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Influence on physiological functions of bovine mammary epithelial cells in 3D cell culture through bacterial stimuli and ketone bodies

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Abbreviations

18SrRNA	18S ribosomal RNA gene
ACTG1	Actin, gamma 1
AKT1	V-akt murine thymoma viral oncogene homolog 1
AMPK	Adenine monophosphate-activated protein kinase
ATP	Adenosine triphosphate
BAX	BCL2-associated X protein
BCA	Bicinchoninic acid
BCL-2	B-cell CLL/lymphoma 2
Bcl-xL	Anti-apoptotic regulator Bcl-xL
BHBA	β-hydroxybutyrate
bp	Base pair
BSA	Bovine serum albumin
C. diff.	*Clostridium difficile*
C3	Complement component 3
CAA	Chloroacetamide
CASP	Caspase
CASP1	Caspase 1
CASP3	Caspase 3
CASP8	Caspase 8
CCL2	Chemokine (C-C motif) ligand 2
CCL20	Chemokine (C-C motif) ligand 20
CCL5	Chemokine (C-C motif) ligand 5
CCR7	Chemokine (C-C motif) receptor 7
CD14	CD14 surface receptor
CD40	CD40 surface receptor
CD68	CD68 surface receptor
cDNA	Complementary DNA
CEBPB	CCAAT/enhancer binding protein β
CO_2	Carbon dioxide
CoA	Coenzyme A
Cq	Cycle of quantification
CSN2	β-Casein
CSN3	κ-Casein
ctr	Control
CXCL3	Chemokine (C-x-C motif) ligand 3
CXCL5	Chemokine (C-x-C motif) ligand 5
CXCL8	Chemokine (C-X-C motif) ligand 8
CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1
CYP1B1	Cytochrome P450, family 1, subfamily B, polypeptide 1
DAB	3,3'-diaminobenzidine
ddH_2O	Double distilled water
DEPC H_2O	Diethylpyrocarbonate water
DMEM/F-12 HAM	Dulbecco's modified eagle's medium nutrient mixture f-12 ham
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DTT	Dithiothreitol
E. coli	*Escherichia coli*
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
eIF4E	Eukaryotic initiation factor 4E
EIF4EBP1	Eukaryotic translation initiation factor 4E-binding protein 1
ELF5	E74-like factor 5
ESI	Electrospray ionisation
EtOH	Ethanol

FAS	Fas cell surface death receptor
FBS	Fetal bovine Serum
FcRN	IgG Fc receptor
FDR	False discovery rate
FOS	FBJ murine osteosarcoma viral oncogene homolog
GAPDH	Glyceraldehyd-3-phosphate dehydrogenase
GR	Glucocorticoid receptor
H_2SO_4	Sulfuric acid
H3F3A	H3 histone, family 3A
HBSS	Hank's balanced salt solution
HC	Hydrocortisone
HP	Haptoglobin
HPLC	High liquid performance chromatography
HRP	Horse radish peroxidase
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IL10	Interleukin 10
IL13RA	Interleukin 13 receptor, alpha 1
IL1-A	Interleukin 1, alpha
IL1-B	Interleukin 1, beta
IL6	Interleukin 6
IRAK1	Interleukin-1 receptor-associated kinase 1
IRAK4	Interleukin-1 receptor-associated kinase 4
IRF3	Interferon regulatory factor 3
ITS	Insulin/Transferrin/Sodium selenite solution
JAK2	Janus kinase 2
KRT8	Cytokeratin 8
LAP	Lingual antimicrobial peptide
LBP	Lipopolysaccharide binding protein
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography-mass spectrometry/mass spectrometry
LF	Lactoferrin
LPO	Lactoperoxidase
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
LY96	Lymphocyte antigen 96
LYS	L-Lysine
LYZ1	Lysozyme 1 K
M	Molecular weight
MAPK1	Mitogen-activated protein kinase 1
MAPK8	Mitogen-activated protein kinase 8
MIQE	Minimum information for publication of quantitative real-time PCR experiments
miRNA	Micro RNA
M-MLV	Moloney murine leukemia virus
MMP1	Matrix metallopeptidase 1
MOI	Multiplicity of infection
mRNA	Messenger RNA
MS	Mass spectrometry
mTOR	Mammalian target of rapamycin
MX1	Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)
MX2	Myxovirus (influenza virus) resistance 2 (mouse)
MYD88	Myeloid differentiation primary response gene 88
m/z	Mass-to-charge-ratio
$NaHCO_3$	Natrium hydrogen carbonate
NCBI	National Library of Medicine
NEB	Negative energy balance

NEFA	Non-esterified fatty acids
NFκB	Nuclear factor κb
NOS2	Nitric oxide synthase 2, inducible
NTC	Non-template control
OD	Optical density
PAMP	Pathogen associated molecular pattern
pbMEC	primary bovine mammary epithelial cells
PBS	Phosphate Buffered Saline
PBS-T	PBS-Tween20
PenStrep	Penicillin/Streptomycin
PI3K	Phosphoinositide 3-kinase
pIGR	Polymeric immunoglobulin receptor
PMN	Polymorphonuclear leukocytes
PRL	Prolactin
PRLR	Prolactin receptor
qPCR	Quantitative polymerase chain reaction
RELA	V-rel reticuloendotheliosis viral oncogene homolog A (avian) (NF-kappa-B p65 subunit)
RIN	RNA integrity number
RNA	Ribonucleic acid
RP-HPLC	Reverse phase high liquid performance chromatography
rpm	Revolutions per minute
RPS6KB1	Ribosomal protein S6 kinase beta-1
rRNA	Ribosomal RNA
RT	Reverse transcription / room temperature
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
RUNX2	Runt-related transcription factor 2
S100A12	S100 calcium binding protein A12
S100A9	S100 calcium binding protein A9
S. aureus	Staphylococcus aureus
SAA3	Serum amyloid A3
SCC	Somatic cell count
SEM	Standard error of the mean
sIgA	Secretory immunoglobulin A
SOTA	Self-organizing tree algorithm
STAT2	Signal transducer and activator of transcription 2
STAT5	Signal transducer and activator of transcription 5
STAT5A	Signal transducer and activator of transcription 5A
STRING	Search tool for the retrieval of interacting genes
TAE	Tris-acetate-EDTA
TAP	Tracheal antimicrobial peptide
TCA cycle	Tricarboxylic acid cycle
TGFβ1	Transforming growth factor, beta 1
Th	Thomson
TIRAP	TCDD-inducible poly(ADP-ribose) polymerase
TLR	Toll-like receptor
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
TM	Annealing temperature
TMB	3,3,5,5-tetramethylbenzidine
TNF	Tumor necrosis factor
TNFR2	Tumor necrosis factor receptor 2
TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A
TNFα	Tumor necrosis factor α
TRAF6	TNF receptor-associated factor 6, E3 ubiquitin protein ligase
TSC	Tuberous sclerosis complex
UBB	Ubiquitine B

WNT4	Wingless-type MMTV integration site family member 4
YWHAZ	Tyrosine 3-monooxygenase / tryptophan 5-mono-oxygenase activation protein zeta polypeptide
YY1	Yin Yang 1

Summary

Primary bovine mammary epithelial cells (pbMEC) are increasingly used as primary cell culture model to investigate the molecular mechanisms of the innate immune response in the bovine mammary gland. High producing dairy cows are often challenged with mastitis pathogens like *Staphylococcus aureus* or *Escherichia coli*. As pbMEC are lining the alveoli of the bovine udder, they contribute to the blood-mammary gland barrier and are already known to be able to recognize pathogens and furthermore, induce specific signaling pathways that result in the production and secretion of chemotactic molecules. However, in order to elucidate molecular mechanisms that are responsible for the functionality of the innate immune response cascade, it is important to employ advanced cell culture models that better guarantee the transferability of *in vitro* data.

