivation.

Cell stimulation

The 22 cultures were reseeded at 30 000 cells per well in one 12-well plate (Greiner) each and cultivated until confluent. Cells from three wells from each plate were then detached with accutase (PAA, Pasching, Austria) and counted manually for an estimate of the mean cell count per well in the other wells of the plate. Medium was removed and fresh medium without FBS, antibiotics and antimycotic was added. Heat-inactivated bacteria were added in a multiplicity of infection (MOI) of 30 colony forming units (cfu) per cell. This MOI was chosen as a typical bacterial load from other experiments with pbMEC (Danowski *et al.*, 2012a; Gunther *et al.*, 2009) to ensure that every culture received the same stimulation per cell. Control wells were left untreated. After 6 h of incubation, two wells each of control and *E. coli* treated cells were sampled from every plate. After 30 h, two wells each of control, *E. coli* and *S. aureus* treated cells, were sampled. After 78 h, two wells each of control and *S. aureus* treated cells were sampled. The incubation times were chosen to cover the often described earlier onset of the immune reaction to *E. coli* and the later reaction to *S. aureus* (Bannerman *et al.*, 2004; Gunther *et al.*, 2011; Petzl *et al.*, 2008). Cells were harvested with the lysis buffer of the Qiagen AllPrep RNA/Protein kit (Qiagen, Hilden, Germany).

Immunocytochemistry

Immunocytochemical staining of the epithelial marker cytokeratin was performed as described in Danowski *et al.* (2012a, b). Briefly, the cells were fixed on the slides and permeabilized in ice cold methanol/acetone (1:1) for 10 min. They were washed three times for 5 min in PBS-Tween (PBST). Endogenous peroxidases were blocked in 1 percent H_2O_2 for 30 min. After washing, background staining was reduced with goat serum (1:10 in PBST, DAKO, Glostrup, Denmark) for 10 min at room temperature. Monoclonal mouse anti-cytokeratin pan antibody clone C-11 (1:400 in PBST, Sigma-Aldrich) was incubated overnight at 4 °C in moist atmosphere protected from light. The negative control wells received goat serum (1:10 in PBST) instead. After washing, horseradish peroxidase (HRP) labelled goat anti-mouse-immunoglobulin (1:400 in PBST, DAKO) was incubated for 1 h. HRP was visualized with 0.01 percent diaminobenzidine and 0.01 percent H_2O_2 in PBST for 15 min at room temperature and protected from light. Nuclei were stained with Haemalaun after Mayer (Roth, Karlsruhe, Germany) for 15 s and developed with tap water. The slides were dehydrated in 50 percent ethanol, 100 percent ethanol and Rotihistol (Roth) for 2 min each and covered with Eukitt (Roth) and a cover slip.

RNA and reverse transcription

The AllPrep RNA/Protein Kit together with the RNAse-free DNAse set (both Qiagen, Hilden, Germany) was used to extract total RNA and protein from the lysed cells and remove DNA contamination following manufacturer's instructions. Concentration and purity of the obtained RNA samples were measured with the Nanodrop 1000 spectrophotometer (Peqlab, Erlangen, Germany) at 260 nm. The integrity of the RNA was analysed with the RNA 6000 Nano Assay kit on Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). For the reverse transcription to cDNA, a total amount of 100 ng RNA was used in a reaction volume of 30 µl containing 100 units of Moloney murine leukemia virus (M-MLV) H(−) reverse transcriptase and 5 × buffer (Promega, Mannheim, Germany), 0.5 mM dNTPs and 0.5 µM Oligo-d(T) primer (Fermentas, St. Leon-Rot, Germany), and 2.5 µM random hexamer primers (Invitrogen by Life Technologies, Darmstadt, Germany). Reverse transcription reaction was run with annealing (21 °C for 10 min), transcription (48 °C for 50 min) and degrading phase (90 °C for 2 min). To check for genomic DNA contamination, an RNA pool from each extraction run was incubated with the same protocol without reverse transcriptase.

PCR primer pairs

Primer pairs (Metabion, Martinsried, Germany) were designed with HUSAR (DKFZ, German Cancer Research Center, Heidelberg) or PrimerBLAST from NCBI (National Center for Biotechnology Information, National Library of Medicine, Bethesda, MD, USA) using mRNA sequences from the NCBI. Specificity of primer pairs was checked via melting curve analysis and gel electrophoresis of the amplified product. PCR efficiencies of the assays were measured with a five-point dilution series of three cDNA samples in qPCR triplicates and calculated as described in Bustin *et al.* (2009). Name and symbol, selected relevant functions taken from the Gene Ontology Annotation (UniProt-GOA) database (Dimmer *et al.*, 2012), NCBI reference sequence number, primer pair sequences and amplicon lengths of the genes measured in RT-qPCR are shown in Supplementary Table S1.

RT-qPCR

A primer-specific preamplification step was carried out to adjust cycle of quantification (Cq) values to the measuring range with the following temperature profile: 95 °C for 3 min followed by 18 cycles of 95 °C for 20 s, 55 °C for 3 min and 72 °C for 20 s. 4 µl cDNA were amplified in a

volume of 20 µl with the iQ Supermix (Bio-Rad, Munich, Germany) and a primer concentration of 25 nM (Metabion, Martinsried, Germany) of each primer pair over 18 cycles. RT-qPCR was done on the microfluidic high-throughput BioMark™ HD system (Fluidigm, San Francisco, CA, USA) (Spurgeon, Jones and Ramakrishnan, 2008). One 48.48 Gene Expression (GE) Dynamic Array chip was used to measure PCR efficiencies of the assays and four 96.96 GE Dynamic Arrays were used to measure gene expression in the samples. One representative and stably expressed sample was chosen as between-chip calibrator and measured repeatedly on all chips. 5 ìl sample premix containing 2.5 µl SsoFast EvaGreen Supermix (Bio-Rad), 0.25 µl of sample loading reagent (Fluidigm), 0.1 µl ROX (diluted 1:3, Invitrogen), 1.25 µl preamplified and 1:9 diluted cDNA and water, as well as 5 µl assay premix containing 2 µl 10 µM primer pairs in the final concentration of 4 ìM, 2.5 µl Assay loading reagent (Fluidigm) and water were prepared and transferred to the primed GE Dynamic Array 96.96. The samples and assays were mixed inside the chip with the Nanoflex IFC controller (Fluidigm). The final concentration of primers in the individual reaction was 400 µM. The temperature profile was 98 °C for 40 s then followed by 40 cycles consisting of 95 °C for 10 s and 60 °C for 40 s. A melting curve of all PCR products was performed after the run to check for specificity. The Cq, where the fluorescence signal crossed the threshold, was detected by the BioMark Data Collection Software 2.1.1. built 20090519.0926 (Fluidigm, San Francisco, CA, USA). RT-qPCR was conducted following the minimum information for the publication of quantitative real-time PCR experiments (MIQE)-Guidelines (Bustin *et al.*, 2009).