Therefore, our aim was to establish an advanced, more *in vivo-like* cell culture model that enables a more native behavior of pbMEC *in vitro*. pbMEC extracted from milk of healthy Brown Swiss cows were non-invasively extracted and used for the generation of a functional 3D cell culture model. The extracellular matrix was mimicked using the extracellular matrix-like scaffold Matrigel® and the cell culture medium was further supplemented with lactogenic hormones, like prolactin and hydrocortisone, and the essential amino acid L-lysine. The 3D cell culture model was further used to conduct two infection studies with pbMEC obtained from Brown Swiss cows. The aim of the first study was to elucidate a set of special molecular biomarkers that ~~might~~ play a key role in the promotion of innate immunity against gram-positive bacteria. Furthermore, those molecular biomarkers should be used to distinguish between high and low responder dairy cows. The term high and low responder cow, hereby refers to the ability of the animal to produce and secrete high specific immunoglobulin concentrations into the milk. The human pathogen *Clostridium difficile* is of great interest in case of the production of immune milk that can be applied to protect patients from the recurrent *Clostridium difficile* associated diarrhea. Therefore, this gram-positive pathogen, was used for the immune treatment *in vitro* and for the preceding immunization of the cows. Within the second immune study, the effect of the metabolite beta-hydroxybutyrate (BHBA) on the innate immune response of pbMEC challenged with the gram-negative mastitis pathogen *Escherichia coli* was investigated. Elevated levels of BHBA normally accumulate in the early phase of lactation, in the case of negative energy balance, and often result in the metabolic disorder ketosis.

We successfully established a 3D cell culture of pbMEC isolated from fresh milk and showed that pbMEC cultured in 3D cell culture showed a polarized phenotype and were hence able to form alveolar-like structures *in vitro*. Furthermore, the cultivation of pbMEC resulted in an enhanced production and secretion of milk and whey proteins. Changes in the gene expression pattern of genes coding for milk proteins and for components of the JAK-STAT and mTOR pathway, were compared between pbMEC cultured in 3D and 2D cell culture. The enhanced functionality of the cells cultured in 3D cell culture was verified by RT-qPCR and LC-MS/MS measurements. Furthermore, within the first infection study, the gene expression profiles of 61 selected immune relevant genes were compared between the high and low responder animals using a RT-qPCR approach (BioMark^{TM} HD 96x96, Fluidigm). Results of this study indicated, that especially the pro-inflammatory cytokines are suitable to serve as molecular biomarkers to select for high responder animals. In the second infection study we were able to determine a declined innate immune response when pbMEC were co-stimulated with *Escherichia coli* and BHBA *in vitro*. The immunosuppressive effect of BHBA on the innate immune response of pbMEC was hereby evaluated using RT-qPCR and ELISA measurements.

Based on these results we concluded that a functional 3D cell culture model of pbMEC was successfully established, which can be further used for immunological and metabolic studies. We are convinced that this 3D cell culture model can be used in future studies to obtain more faithful, valid and representative data, as this cell culture approach represents a more *in-vivo* like model of the functional bovine mammary gland.

Zusammenfassung

Zur Erforschung der molekularen Mechanismen der innaten Immunantwort im bovinen Euter werden vor allem primäre bovine Euterepithelzellen (pbMEC) verwendet. Da pbMEC die Alveolen des bovinen Euters auskleiden, stellen sie die erste zelluläre Abwehr gegen mögliche Pathogene dar und bilden somit die Blut-Euter-Schranke. In früheren Studien wurde bereits gezeigt, dass pbMEC Pathogene erkennen und spezifische Signaltransduktionswege induzieren können, die zur Produktion und Sekretion von chemotaktischen Molekülen führen. Es ist allerdings von großer Bedeutung abgewandelte Zellkulturmodelle, für die Erforschung zugrundeliegender molekular-physiologischer Mechanismen der innaten Immunantwort, zu etablieren. Dies könnte die Übertragbarkeit der Daten, die in einem *in vitro* Modell generiert wurden, erleichtern.

Unser Ziel war es ein funktionales 3D Zellkulturmodell der pbMEC zu generieren, welches versucht die Umgebung der pbMEC möglichst realitätsgetreu nachzuahmen. Hierfür wurden pbMEC aus der Milch von Brown Swiss Kühen isoliert und auf dem, die extrazelluläre Matrix nachahmendem Gerüst, Matrigel®, kultiviert. Den unterschiedlichen Zellkulturmedien-Kompositionen, wurden zudem noch die laktogenen Hormone Prolaktin und Hydrokortison und die essentielle Aminosäure L-Lysin zugesetzt. Basierend auf diesem funktionalen 3D Zellkultur Ansatz wurden zwei Infektionsstudien an isolierten pbMEC durchgeführt. Im Rahmen der ersten Studie sollten potentielle molekulare Biomarker gefunden werden, die eine Schlüsselrolle in der innaten Immunantwort gegen gram-positive Bakterien, einnehmen. Zudem sollten die molekularen Biomarker dazu beitragen, laktierende Kühe in „High Responder“ und „Low Responder“ zu unterteilen. Die Begriffe High und Low Responder definieren sich hierbei durch die Menge an spezifischem Immunglobulin A, das die Kuh durch eine vorangegangene Immunisierung produzieren und in die Milch abgeben kann. Im Rahmen dieser ersten Immunstudie wurde der gram-positive Erreger *Clostridium difficile* sowohl für die Immunisierung der Tiere als auch für die *in vitro* Immunstudien verwendet. Der gram-positive Erreger ist von großem Interesse für die Produktion von Immun-Milch, die zur Prävention von *Clostridium difficile* assoziierten Durchfallerkrankungen eingesetzt werden soll. Die zweite Studie befasste sich mit den Auswirkungen des Ketonkörpers Beta-Hydroxybutyrat (BHBA) auf die innate Immunantwort der pbMEC, welche mit dem gram-negativen Mastitis-Erreger *Escherichia coli* induziert wurden. Erhöhte BHBA Konzentrationen akkumulieren vor allem in der frühen Phase der Laktation und führen im Falle einer negativen Energiebilanz zur Entstehung der Stoffwechselerkrankung Ketose

Wir konnten erfolgreich ein 3D Zellkulturmodell von, aus frischer Milch isolierten, pbMEC etablieren und zeigen, dass pbMEC, die in 3D Zellkultur kultiviert wurden, einen polarisierten Phänotyp aufwiesen und dadurch in der Lage waren Alveolen-artige Strukturen *in vitro* zu bilden. Zudem resultierte die Kultivierung der pbMEC in 3D Zellkultur in einer verstärkten Produktion und Sekretion von Milch- und Molkenproteinen. Die Änderungen im Genexpressionsprofil von Genen, die für Milchproteine, aber auch für Komponenten des JAK-STAT und mTOR Signaltransduktionswegs kodierten, wurden zwischen pbMEC, die in 3D und 2D Zellkultur kultiviert wurden, verglichen. Die verbesserte Funktionalität der pbMEC in 3D Zellkultur, konnte durch RT-qPCR Experimente und LC-MS/MS Messungen nachgewiesen werden. In der ersten immunologischen Studie, die mit Hilfe des 3D Zellkulturmodells durchgeführt wurde, konnten durch ein RT-qPCR Verfahren (BioMark™ HD 96x96, Fluidigm), 61 Expressionsprofile von ausgewählten Immungenen zwischen den High und Low Responder Tieren abgeglichen werden. Es konnte gezeigt werden, dass sich vor allem die pro-inflammatorischen Zytokine als potentielle molekulare Biomarker eignen. Im Rahmen der zweiten immunologischen Studie konnte mit Hilfe von RT-qPCR und ELISA Messungen ein immunsuppresiver Effekt durch die Co-Stimulation der pbMEC mit *Escherichia coli* und BHBA gezeigt werden.

Basierend auf diesen Ergebnissen konnte die erfolgreiche Entwicklung eines funktionalen 3D Zellkulturmodells der pbMEC beschrieben werden. Dieses 3D Zellkulturmodell, das die *in vivo* Situation der pbMEC besser repräsentiert, erscheint sehr vielversprechend für die zukünftige Generierung von vertrauensvollen und *in vivo* nahen Forschungsergebnissen.

1 Introduction

1.1 Common metabolic disorders and infectious diseases of dairy cows

Improved feeding conditions and the application of modern breeding techniques, resulted in a constantly increasing milk yield of dairy cows. However, high producing dairy cows, often experience metabolic disorders and/or mammary gland infections, not only affecting the animal's welfare, but also milk yield and quality and hence profitability of the dairy cow [1]. As the milk production rapidly increases after calving, during the first weeks of lactation, high producing dairy cows often experience a state of negative energy balance (NEB), as they are not able to produce enough energy for maintenance, growth and milk production through feed intake and digestion [2,3]. As not enough glucose is available the body fat reserves of the animal are mobilized, resulting in the increase of non-esterified fatty acids (NEFA). Those NEFA are further metabolized in order to produce acetyl-CoA via beta-oxidation in the liver. In case of NEB, gluconeogenesis takes place in the liver, wherefore oxaloacetate that is known to normally be the input substrate of the tricarboxylic acid cycle (TCA), is required. Therefore, oxaloacetate is not any more available for the removal of acetyl-CoA via the TCA (Figure 1). Acetyl-CoA hence accumulates and is further metabolized via ketogenesis to ketone bodies. This leads to increased concentrations of the ketone bodies, acetoacetate, acetone and BHBA in the blood plasma of ketotic dairy cows, leading to the development of subclinical (1.4 mM BHBA) or clinical ketosis (2 to 3 mM BHBA) [2–5]. High producing dairy cows are not only affected by metabolic disorders, but also by inflammatory diseases of the bovine mammary gland, like mastitis. As a co-occurrence of both diseases has been detected several times in the past, it is already discussed whether it could be possible that the metabolic disorder ketosis correlates with an increased risk of clinical mastitis. The disease mastitis is a quite common disease among dairy cows, as they're often challenged with the two major mastitis pathogens *Escherichia coli (E. coli*) and *Staphylococcus aureus* (*S. aureus*). Those pathogens manage, despite of the defense capabilities of the bovine mammary gland, to cross the teat and blood-mammary gland barrier [6,7]. Depending on the mastitis pathogen, the cows either endure subclinical (gram-positive pathogens) or clinical mastitis (gram-negative pathogens), whereby clinical mastitis is defined by acute symptoms of inflammation in the milk collecting cistern and the teat, by a reduced milk production and an elevated somatic cell count (SCC) [1]. Whereas subclinical mastitis is only characterized by an elevated SCC and reduced milk yield in the

absence of typical inflammatory symptoms [1,3]. As mastitis and ketosis are affecting dairy cow welfare, as well as their profitability, it is of common interest to elucidate underlying mechanisms responsible for the functionality of the innate immune response of the bovine mammary gland and how it is influenced by several metabolites [1,8]. So far, the effect of the main ketone body BHBA on the defense capability of the bovine mammary gland is mainly unknown.