Data analysis of RT-qPCR

Melting Curve Analysis Software 1.1.0. built 20100514.1234 (Fluidigm) and Real-time PCR Analysis Software 2.1.1. built 20090521.1135 (Fluidigm) were used to determine the valid PCR reactions. Invalid reactions were not used for later analysis and treated as missing data. Owing to loss of measurement precision, Cq values higher than 30 were treated as missing data and values between 25 and 30 were replaced by 25. Raw Cq values were processed with Genex 5.3.2 (MultID Analyses AB, Gothenburg, Sweden), using interplate calibration and reference gene normalization. Actin gamma 1 (ACTG1), keratin 8 (KRT8) and H3 histone, family 3A (H3F3A) were identified as suitable reference genes with the Normfinder tool within Genex 5.3.2. (MultID). The subtraction of reference gene Cq value index from target gene Cq value yielded the dCq value. Genex 5.3.2 (MultID) was also used for principal component analysis (PCA). All other statistical calculations were conducted with SigmaPlot 11 (Systat, Chicago, IL, USA) or SPSS Statistics Standard 21.0 (IBM, Armonk, NY, USA). Fold change in expression was calculated with the 2^{-ddCq} method (Livak and

Schmittgen, 2001). Independent *t*-tests were used to compare basal expressions and fold changes in expression between breeds ($P < 0.05$). Paired *t*-tests or signed rank tests on dCq values were used to find differentially expressed genes between treatment and control. Graphs were drawn with SPSS (IBM) or SigmaPlot 11 (Systat). It must be noted that no correction for multiple testing was imposed on the *P*-values. This study is of descriptive and explorative character only, not of a diagnostic one. Such a correction would have been too stringent and masked many of the differences.

Protein quantification with ELISA

Total protein content in the extracted cell protein was determined with the bicinchoninic acid (BCA) assay (Smith *et al.*, 1985) and measured with a photometer (Tecan, Männedorf, Switzerland). Lactoferrin (LF) was measured with the ELISA protocol and reagents used by Danowski *et al.* (2012b). Cell protein was diluted 1:1 in PBST and measured in duplicates. Interleukin-10 (IL-10) was determined using the ELISA protocol from Groebner *et al.* (2011) with minor modifications: the capture antibody mouse anti-bovine IL-10 antibody clone CC318 (AbD Serotec, Düsseldorf, Germany) was used at 5 µg/ml and the detection antibody biotinylated monoclonal mouse anti-bovine IL-10 antibody clone CC320 (AbD Serotec) was used at 1 µg/ml and incubated for 2 h. Samples were diluted 1:50 in PBST. Serum amyloid A (SAA) was measured in 30 h *E. coli* treated and control samples with the PHASE Serum amyloid A Multispecies ELISA kit (TriDelta, Maynooth, Ireland) according to manufacturer's instructions. Samples were diluted 1:67 in PBST.

Data analysis of ELISA

LF contents were calculated from the standard curve (Magellan data analysis software, Tecan, Männedorf, Switzerland). They were normalized to the total protein content of the sample and presented as ng LF/µg cell protein. A paired *t*-test in SigmaPlot 11 (Systat, Chicago, IL, USA) was used to test for differential expression of LF between treated and control samples at each time point ($P < 0.05$). Independent *t*-tests were used to compare treated and control levels between breeds. Owing to a lack of a commercial standard, relative IL-10 concentration was determined by normalizing the OD to the total protein content of the sample. To avoid interplate bias we gave the ratio of normalized ODs of treated and control samples that were each measured together on the same plate, multiplied by 100, this yielded IL-10 in % of control. SAA contents were determined with the standard curve as indicated in the manual. A paired *t*-test or signed rank test in SigmaPlot (Systat, Chicago, IL, USA) was used to compare SAA content in 30 h *E. coli* treated and control samples ($P < 0.05$). An independent *t*-test was used to compare breeds ($P < 0.05$).

Results

Cell culture and immunocytochemistry

An average of 5.98 million cells per animal with a range of 1–19 million cells was harvested for storage in liquid nitrogen. All the cultures showed a clear and continuous staining for cytokeratin, whereas the negative controls remained unstained. No cell types other than epithelial-like cells could be detected. All cultures showed the typical cobblestone-like shape with varying cell sizes. An example is shown in Supplementary Figure S2.

Gene expression

Table 1 shows the normalized basal expression of 16 innate immune genes in the untreated control samples after 6, 30 and 78 h incubation. These 16 genes were differentially expressed between breeds at one time point at least. CXCL8, LPO, CD68, CASP8, TLR2, TLR4 and MX2 were differentially expressed at all three time points. Six genes of the TLR pathway were differentially expressed at 6 h. Notably in CASP8, CXCL8, TLR2 and TLR4, the ancient breeds had lower Cq values and therefore higher expression levels than the modern breeds. WP had higher expression levels of CCL5, IL10, MX1 and MX2 than the other breeds. It also had a higher CCL20, CD68 and LPO expression than RH.