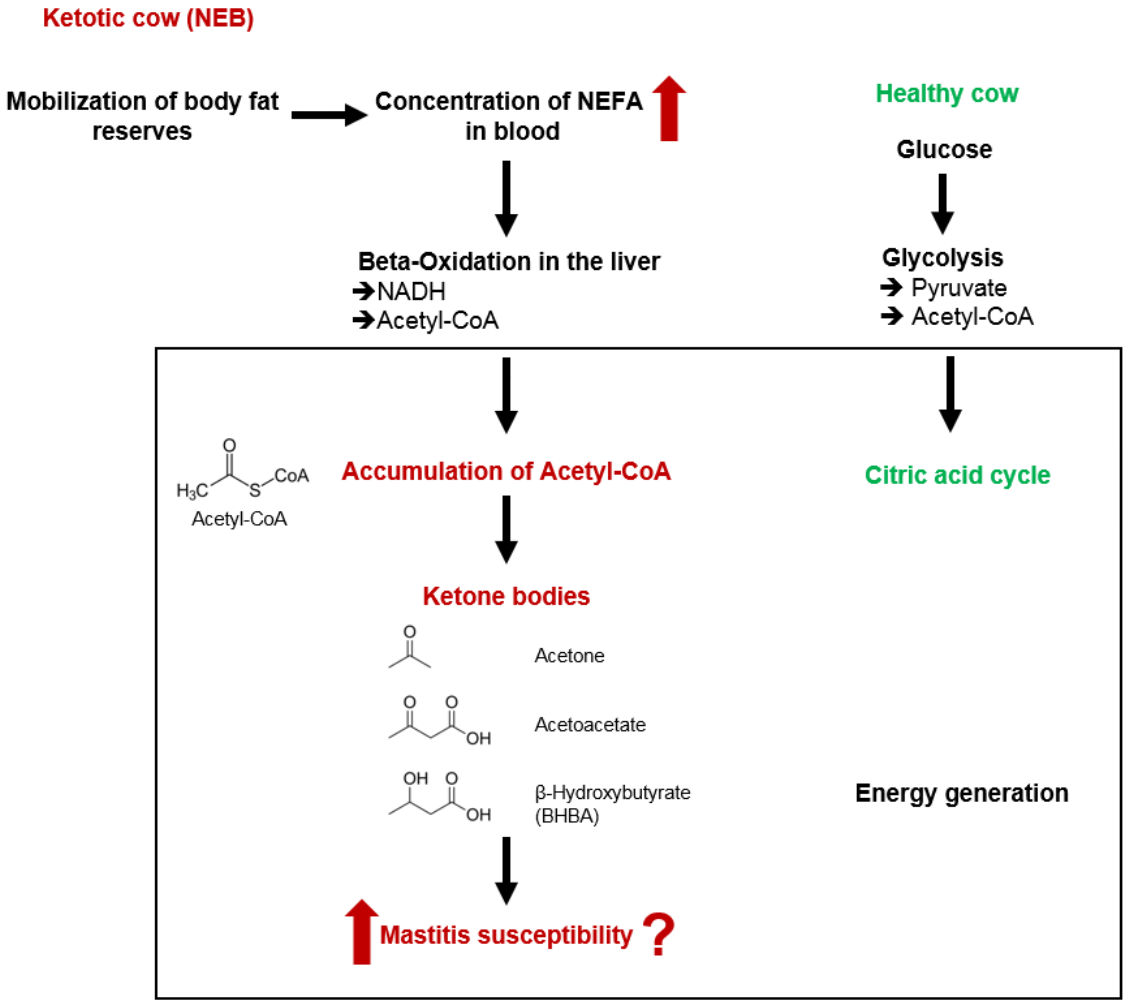


Figure 1: Metabolic pathway of cows in negative energy balance:
In negative energy balance body fat reserves are mobilized resulting in increased plasma concentrations of non-esterified fatty acids (NEFA). In the liver, acetyl-CoA is formed via the β-oxidation pathway and accumulates due to an insufficient availability of oxaloacetate for the tricarboxylic acid (TCA) cycle. Instead of entering the TCA cycle (healthy cow), acetyl-CoA is metabolized via ketogenesis [4] and the ketone bodies acetone, acetoacetate and BHBA are built.

1.2 The bovine mammary gland

1.2.1 Milk production and defense mechanisms

The mature, lactating bovine mammary gland is an apocrine gland, consisting of 4 separate quarters. Each quarter consist of a teat canal associated with ducts and alveoli [9,10]. The alveoli, the milk producing subunits of the gland are located in the glandular tissue. Milk ducts lead to the gland cistern, representing the storage place for the milk. The gland cistern leads to the teat cistern and the teat canal that can be closed between the milkings due to the teat sphincter. The main task of the bovine mammary gland is milk production to supply the newborn calf with water, nutrients and immunoglobulins. Additionally, the teat itself functions as defense barrier against invading mastitis pathogens (Figure 2). Through the teat sphincter, the teat canal is tightly closed during the milkings and furthermore, the teat canal is lined by keratin that avoids the migration of pathogens into the teat canal and further contains bactericidal proteins and fatty acids [11–13]. At the transition of the teat canal to the teat cistern the Rosette of Furstenberg is located, a connective tissue containing immune cells, like macrophages [10]. The pbMEC which are lining the alveoli form the first cellular barrier before the pathogen is able to enter the blood circulation [6,7]. Therefore, the pbMEC mainly contribute to the innate immune system of the bovine mammary gland [14–17].

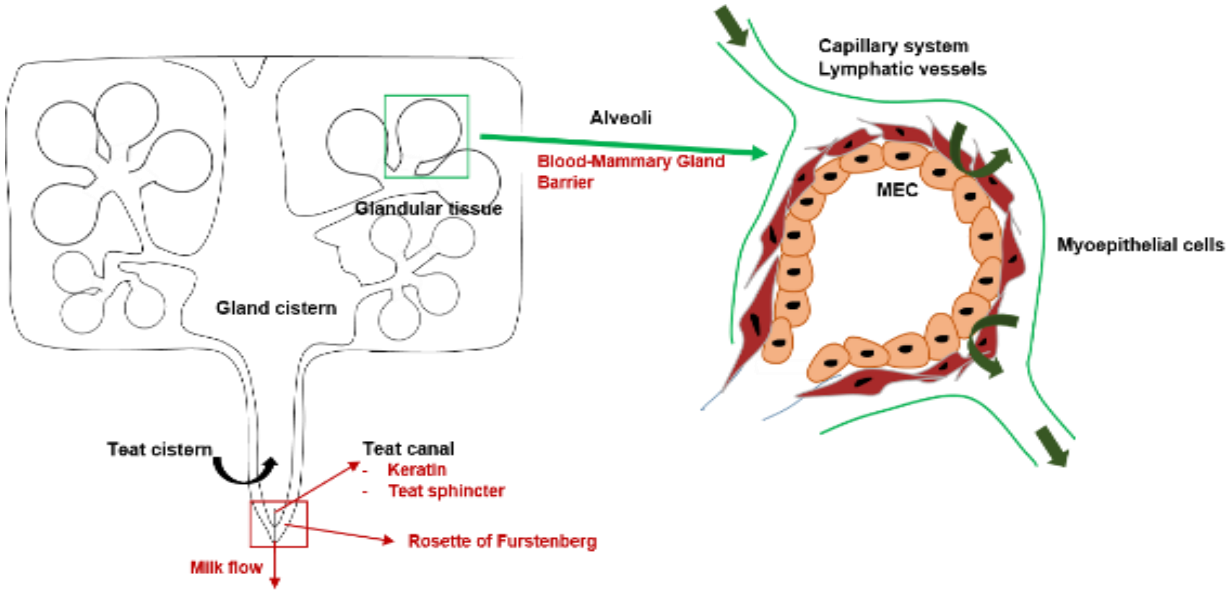


Figure 2: The bovine mammary gland - structure and defense mechanisms:
(Left) One quarter of the bovine mammary gland is exemplarily shown. Each quarter contains glandular tissue that is mainly built through the alveolar cells [green box]. The bovine mammary gland further consists of the gland cistern and the teat cistern where part of the milk is stored between the milking. Each quarter of the gland owns its particular defense barriers against potential pathogens, like the milk flow, the Rosette

of Furstenberg and the teat canal that is coated with keratin and can be tightly closed through the teat sphincter. **(Right)** The alveoli are lined by primary bovine mammary epithelial cells, the milk producing cells of the gland, so that a lumen is formed. The mammary epithelial cells are surrounded by contractile myoepithelial cells, blood capillaries and lymphatic vessels and are therefore known to build the blood-mammary gland barrier.

1.2.2 Primary bovine mammary epithelial cells

pbMEC are situated on a basal-membrane and are lining the lumen of the alveoli [10]. They are further surrounded by contractile myoepithelial cells, blood capillaries and lymphatic vessels, building the blood-mammary gland barrier [6,7] (Figure 2). It has already been proven that pbMEC are not only responsible for the milk protein production and secretion into the alveolar lumen, but also for the transepithelial transport of immunoglobulins into the milk [18]. Additionally, they bear toll-like receptors (TLR) on their cell surface enabling the pbMEC to recognize invading bacteria through pathogen associated molecular patterns (PAMP), like lipopolysaccharide (LPS) or lipoteichoic acid (LTA) [17,19–21].

1.2.2.1 Lactation and induction of milk protein production

In order to induce lactogenesis, the alveolar cells have to proliferate and further differentiate before the synthesis and secretion of milk constituents can appear [9]. For the synthesis of milk and whey proteins it is necessary that non-essential and essential amino acids are supplied to the gland by the bloodstream [22]. Inside the secretory epithelium, the amino acids are then used for the synthesis of αS1-casein, αS2-casein, β-casein, κ-casein and the whey proteins α-lactalbumin and β-lactoglobulin [22]. The biosynthesis of the proteins is either induced by essential amino acids, like L-lysine (LYS) or by hormones like prolactin (PRL) [23–26]. Those molecules are able to induce intracellular signaling cascades, resulting in the activation of nuclear transcription factors and hence transcriptional initiation of the expression of genes coding for milk proteins [24,27]. As endocrine factors seems to play an important role in the induction of gene expression, previous studies with mammary epithelial cell cultures, and also *in vivo* experiments with rodents, showed that especially insulin, PRL and hydrocortisone (HC) are required to induce milk protein synthesis [22]. Besides the primary lactogenic hormone, PRL, that is known to have a positive effect on the proliferation and differentiation of mammary epithelial cells, as well as on the induction of the JAK-STAT signaling pathway, leading to the stimulation of milk protein gene expression, the glucocorticoid HC, is also known to be an essential lactogenic hormone [23,24,27,28]. HC and PRL are responsible for the differentiation of the alveolar system and they therefore together synergistically regulate the milk protein gene expression [23,29]. Furthermore, it has been shown that the essential amino acid LYS, is able to induce the mTOR signaling pathway by activating the

mitogen-activated protein kinase 1 (MAPK1) [25]. The mammary epithelium bears the prolactin receptor (PRLR), a member of the cytokine receptor superfamily that associates with the janus kinase (JAK) family of tyrosine kinases and therefore is able to directly respond to the endocrine PRL signal (Figure 3). The extracellular recognition of PRL, leads to the activation of JAK2 by transphosphorylation and hence to the phosphorylation of the PRLR. The phosphorylation of the PRLR enables the binding of the signal transducer and activator of transcription 5A (STAT5A) protein via Src homology (SH2) domains. Furthermore, the tyrosine of STAT5A is phosphorylated, resulting in the dimerization of two STAT5A proteins and the translocation of the dimeric complex into the nucleus. There, the transcription of the milk protein genes is induced, as it has been shown that all milk proteins contain at least one binding site for STAT5A [23,27,30]. E74-like factor 5 (ELF5) further enhances the transcriptional activity of STAT5A and therefore also plays an important role in the expression of milk protein genes as well as in the development of the mammary gland by the regulation of the terminal differentiation of alveolar cells [31–33]. The transcripts are then further processed by the translation initiation complex, consisting of the mammalian target of rapamycin (mTOR) (Figure 3). The mTOR signal transduction acts as a regulator of protein synthesis on transcriptional and translational level, whereby the inducing signals originate from essential amino acids, nutrients and mitogens [30]. The cationic amino acid transporter (CAT1) is mainly responsible for the transport of cationic amino acids, like LYS, into mammary epithelial cells, resulting in the activation of kinases that phosphorylate and hence activate mTOR [30]. The activation of mTOR leads to the phosphorylation of the downstream effector molecules ribosomal protein S6 kinase veta-1 (RPS6KB1) and eukaryotic translation initiation factor 4E-binding protein 1 (EIF4EBP1). The phosphorylation of EIF4EBP1 induces the dissociation of eukaryotic initiation factor 4E (eIF4E) from the mTOR protein complex. The eIF4E complex in turn associates with other eukaryotic translation initiation factors, so that a complex is formed that in turn binds to the 40S ribosomal subunit, enabling the formation of the translation initiation complex. In addition, the activated RPS6KB1 phosphorylates the ribosomal protein S6 promoting the initiation of translation [30,34–36].

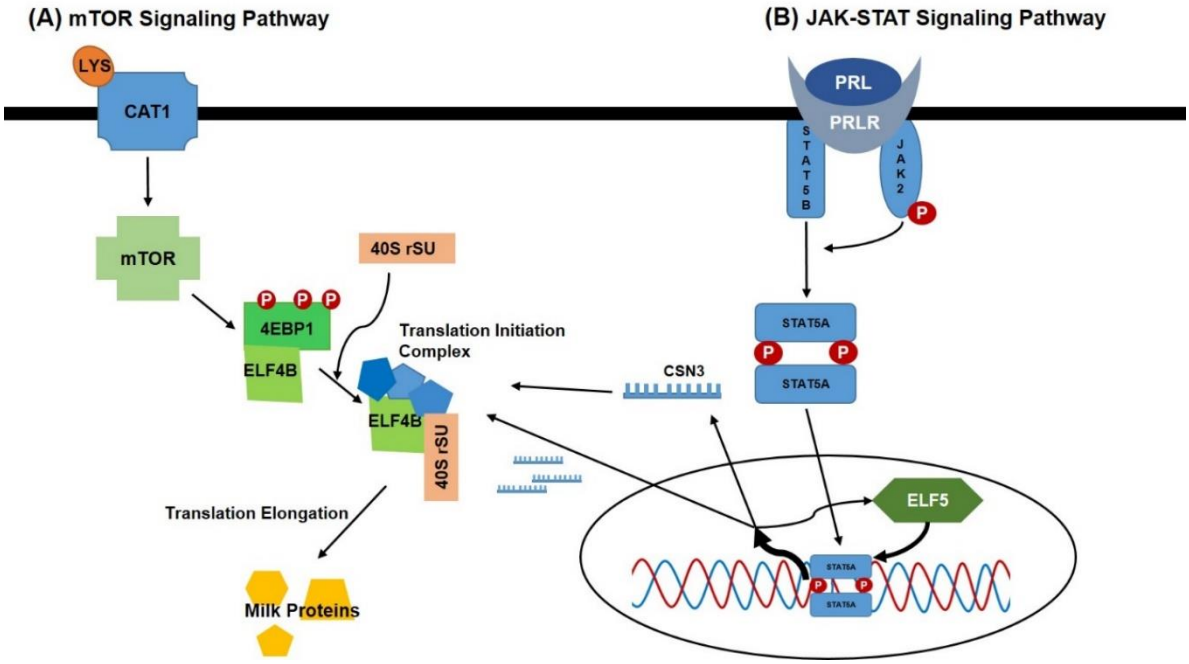


Figure 3: Schematic description of the JAK-STAT and mTOR signaling pathway:
(A) The mTOR signaling pathway can be induced through several external signals, like essential amino acids that are recognized by the CAT1 receptor, resulting in a phosphorylation cascade that enables the formation of the translation initiation complex with the 40S ribosomal subunit (40S rSU) and hence the protein biosynthesis of milk proteins. **(B)** The JAK-STAT signaling pathway is induced by the extracellular recognition of PRL through the PRLR, resulting in the activation of downstream effector molecules by phosphorylation. Hereby the STAT5A protein is phosphorylated and dimerizes. This transcription factor complex, which is further activated by ELF5, translocates into the nucleus and activates the transcription of milk protein genes. The resulting milk protein mRNAs are then released into the cytoplasm and translated into milk proteins due to the translation initiation complex of the mTOR pathway.