Tables 2 and 3 show the relative fold changes in gene expression of innate immune genes between control and treated cells. Only genes that were differentially expressed in one breed ($P < 0.05$) or were at least 1.5-fold up-regulated are presented. Table 2 shows the fold changes in gene expression after 6 and 30 h exposure to *E. coli*. After 6 h, HLD had lower fold changes than BS in complement component 3 (C3) and caspase 8 (CASP8), lower fold changes than RH in chemokine (C-C motif) ligand 2 (CCL2) and lymphocyte antigen 96 (LY96) and lower fold changes than WP in lactoperoxidase (LPO). C3, chemokines and cytokines were strongly up-regulated. Antimicrobial peptides were only up-regulated in the modern breeds. S100 and MX genes were more differentially expressed in the modern breeds. The most regulated gene after 6 h exposure to *E. coli* was SAA3 with nearly 290-fold in RH. After 30 h exposure to *E. coli*, BS had higher fold changes than RH in CD14. C3, chemokines, cytokines and antimicrobial peptides were strongly up-regulated. With the two *E. coli* treatments, more of the antimicrobial peptides were up-regulated in BS than in the other breeds. After 6 h exposure to *E. coli* there was no up-regulation of these in the ancient breeds. The S100 and MX genes were only up-regulated in the modern breeds. The most regulated gene after 30 h exposure to *E. coli* was SAA3 with 1900-fold in RH. Table 3 shows the fold changes in gene expression after 30 and 78 h exposure to *S. aureus*. There were no breed differences after 30 h exposure to *S. aureus*. The only differentially expressed genes were the antimicrobial peptides LPO and LYZ1 in WP and TLR4 in BS. After 30 h exposure to *S. aureus*

LYZ1 had the highest significant fold change with 1.6 in WP. After 78 h exposure to *S. aureus*, HLD differed from BS in transforming growth factor β1 (TGFβ1). They were both down-regulated and differed from RH which was up-regulated. LY96 was slightly elevated in HLD compared with WP and RH. After 78 h exposure to *S. aureus,* the highest significant fold change was found in LF in RH with 1.6. SEM was generally very high. In general, the modern breeds had a higher number of regulated genes than the ancient breeds (Tables 2 and 3). Figure 2 shows the PCA on the dCq values of the control samples (Figure 2a) and the ddCq values, the differences between control and treated dCqs (Figure 2b). Each symbol represents all data of all respective samples from one animal. A visual clustering can be observed in the basal expression (Figure 2a): RH and BS form two subgroups in the lower half of the picture. WP and HLD are mixed together, but separated from the modern breeds in the upper half of the graph. No such separation is visible in the PCA on the ddCqs of gene expression.

Protein production

LF content in total cell protein is shown together with the inversed expression of its gene (20-dCq), so that higher bars represent higher gene expression (Figure 3). While an up-regulation in the gene expression could be observed in most *E. coli* treatments and after 78 h with *S. aureus*, only RH and WP had a significant protein increase with 30 h exposure to *E. coli*. BS even showed a down-regulation in LF protein with 30 h exposure to *S. aureus*. BS had higher gene expression levels than RH and HLD in 78 h control cells. HLD had higher control and *S. aureus* treated LF protein levels after 30 h compared with WP.

IL-10 was determined relatively as IL-10 in % of control and is shown together with the fold change of its gene expression (Figure 4). There were no significant breed differences. While there was an often significant up-regulation in IL10 gene expression (see Tables 2 and 3) the rise in protein production was not consistent throughout the breeds and the treatments. In BS, there was a qualitative increase of approximately 50 and 25 percent of IL-10 protein after 30 and 78 h exposure to *S. aureus*, respectively. RH had a qualitative increase of about 60 percent with 30 h exposure to *E. coli*. WP showed no visible changes compared with controls. In HLD, there was about 50 percent more IL-10 with 6 h *E. coli* and 78 h *S. aureus* treatments, as well as about 100 percent more with 30 h *S. aureus* treatment. SEM of the protein data was considerably high.

SAA content was measured in control and *E. coli* treated cells after 30 h stimulation and is shown together with the inversed expression of its gene (20-dCq), so that higher bars represent higher gene expression (Figure 5). Gene expression was significantly increased by the treatment, but only in BS this was also true for the protein production. BS and RH differed significantly from HLD in basal SAA levels (control). However, only BS differed significantly from HLD in *E. coli* treated SAA levels.

Table 1. Basal mRNA expression (mean dCq and SEM) of innate immune genes in pbMEC from four cattle breeds, unstimulated control after 6, 30 and 78 h.

Gene			6 h				30 h				78 h			
			Breed				Breed				Breed			
			BS	RH	WP	HLD	BS	RH	WP	HLD	BS	RH	WP	HLD
Chemokines														
CCL20	Mean		12.21_a	15.40_a	11.94_a	13.96_a	13.01_a	15.05_a	12.79_a	13.83_a	13.71_{ab}	15.83_a	12.47_b	14.64_{ab}
	SEM		0.63	1.24	1.63	1.14	0.60	0.95	0.93	0.73	0.78	0.90	1.43	0.90
CCL5	Mean		15.12_a	15.19_a	12.44_b	15.60_a	15.01_a	14.79_a	13.48_a	15.29_a	14.49_{ab}	14.74_{ab}	13.15_a	15.37_b
	SEM		0.67	0.73	0.63	0.80	0.72	0.66	0.72	0.83	0.47	0.46	0.46	0.83
CXCL8	Mean		10.36_{ab}	11.51_a	9.22_b	9.25_b	10.96_a	11.37_a	9.45_a	10.07_{ab}	11.07_{ab}	12.36_a	9.76_b	10.60_b
	SEM		0.50	0.29	0.74	0.48	0.57	0.29	0.44	0.38	0.42	0.22	0.75	0.55
Cytokines														
IL6	Mean		7.22_a	6.88_a	8.92_a	8.03_a	7.66_a	7.52_a	9.56_b	7.92_{ab}	8.39_a	8.59_a	11.06_b	9.20_{ab}
	SEM		0.35	0.45	0.94	0.88	0.26	0.52	0.89	0.62	0.18	0.66	0.87	0.88
IL10	Mean		15.12_a	15.22_a	12.05_b	15.09_a	14.86_a	14.77_a	13.05_a	14.86_a	14.37_a	14.77_a	12.46_b	14.87_a
	SEM		0.68	0.68	0.76	0.77	0.67	0.66	0.67	0.87	0.44	0.44	0.38	0.98
Antimicrobial peptides														
LF	Mean		9.44_a	10.45_a	9.70_a	9.12_a	8.06_a	9.38_a	8.92_a	8.66_a	5.56_a	7.93_b	6.21_{ab}	7.11_b
	SEM		0.66	0.24	0.55	0.38	0.67	0.56	0.59	0.49	0.51	0.84	0.85	0.33
LPO	Mean		15.15_{ab}	15.97_a	14.20_b	15.37_{ab}	15.09_{ab}	15.87_a	14.34_b	15.26_{ab}	15.30_{ab}	15.62_a	13.96_b	15.06_{ab}
	SEM		0.63	0.28	0.50	0.18	0.53	0.36	0.63	0.16	0.42	0.50	0.67	0.27
Scavenger receptor														
CD68	Mean		13.28_{ab}	13.66_a	12.49_b	12.42_b	13.56_a	13.73_a	12.66_b	13.07_{ab}	14.16_{ab}	14.46_a	13.24_b	13.58_{ab}
	SEM		0.25	0.01	0.25	0.50	0.19	0.19	0.33	0.43	0.23	0.27	0.39	0.40
TLR pathway														
CASP8	Mean		7.61_a	7.82_a	6.62_b	6.64_b	7.90_a	7.90_a	6.85_b	7.01_b	7.76_{ab}	8.24_a	7.04_c	7.12_{bc}
	SEM		0.17	0.16	0.15	0.21	0.31	0.25	0.09	0.20	0.13	0.28	0.21	0.22
LBP	Mean		16.99_a	16.45_{ab}	16.60_{ab}	15.56_b	15.91_a	15.93_a	15.38_a	15.62_a	14.72_a	15.16_a	14.69_a	14.74_a
	SEM		0.44	0.35	0.21	0.56	0.55	0.26	0.34	0.51	0.40	0.58	0.91	0.43
LY96	Mean		4.92_a	5.53_b	4.43_{ac}	4.10_c	4.91_a	5.29_a	4.48_a	4.30_a	5.45_a	5.51_a	4.56_a	4.44_a
	SEM		0.08	0.29	0.18	0.21	0.13	0.63	0.20	0.28	0.34	0.52	0.27	0.27
MYD88	Mean		7.40_a	7.38_{ab}	7.18_{ab}	6.88_b	6.89_a	7.24_a	6.96_a	6.82_a	6.38_a	7.05_a	6.73_a	6.73_a
	SEM		0.18	0.15	0.19	0.17	0.20	0.39	0.19	0.21	0.18	0.38	0.08	0.29
TLR2	Mean		14.54_a	15.21_a	14.44_{ab}	13.47_b	14.04_{ab}	14.73_a	13.75_b	13.25_b	13.68_{ab}	14.57_a	13.34_b	13.02_b
	SEM		0.17	0.36	0.48	0.33	0.37	0.32	0.23	0.27	0.36	0.33	0.19	0.38
TLR4	Mean		8.87_{ab}	9.37_a	8.10_c	8.20_{bc}	8.76_{ab}	9.25_a	7.87_c	8.09_{bc}	7.91_a	9.14_a	7.58_a	7.97_a
	SEM		0.20	0.16	0.30	0.29	0.30	0.17	0.25	0.21	0.32	0.28	0.31	0.18
Others														
MX1	Mean		6.14_{ab}	6.54_{ab}	4.37_a	7.03_b	6.52_a	7.47_a	4.69_a	7.49_a	7.15_a	7.42_a	5.20_a	7.16_a
	SEM		0.34	0.88	0.54	1.27	0.41	0.89	0.87	1.48	0.71	0.68	0.27	1.56
MX2	Mean		11.59_a	11.42_a	8.22_b	11.61_a	11.29_{ab}	12.21_a	8.84_b	12.38_a	11.86_{ab}	12.59_a	9.20_b	12.05_{ab}
	SEM		0.72	0.97	0.63	1.24	0.57	1.06	1.08	1.28	0.84	0.96	0.45	1.33

Note: BS = Brown Swiss, RH = Red Holstein, WP = White Park, HLD = Highland. Means with different subscript letters are significantly different between the breeds ($P < 0.05$).

Table 2. Fold changes of the normalized relative gene expression of innate immune genes in pbMEC from four cattle breeds after 6 h and 30 h stimulation with *E. coli*.