1.2.2.2 Innate Immunity of the bovine mammary gland – Toll-like receptor pathway

Innate immunity is directly induced upon pathogen recognition. The pathogen is thereby recognized via PAMP by germline-encoded receptors of the innate immune system [37,38]. For the recognition of gram-positive and gram-negative pathogens, different TLR are required. Gram-positive pathogens, like *S. aureus*, are mainly recognized by TLR1/TLR2 heterodimers, as this receptor is able to bind LTA, the main cell wall constituent of gram-positive bacteria [39]. By contrast, gram-negative pathogens, like *E. coli*, are recognized by TLR4 homodimers that are able to bind LPS, the main constituent of the outer membrane of gram-negative bacteria, via the co-factor cluster of differentiation 14 (CD14) [37,39]. TLR4 signal transduction is mediated by co-factors like CD14, lipopolysaccharide binding protein (LBP) and lymphocyte antigen 96 (LY96). Both TLR1/TLR2 and TLR4 utilize the myeloid differentiation primary response gene 88 (MYD88) dependent pathway [37,39]. Upon activation of TLR4 or TLR1/TLR2, the toll/interleukin 1 receptor homology domain adaptor protein (TIRAP) mediates protein-protein interactions between the TIR domain bearing TLR and their TIR domain bearing adaptor protein MYD88 (Figure 4). Activated

MYD88 initiates the downstream signaling cascade by recruiting interleukin-1 receptor-associated kinase 4 (IRAK4) and IRAK1. IRAK1 further phosphorylates TNF receptor associated factor 6 (TRAF6), leading to the phosphorylation of Iκκ - β and hence the translocation of the transcription factor nuclear factor κB (NFκB) into the nucleus [39,40]. The transcription factor NFκB is known to induce the gene expression of several chemokines (CCL2, CCL5, CCL20, CXCL5, CXCL8, CXCL3), inflammatory cytokines (IL1-A, IL1-B, IL6, IL10, TNFα, TGFβ1), acute phase proteins (SAA3, S100A9, S100A12) and antimicrobial peptides (LF, LYZ1, LPO, TAP, LAP). All in all it is discussed that the master transcription factor NFκB regulates the expression of more than 200 different immune genes [21] The induced chemotactic gradient results in the recruitment of leukocytes, like macrophages and neutrophils, to the site of infection, whereby the antimicrobial peptides directly combat the invading pathogens due to their bactericidal properties [17,19,20].

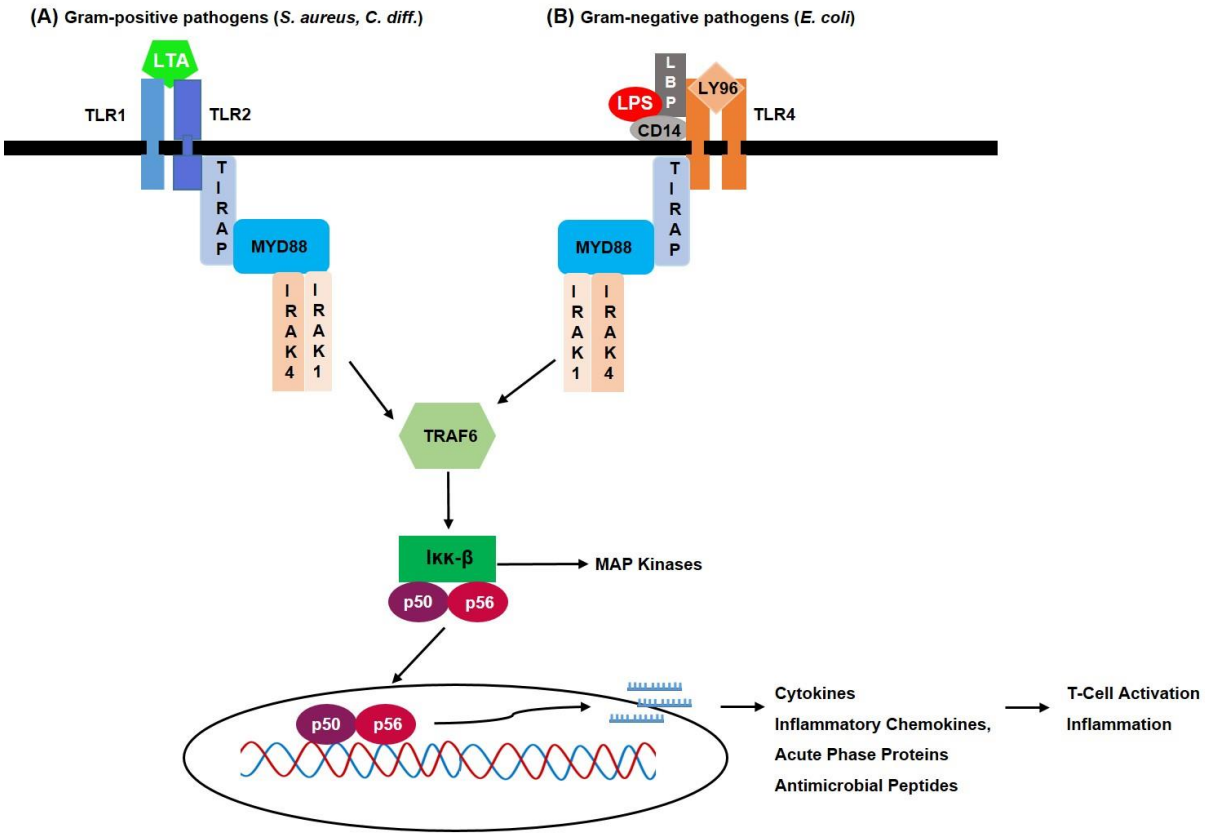


Figure 4: MYD88 dependent TLR4 and TLR1/TLR2 signaling upon pathogen recognition:

Gram-positive pathogens **(A)** are recognized through their cell wall component LTA by TLR1/TLR2 heterodimers. Gram-negative pathogens **(B)**, by contrast are recognized through the LPS present in their outer membrane by co-factors of TLR4, resulting in homodimerization of TLR4. TLR4 and TLR1/TLR2 downstream signaling is in this case initiated by the TIR domain containing adaptor protein MYD88, resulting in the activation and translocation of NFκB (p50-p56) into the nucleus. The transcription factor NFκB finally induces the transcription of genes coding for cytokines, inflammatory chemokines, acute phase proteins and antimicrobial peptides, leading to the recruitment of other immune cells and the activation of adaptive immunity.

1.3 Utilization of the immune response capability of dairy cows in animal science and human medicine

It is already known that bovine colostrum and milk are rich in immunoglobulins. The immunoglobulins are of great importance for the homologous transfer of passive immunity from the mother to the calf [41]. It has however been shown that dairy cows do have a quite diverse immune response and therefore exhibit a distinctly different magnitude of immune response against invading pathogens [42,43]. Dairy cows with high immunoglobulin levels in blood and milk have been shown to exhibit a lower disease occurrence as well as a more robust immune response [42,42,43]. Understanding the mechanisms behind the phenomenon of animals with high or low immunoglobulin levels in blood and milk could be beneficial in order to improve milk quality and hence profitability of the dairy cows, while reducing the extensive use of antibiotics for the treatment of diseases like mastitis [8,43,44]. However, the investigation of the underlying genetic level, responsible for the capability of dairy cows to combat pathogens, is not only of interest in case of animal science, but also in the case of the production of human medical products out of bovine milk [41]. A lot of research is done in order to realize the idea of an effective heterologous passive transfer of immunity via milk derived products [45–47]. This is of great interest especially for the prevention of gastrointestinal diseases in humans [41]. Researchers therefore aim to increase the amount of pathogen specific immunoglobulin G (IgG) and secretory immunoglobulin A (sIgA) in bovine milk through repeated immunization of the pregnant or lactating dairy cow against one or even more antigens, representing pathogens of bacterial or viral origin [41]. The repeated immunization stimulates plasma cells to produce specific IgG and sIgA, which are both transported from the blood serum into the milk by specialized transepithelial receptors. IgG is transported by the neonatal Fc receptor (FcRn) and IgA by the polymorphic immunoglobulin receptor (pIgR) [18]. Both are located on the basolateral surface of the mammary epithelial cells [18]. Especially IgA in its secretory form is of great interest in case of the prevention from gastrointestinal diseases. sIgA is known to neutralize pathogens in the intestinal lumen by neutralizing bacterial enterotoxins, and prevents the adhesion of enteropathogenic bacteria to mucosal epithelial cells [48,49]. Furthermore, especially sIgA, due to the secretory piece component, is quite resistant against gastrointestinal digestion so that most of the specific immunoglobulins obtained from so called immune milk products will reach the gastrointestinal tract and remain intact [50]. The high yield of specific immunoglobulins required to guarantee an effect of heterologous passive immunization, demands the pre-selection of animals that are high responders and hence show a good and fast immune response and a high immunoglobulin yield in milk, upon vaccination [42,43]. This implies a new research field that aims to elucidate the