Gene		*E. coli* 6 h BS	RH	WP	HLD	*E. coli* 30 h BS	RH	WP	HLD
Complement system									
C3	Fold change	4.4**$_a$	4.2**$_{ab}$	6.1*$_{ab}$	1.9$_b$	8.6***	11.1**	6.8*	11.9*
	SEM	0.9	1.1	2.1	0.5	1.8	3.7	2.3	4.0
Chemokines									
CCL2	Fold change	28.8**$_{ab}$	10.8***$_a$	27.5*$_{ab}$	4.4$_b$	43.9***	27.3**	26.8*	18.2**
	SEM	12.3	1.9	11.8	1.0	13.1	9.0	12.1	9.2
CCL5	Fold change	24.1	4.0*	4.8*	2.0*	51.6**	26.6*	55.4*	3.9*
	SEM	18.3	1.7	1.5	0.5	33.3	14.1	37.7	1.3
CCL20	Fold change	39.4**	36.8**	110.8*	17.7*	50.2***	119.6*	74.5*	37.9*
	SEM	17.1	13.8	65.5	7.6	11.1	75.8	42.2	20.5
CXCL5	Fold change	8.1**	8.0***	11.7*	3.9*	7.8***	9.0**	7.2*	6.4*
	SEM	2.1	1.6	4.0	1.0	1.3	2.6	2.3	2.0
CXCL8	Fold change	20.1*	21.2***	33.8*	7.0*	21.9***	26.7**	16.0*	11.2*
	SEM	7.7	8.3	13.4	3.1	7.5	9.5	6.1	4.2
Inflammatory cytokines									
IL6	Fold change	3.6*	3.4**	7.7*	2.1*	5.3***	5.1*	11.2*	3.2*
	SEM	1.1	0.6	3.00	0.4	1.2	1.7	5.7	0.8
IL10	Fold change	18.8*	3.3*	4.1	1.7	53.1**	25.2*	57.5	3.5
	SEM change	11.0	1.1	1.8	0.4	35.1	13.3	33.8	1.2
TGFβ1	Fold change[1]					1.7	0.9	0.9	1.4
	SEM					0.7	0.0	0.1	0.4
TNF	Fold change	21.9**	21.6	54.0*	6.0*	21.5***	40.6**	65.1	13.1**
	SEM	7.0	9.0	32.7	2.0	4.1	17.2	35.7	6.5
Antimicrobial peptides									
LAP	Fold change	2.8**	1.5	6.8	2.1	19.5**	4.6	84	25.2
	SEM	0.6	0.5	2.7	0.8	8.0	1.0	53.5	14.3
LF	Fold change	3.2**	3.1*	2.8	1.7	8.6**	12.0**	9.3*	7.3*
	SEM	0.6	1.0	0.9	0.4	2.3	4.2	4.0	2.7
LPO	Fold change	1.9$_{ab}$	0.9$_{ab}$	2.9$_a$	0.9$_b$	4.2*	1.7	9.2*	2.2
	SEM	0.4	0.1	0.8	0.2	1.2	0.2	4.1	0.7
LYZ1	Fold change	2.1	2	3.7	1.9	40.2**	12.7*	32.1*	13.4*
	SEM	0.5	0.6	1.0	0.5	27.6	6.5	14.2	4.6
TAP	Fold change	54.8*	1.3	33.5	8.5	105.7**	11.8*	234.8	47.8
	SEM	32.8	0.1	12.9	6.1	57.9	1.7	117.8	41.0
Acute phase proteins									
SAA3	Fold change	98.7	289.4*	418.2	10.8	618.2**	1912.3**	1769.1*	69.9*
	SEM	90.1	263.4	172.5	3.7	272.4	1445.1	1076.6	39.1
TLR signalling									
CASP8	Fold change	1.3$_a$*	1.1$_{ab}$	1.2$_{ab}$	1.0$_b$	1.4*	1.3*	1.2	1.4
	SEM	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2
CD14	Fold change	1.3	1.1	1.2	1.8	1.8$_a$*	1.0$_b$	1.1$_{ab}$	1.8$_{ab}$
	SEM	0.1	0.1	0.1	0.4	0.3	0.1	0.2	0.4
LBP	Fold change	2.5	2.2	1.4	1.2	1.2	1.4	1.2	2.7
	SEM	0.7	0.7	0.4	0.3	0.1	0.4	0.5	0.2
LY96	Fold change	1.0$_{ab}$	1.1$_a$*	0.9$_{ab}$	1.0$_b$				
	SEM	0.0	0.0	0.1	0.0				
TLR2	Fold change	5.7	1.7***	6.9*	1.5	5.0*	2.0*	2.8	2.2*
	SEM	2.1	0.1	3.3	0.2	2.1	0.3	0.9	0.4
Others									
MX1	Fold change	2.8	1.6*	1.2	1.5	4.4*	3.5**	3.1	2.9
	SEM	1.4	0.2	0.3	0.4	1.4	0.8	1.1	1.3
MX2	Fold change	11.6*	2.0*	1.5	1.8	8.6***	8.3*	6.5	3.9
	SEM	7.6	0.5	0.4	0.6	2.3	2.8	3.3	1.6
S100A9	Fold change	3.9**	2.4	8.6*	2.1	12.0***	12.6*	14.2	20.4
	SEM	0.8	0.9	4.0	0.6	4.1	4.1	6.0	15.9
S100A12	Fold change	2	MD[2]	6.5	2.6	4.2**	2.1	5.1	1.7
	SEM	0.5	MD	3.1	1.0	0.9	1.2	1.4	0.5
Regulated genes[3]		14	14	10	6	22	18	11	12

Note: BS = Brown Swiss (*n* = 6), RH = Red Holstein (*n* = 6), WP = White Park (*n* = 5), HLD = Highland (*n* = 5); Stars indicate significant differences between treated and control dCq: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Fold change means with different subscript letters differ between breeds ($P < 0.05$).

[1]Empty genes: no significant breed differences in expression fold changes and no fold changes >1.5 at this time point.

[2]Missing data.

[3]$P < 0.05$ for dCq difference between treatment and control.

Table 3. Fold changes of the normalized relative gene expression of innate immune genes in pbMEC from four cattle breeds after 30 h and 78 h stimulation with *S. aureus*.

Gene		*S. aureus* 30 h				*S. aureus* 78 h			
	Treatment	Breed				Breed			
		BS	RH	WP	HLD	BS	RH	WP	HLD
Complement system									
C3	Fold change[1]					1.1	1.6	1.1	1.2
	SEM					0.1	0.4	0.1	0.1
Chemokines									
CCL2	Fold change					1.0	1.3	1.3	1.6
	SEM					0.1	0.1	0.2	0.3
CCL5	Fold change					1.5*	1.6	1.3	1.2
	SEM					0.2	0.4	0.6	0.3
CCL20	Fold change					1.7	0.9	3.3	1.5
	SEM					0.6	0.2	1.3	0.4
CXCL8	Fold change					1.3	1.2	1.9	1.1
	SEM					0.2	0.1	0.6	0.2
Inflammatory cytokines									
IL10	Fold change					1.5*	1.5	1.0	1.1
	SEM					0.2	0.5	0.4	0.3
TGFβ1	Fold change					0.9^*_b	1.2^*_c	0.9_{abc}	0.8_a
	SEM					0.0	0.1	0.1	0.0
TNF	Fold change					1.2	1.2	4.5	1.8
	SEM					0.3	0.1	2.9	1.1
Antimicrobial peptides									
LAP	Fold change					1.2	MD[2]	5.1	1.9
	SEM					0.2	MD	3.4	0.8
LF	Fold change					1.0	1.6*	1.3	1.1
	SEM					0.1	0.2	0.4	0.1
LPO	Fold change	1.0	1.2	1.2*	1	1.2	0.9	1.7	1.5
	SEM	0.3	0.2	0.2	0.1	0.2	0.1	0.7	0.4
LYZ1	Fold change	1.1	0.9	1.6*	1.9	1.6	1.2	2.9	1.2
	SEM	0.1	0.2	0.3	0.5	0.5	0.2	1.2	0.1
TAP	Fold change	0.9	1.1	1.5	0.9	5.2	0.7*	6.9	1.5
	SEM	0.3	0.4	0.3	0.2	4.3	0.2	3.7	0.8
Acute phase proteins									
SAA3	Fold change	1.7	2.5	3	1.2	5.1	3.1	5.4	1.2
	SEM	0.5	1	1.7	0.3	3.8	1.5	4.3	0.2
TLR signalling									
LBP	Fold change					1.0	1.4	0.9	1.8
	SEM					0.2	0.4	0.2	1.0
LY96	Fold change					1.1_{ab}	1.0_b	1.0_b	1.1^*_a
	SEM					0.0	0.1	0.0	0.0
TLR2	Fold change					1.0	1.0	1.3	1.9
	SEM					0.1	0.1	0.2	1.1
TLR4	Fold change	1.3*	1.0	1.0	1.1	1.0	1.2*	1.0	1.0
	SEM	0.2	0.1	0.1	0.0	0.1	0.1	0.1	0.0
Scavenger receptor									
CD68	Fold change					1.1	1.3	1.2	1.7
	SEM					0.2	0.2	0.3	0.5
Others									
MX1	Fold change	2.1	1.2	1.0	1.7	1.7	1.2	1.1	0.8
	SEM	0.7	0.4	0.3	0.3	0.4	0.2	0.3	0.2
MX2	Fold change	1.9	1.9	1.1	1.9	2.9	1.3	1.4	1.0
	SEM	0.7	1.3	0.4	0.4	1.3	0.2	0.5	0.2
S100A9	Fold change					1.3	1.3	2.2	1.3
	SEM					0.2	0.2	0.6	0.2
S100A12	Fold change					1.3^*_a	0.8_b	1.2_{ab}	1.6_{ab}
	SEM					0.1	0.2	0.2	0.5
Regulated genes[3]		1	0	2	0	4	4	0	1

Note: BS = Brown Swiss (*n* = 6), RH = Red Holstein (*n* = 6), WP = White Park (*n* = 5), HLD = Highland (*n* = 5); Stars indicate significant differences between treated and control dCq: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Fold change means with different subscript letters differ between breeds ($P < 0.05$).

[1] Empty genes: no significant breed differences in expression fold changes and no fold changes >1.5 at this time point.

[2] Missing data.

[3] $P < 0.05$ for dCq difference between treatment and control.
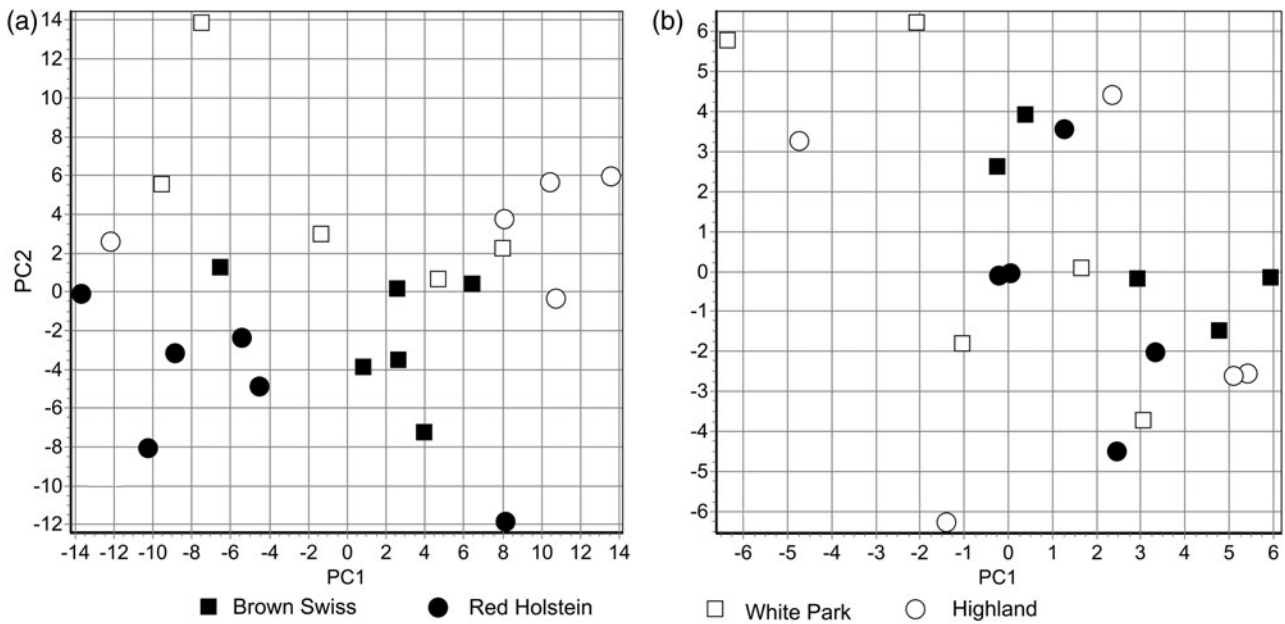
Figure 2. PCA of (a) dCq values (basal expression of unstimulated control) and (b) ddCq values (difference between treated and control dCq) of 28 target genes in pbMEC from four cattle breeds after stimulation with *E. coli* and *S. aureus*. Each symbol represents all respective samples of one animal.