molecular mechanisms responsible for the high or low immune responsiveness of dairy cows towards pathogens and vaccination. Markers have to be explored that can help to distinguish between high and low responder animals before the start of immune milk production. Hereby, the bovine mammary epithelial cells, as well as the blood lymphocytes, are in the research focus. The results obtained *in vitro* should of course be representative and directly transferable to the whole organ or organism. Therefore, new advanced cell culture techniques, despite of the commonly used 2-dimensional (2D) culture techniques, are required that are able to better resemble the *in vivo* situation.

1.4 Advanced cell culture techniques for the investigation of molecular processes

1.4.1 Restrictions of 2-dimensional cell culture methods

2D culture methods are routinely used in most of the laboratories all over the world. As the cells are just grown on the plastic surfaces of tissue culture plates, supplemented with the required cell culture medium, it is a quite cheap and easy method [51,52]. Normally, cells cultivated under those conditions only build monolayer cultures, not really representing the cellular architecture they exhibit *in vivo* [53]. In the organism, cells are normally surrounded by a complex environment consisting of extracellular matrices (ECM), lymph vessels, capillary tissue and other cell types [51,54,55]. The tissue-like architecture can normally not be recapitulated under the simplified conditions of 2D cell culture [51,52,56,57]. However, 2D cell culture systems are more and more used to investigate underlying molecular processes that normally take place *in vivo*, like the immune response, changes in gene expression and the metabolic response of cells [51,55,58,59]. Certainly, the transferability of the obtained data to an organ or organism is controversially discussed, especially as it has been shown that cells cultured in 2D cell culture lack specialized cell-cell and cell-matrix contacts that are important in the case of cellular signaling, transport processes and cell differentiation [24,51,52]. Therefore, researchers aim to establish more advanced cell culture models that might be able to better recapitulate the *in vivo* situation of the respective cell types [51,53].

1.4.2 The benefits of 3-dimensional cell culture methods

The term 3D cell culture in general defines that different cell types are no longer cultivated on plastic surfaces of tissue culture plates, but more over in or on scaffolds that mimic the ECM of the cell type of interest [51]. Therefore, naturally occurring materials like collagen, laminin, fibronectin and others are either purified from animal tissue or tumors (Engelbreth Holm Swarm Mouse Sarcoma), or are fabricated from synthetic polymers, in order to build a non-toxic and biocompatible scaffold [51,54,60–63]. Despite of the problems connected with the 3D cell culture approach, like the development of a cheap, inert, porous scaffold, customized for the requirements of the different cultured cells, it has already been shown that primary cells cultured in 3D cell culture behaved more *in vivo*-like than primary cells that were cultivated in 2D cell culture [55,58]. Pampaloni et al. (2007) described that cells cultured in 3D cell culture developed specialized cell-cell and cell-matrix contacts, showed an increased cell viability, differentiated into cells with an *in vivo*-like morphology and showed a more *in vivo*-like gene expression profile and immune and stress response [51]. This indicated that cells cultivated in 3D cell culture behave more natively and therefore, might be able to provide more relevant research results that could be beneficial to close the gap between 2D cell culture and the physiological tissue, reducing the need of animal testing in the future [51,64].

1.4.3 The importance of the cellular environment for engineering cellular behavior and functionality

The ECM of cells is a complex mixture of proteins and sugars that is known to influence cellular behavior, function and identity [65,66]. Furthermore, it also plays an important role as communicating structure with signaling properties and contributes to the microenvironment of the cultivated cells, as it is able to retain growth factors, cytokines and other small molecules [65,67,68]. The specific composition and the biochemical and defined geometrical structure are quite difficult to reproduce for the 3D cell culture approaches. However, the presence of an ECM-like scaffold is mandatory for the proper functionality of cells [51]. The adhesion of cells to the ECM is enabled by macromolecules, like integrins and proteoglycans, present in the ECM [69]. The adhesion is important for the elasticity of the cell layer, cell migration and cellular processes like cell proliferation, differentiation and metabolism [65]. As the attachment of the cells to the ECM-like scaffold contributes to the establishment of a functional cytoskeleton and hence a polarized phenotype, the scaffold also facilitates the development of cell-cell contacts via the cytoskeleton [69–71]. Therefore, cells cultivated on an ECM-like scaffold *in vitro* are able to better

regulate intracellular processes through the transduction of extracellular signals into the cells [51,65].

2 Aim of the study

Within this thesis, we wanted to establish a functional 3D cell culture model of primary bovine mammary epithelial cells that were non-invasively isolated out of fresh cow milk. This 3D cell culture model should be further used to investigate the effect of different immune stimulatory agents, like the pathogens *C. diff.* and *E. coli* and the metabolite BHBA, on the innate immune response capacity of pbMEC. Our goal was to distinguish animals with significant different immune responses, based on their gene expression level, due to a special set of molecular biomarkers. These expressed biomarkers are part of the downstream signaling process of the innate immune response. Furthermore, we wanted to show, the dependency and correlation between ketosis markers and the increased mastitis susceptibility in high producing dairy cows. Hereby, especially the predominant ketone body BHBA and its effect on the physiological function of the pbMEC was in the research focus.

3 Materials and Methods

3.1 Experimental set-ups

3.1.1 Experimental set-up I – Establishment of a 3D cell culture model of pbMEC

For experimental set-up I, pbMEC isolated from milk were used to establish a 3D cell culture model. pbMEC were therefore cultivated on Matrigel® coated 6-well culture plates and furthermore supplemented with cell culture medium containing lactogenic substances, like the hormones PRL and HC and the essential amino acid LYS. The gene expression of selected milk protein genes and of genes coding for components of the JAK-STAT and mTOR signaling pathway, was evaluated. Additionally, secreted proteins in cell culture supernatants were detected with LC-MS/MS measurements (Appendix I).

3.1.2 Experimental set-up II –RT-qPCR for the elucidation of molecular biomarkers

Within the experimental set-up II, we aimed to elucidate molecular biomarkers within a set of 61 candidate immune genes by RT-qPCR (BioMark™ HD 96x96, Fluidigm), in order to distinguish between cows with a good immune response (high responder) and cows with a bad immune response (low responder). Therefore, pbMEC obtained from cows immunized against the non-mastitis pathogen *C. diff.*, were challenged with the formalin inactivated gram-positive pathogen in cell culture (Appendix II).