Discussion

Breed comparison

On the level of basal expression in the PCA, there was a visible separation of ancient from modern breeds and within the two modern breeds. The higher basal expression of the components of the TLR pathway in the ancient breeds could be responsible for an earlier recognition of invading pathogens and therefore lead to an earlier and more effective immune response. The same could be true for the higher basal levels of SAA protein in the ancient breeds which could have a protective effect against
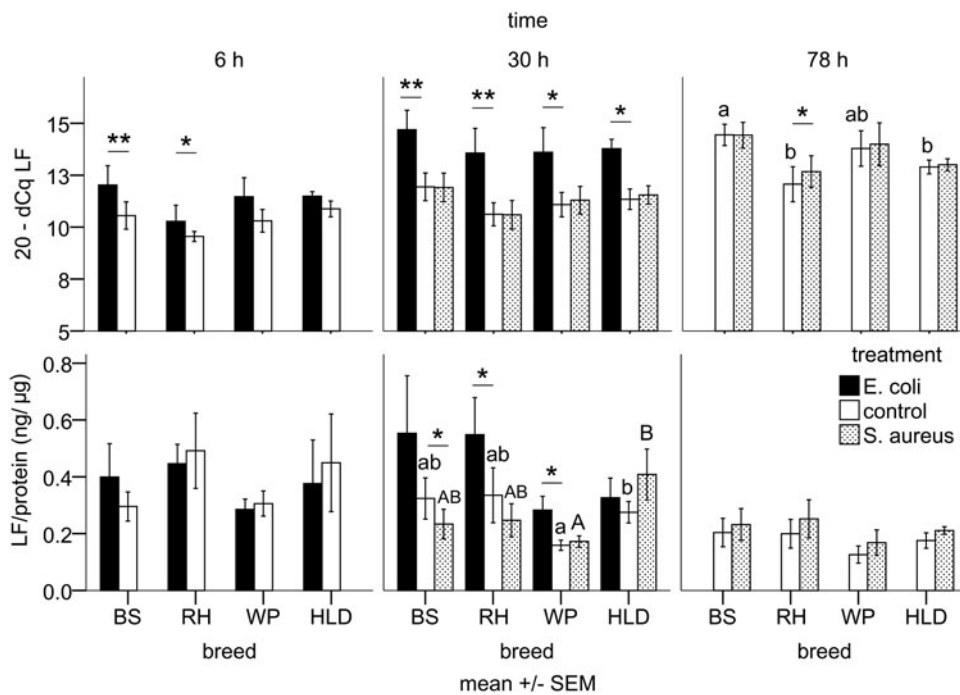
Figure 3. Relative gene expression and LF content in ng/μg cell protein in pbMEC from ancient (WP, HLD; $n = 5$) and modern (BS, RH; $n = 6$) cattle breeds stimulated with *E. coli* (6 and 30 h) and *S. aureus* (30 and 78 h). Stars indicate significant differences between the treatments, letters indicate significant differences of *S. aureus* treated (upper case letters) and control levels (lower case letters) between the breeds ($P < 0.05$). BS = Brown Swiss, RH = Red Holstein, WP = White Park, HLD = Highland cattle.
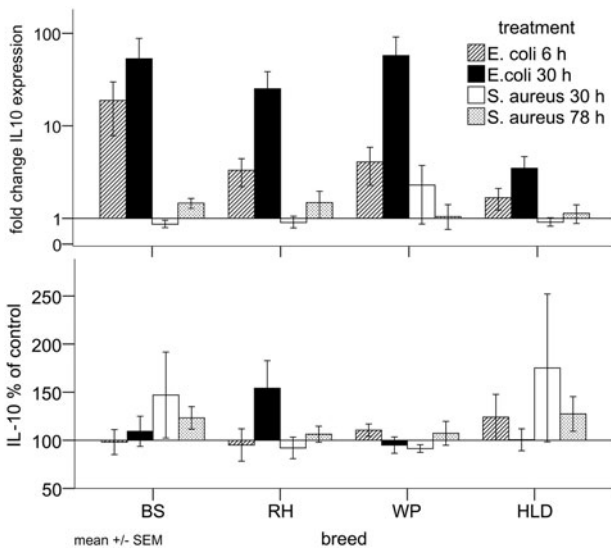
Figure 4. Fold change of IL10 expression and relative IL-10 content in % of untreated control in total cell protein of pbMEC from ancient (WP, HLD; $n = 5$) and modern (BS, RH; $n = 6$) cattle breeds stimulated with *E. coli* (6 and 30 h) and *S. aureus* (30 and 78 h). BS = Brown Swiss, RH = Red Holstein, WP = White Park, HLD = Highland cattle.

pathogens, as SAA is an opsonising agent (Shah, Hari-Dass and Raynes, 2006). Interestingly, basal LF protein levels were lower in the ancient breeds, but differed significantly only between WP and HLD. WP and HLD also differed in basal expression of MX1 and CCL5. So the breeds seem to be all different from each other and cannot just be grouped together in "modern" and "ancient". It is difficult to interpret the fold changes of gene expression, as the SEM were considerably high and led to weak
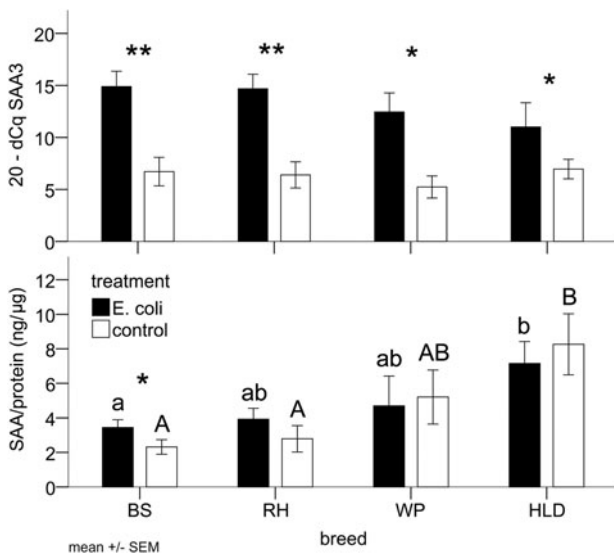

Figure 5. Relative expression of the SAA3 gene and SAA content in ng/μg cell protein in pbMEC from ancient (WP, HLD; $n = 5$) and modern (BS, RH; $n = 6$) cattle breeds stimulated with *E. coli* for 30 h. Stars indicate significant differences between treatments, different letters indicate significant differences of treated (lower case letters) and control cells (upper case letters) between breeds ($P < 0.05$). BS = Brown Swiss, RH = Red Holstein, WP = White Park, HLD = Highland cattle.

significances for visibly high fold changes. In addition, the PCA on ddCq did not reveal any clustering of the animals. However, this set aside, there was a higher number of significantly up-regulated genes in the modern breeds, especially for the antimicrobial peptides, the TLR pathway and the MX genes. HLD had the lowest fold changes in SAA3 expression, but the highest basal levels of SAA protein after 30 h. Although the whole picture is diffuse and complex, it seems as if in those parts of the immune system where we found a difference between the breeds, a higher basal expression led to a lower response. Kandasamy *et al.* (2012) tested the extent of the immune response of cows that had before been classified as "high-" and "low-responder" animals to an intramammary *E. coli* challenge. They found that the weaker immune response of low-responder animals was more effective and led to a shorter resolution phase of the inflammation. Hence, a strong immune response is not necessarily a benefit for the animal. Another prominent example for this phenomenon is the well-studied tolerance of the *Bos indicus* Sahiwal cattle to the indigenous protozoan parasite *Theileria annulata*. Compared with Holstein calves *in vivo* (Glass *et al.*, 2005) they showed fewer clinical symptoms, recovered from a higher dose of pathogen and had lower acute phase protein levels. In another comparison with Sahiwal cattle, macrophages from Holstein cattle showed higher up-regulation of inflammatory and immune response genes (Glass *et al.*, 2012).

To our knowledge, there are no studies on the intra-mammary immune system of ancient cattle breeds such as WP and HLD. There has been evidence that the immune system of modern breeds shows differences in details, but overall is highly conserved (Bannerman *et al.*, 2008a, 2008b), which is in accordance with our results. The *in vivo* response of Holstein and Jersey cows to *E. coli* differed only in the time point of milk cytokine and somatic cell count (SCC) increase, not in overall levels (Bannerman *et al.*, 2008a). To an *S. aureus* challenge Holstein and Jersey animals also responded with similar overall levels of milk SCC and cytokines except for neutrophils and N-acetyl-beta-D-glucosaminidase (NAGase) activity (Bannerman *et al.*, 2008b). Different LF contents in milk have already been observed between Holstein, Jersey and Simmental cows (Krol *et al.*, 2010) as well as between dairy and beef cattle (Tsuji *et al.*, 1990), which adds to our findings of different LF contents in pbMEC. There are several polymorphisms located in the LF gene in different cattle breeds that could be the reason for differential LF expression and production (O'Halloran *et al.*, 2009). The different SAA contents in our pbMEC can be compared with a study where after an LPS challenge SAA in blood serum increased more rapidly in Angus than in Romosinuano steers (an indigenous Colombian breed) and remained at higher levels for 8 h (Carroll *et al.*, 2011). Although in the cells from our ancient breeds the absolute levels of SAA protein were higher than in the modern breeds, there was no significant rise after pathogen

stimulation. Cattle breed differences in gene expression and protein production of the immune system have not been systematically studied so far, but our findings and the above-mentioned studies show that there is evidence for such diversity.

The considerable animal differences within each breed, reflected by the high SEMs and by the wide spread of the symbols representing animals in the PCAs, could be explained by the existence of a substantial between-cow variation in the immune response which has already been shown for Holstein cattle *in vitro* and *in vivo* (Kandasamy *et al.*, 2012). It could be caused by genetic polymorphisms that are linked to a certain breed, but could also be spread all over the cattle population. Furthermore, it has been suggested that a proportion of unexplained phenotypic variation in the dairy cow is because of epigenetic regulation (Singh *et al.*, 2010).

General remarks about the immune response

C3, chemokines, inflammatory cytokines and the inflammation marker SAA3 experienced a strong up-regulation by *E. coli* in all the breeds. The antimicrobial peptides were also strongly up-regulated after 30 h in *E. coli* treated cells. This confirms that our pbMEC continued to exert sentinel functions to trigger the innate immune response upon pathogen recognition as well as an active defence by attacking and opsonising bacterial cells. Interestingly, in our study the TLR pathway was not as markedly regulated, although it is one of the starting points of the immune signalling cascade and has been shown to be a source for potential mastitis resistance (Griesbeck-Zilch *et al.*, 2009). However, in another study the regulation of TLRs in pbMECs was similarly weak, but the authors still concluded that there was a functioning and locally effective immune system (Strandberg *et al.*, 2005). We also found a regulation of the genes we had termed as "others". The calcium-binding, pro-inflammatory, regulatory and anti-oxidant S100 calcium-binding proteins A9 (S100-A9) and A12 (S100-A12) seem to be a class of protective and defence proteins (Hsu *et al.*, 2009) that act in addition to LF, lysozyme 1 (LYZ1), LPO and the β-defensins lingual antimicrobial peptide (LAP) and tracheal antimicrobial peptide (TAP). The antiviral myxovirus (influenza virus) resistance 2 (mouse) gene (MX2) has a yet unknown role in mastitis and remains a subject of further research.

Pathogen comparison

It has previously been shown that *S. aureus* elicits a different and often weaker immune response than *E. coli in vitro* (Griesbeck-Zilch *et al.*, 2008) and *in vivo* (Petzl *et al.*, 2008). The dose of inoculum could have been too low so that the cells did not receive enough signals to trigger the response. Our results can be compared with a similar study with pbMEC from milk and the same strains of pathogens

(Danowski *et al.*, 2012a): in that study, too, the immune response to *S. aureus* was much weaker than to *E. coli*. Our data support the hypothesis that the often subclinical and chronic outcome of *S. aureus* mastitis is caused by this reduced reaction of the mammary immune system.

Gene expression and protein comparison

LF gene expression was generally better reflected by the ELISA measurements than the other two proteins. Although IL10 gene expression was significantly up-regulated in the two modern breeds there was no consistent rise of the protein in cell content. SAA3 expression was up-regulated by *E. coli* after 30 h, but the protein levels reflected that only in BS and RH. For all these three genes (in SAA for the SAA encoding-gene SAA2) microRNAs have been identified that could lead to a differential expression, translation and massive variation in protein levels (Longley, Steel and Whitehead, 1999; Sharma *et al.*, 2009; Liao, Du and Lonnerdal, 2010). These microRNAs could also be differentially expressed between the breeds and determine the breed differences in mRNA expression. LF was also secreted into the media, but the concentrations were mostly below the measuring range (data not shown). This and a delay between mRNA expression and protein synthesis of the three genes could also account for the differences.

Conclusions

To our knowledge this is the first time that the mammary immune system of the ancient WP and HLD cattle was studied *in vitro*. The four breeds BS, RH, WP and HLD were found to differ in parts of the gene expression and protein production. A higher basal expression of some genes and proteins in the cells from the ancient breeds seemed to lead to a lower immune response after pathogen recognition. However, the main immune system pathways that were activated were the same, indicating that the complex network of immune response is to some extent conserved between the *Bos taurus* breeds. With this experimental setup it is possible to study other breeds and other pathogens in the same way, especially with the non-invasive pbMEC extraction from milk which is suitable for the sampling of valuable animals of rare breeds. We confirmed the existence of previously described substantial cow-to-cow variation in immune response. The classification of high- and low-responder animals and the underlying genetic and epigenetic mechanisms remain subject to further analysis.

Acknowledgments

Conflict of Interest statement

The authors declare that there is no conflict of interest.

Supplementary material

Supplementary materials of this paper is available at http://journals.cambridge.org/agr

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