3.1.3 Experimental set-up III – Effect of BHBA on the innate immune response of pbMEC

The third experimental set-up aimed to elucidate whether the ketone body BHBA interferes with the innate immune response of pbMEC against the gram-negative mastitis pathogen *E. coli*. The gene expression profiles of representative target genes were investigated using RT-qPCR measurements on the CFX384™ Real-Time System (Bio-Rad Laboratories GmbH) and the results were further proofed at protein level via ELISA measurements (Appendix III).

3.2 Animals

For the non-invasive isolation of pbMEC from milk, healthy, first lactating Brown Swiss cows were either purchased (experimental set-up II) or, in case of the 3D cell culture and metabolic/immunological study (experimental set-up I and III), obtained from Veitshof Research Station (Technische Universität München, Freising, Germany). The milk and cell sampling took

place in mid lactation (day 100 – 200), whereby only healthy animals with a somatic cell count (SCC) below 200,000 cells per milliliter were considered for the experiments. The health status of the animals was monitored daily during the milk collection and once a month from the Landeskuratorium der Erzeugerringe für tierische Veredelung in Bayern e. V..

3.3 *Clostridium difficile* vaccine and immunization scheme

Nine first lactating Brown Swiss cows that were sampled for experimental set-up II (Appendix II), were immunized during a time-period of 31 weeks, with the MucoCD-N and MucoCD-I vaccine against *C. diff.* (IDT Biologika GmbH, Dessau-Rosslau, Germany) according to a strict immunization scheme. The animals were consequently immunized with the nasal MucoCD-N vaccine every second week and at the same time alternating perkutan in the area of the mammary lymph nodes or intercutan with the MucoCD-I vaccine. The vaccination procedure was approved by the government of Upper Bavaria (AZ. 55.2-1-54-2532.6-17-2012). The health status of the animals was routinely monitored prior and during vaccination as described above. Furthermore, the stool of the animals was tested for *C. diff.* prior to immunization. Feces of all animals were *C. diff.* negative (Leiden University, Medical Center).

3.4 3D cell culture of pbMEC

3.4.1 Matrigel® coating of cell culture plates

In order to establish a physiological functional 3D cell culture model of pbMEC, Matrigel® (growth factor reduced, Corning Inc., Corning, New York, USA), a commercially available scaffold, mimicking the ECM of epithelial cells, was used. Matrigel® is a naturally occurring material that is extracted from the Engelbreth-Holm Swarm Mouse Sarcoma and is therefore rich in ECM proteins like laminin (60 %), collagen IV (30 %), entactin (8 %), heparin sulphate proteoglycan and several growth factors. The 6-well culture plates (Maxisorp, Nunc®, Sigma-Aldrich) for the 3D cell culture approaches were coated with 2.4 mg/ml Matrigel® previous to the extraction of pbMEC. In brief, all laboratory equipment needed for the coating procedure (culture plates, pipettes, falcons, Eppendorf tubes, medium) had to be refrigerated, as the thermosensitive material Matrigel® was only liquid at approximately 4 °C – 8 °C, but polymerized at higher temperatures. The 2.4 mg/ml Matrigel® mixture was obtained by diluting the thawed Matrigel® with chilled, pure DMEM F-12 Ham medium. In order to coat the whole surface of one well of a 6-well culture plate, a total volume of 720 µl of the Matrigel® mixture was required. The Matrigel®-DMEM F12-Ham mixture polymerized for 30 min at 37 °C before it was inoculated with a cell suspension of the isolated

pbMEC. Previous to the inoculation the pbMEC were re-suspended in DMEM F12-Ham cell culture medium, supplemented with 10 % FBS (gibco® Lifetechnologies GmbH, Darmstadt, Germany), 176.7 IU/ml penicillin, 0.176 mg/ml streptomycin, 0.088 mg/ml gentamicin, 4.4 µg/ml amphotericin B and 5 ml ITS liquid media supplement (proliferation medium, Sigma-Aldrich).

3.4.2 Milk cell extraction method

The pbMEC were extracted out of fresh cow milk according to a protocol established from Buehring (1990) [72] and further optimized by Sorg et al. (2013) [38] and Danowski et al. (2013) [73]. In order to guarantee the purity of the cell extracts, the protocol was further optimized due to the implementation of a second filtration step during the washing procedure with 1x Hanks Balanced Salt Solution (HBSS, Sigma-Aldrich). For the inoculation of one well of a 6-well culture plate, one liter of fresh milk was defatted (1850 x g, 10 min, 4 °C) and the cell pellet, consisting of somatic cells, was washed for several times with 1x HBSS (washing buffer) (600 x g, 5 min) (Figure 5). The washing buffer was further supplemented with the antibiotics penicillin (176.7 IU/ml), streptomycin (0.176 mg/ml) and gentamicin (0.088 mg/ml) (Sigma-Aldrich) and the antimycotic amphotericin B (8.3 µg/ml) (Sigma-Aldrich). Additionally, two filtration steps with filter units (cell strainer, BD Biosciences, Erembodegem, Belgium) owing different pore sizes (100 µM, 40 µM) were part of the washing procedure, aiming to eliminate cell aggregates and potential contaminations from the cell suspension. The obtained cell pellet was re-suspended in proliferation medium, inoculated onto one well of a Matrigel® coated 6-well culture plate (Maxisorp, Nunc®, Sigma-Aldrich), and cultivated under standardized cultivation conditions (37 °C, 5 % CO_2) until reaching confluency.

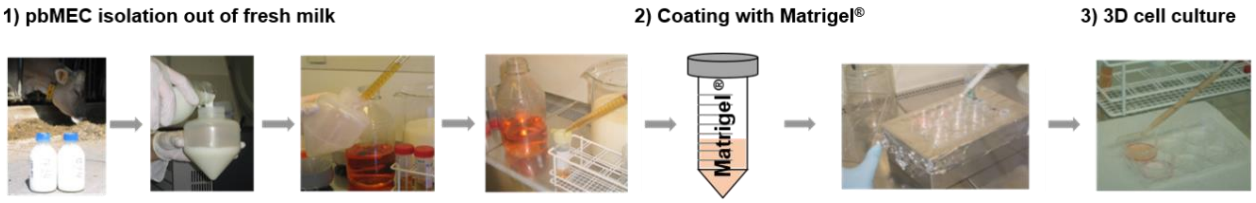


Figure 5: Isolation and 3D cell culture of pbMEC.

(1) pbMEC were extracted out of fresh milk using a milk centrifugation method. The remaining cell pellet was washed with 1xHBSS supplemented with antibiotics and antimycotics for several times and filtered through filter devices. **(2)** 6-well culture plates were coated with the thermosensitive Matrigel® prior to **(3)** the inoculation with the pbMEC cell suspension.

3.4.3 Cell cultivation

For experimental set-up I, pbMEC were either grown in 2D cell culture on the plastic surface of 6-well culture plates, or they were cultured in 3D cell culture on Matrigel® coated 6-well culture plates. The 2D cell culture approaches served as control in order to investigate differences between the 2D cell culture and the 3D cell culture approach. pbMEC were extracted and cultured as described in 3.4.2. The growth promotion and morphology of the pbMEC was monitored regularly, especially, before the proliferation medium that was changed twice a week, was renewed. After reaching confluency the pbMEC were sub-cultivated once using 0.25 % trypsin/EDTA solution (Sigma-Aldrich). The cell pellet (600 x g, 5 min) was re-suspended in fresh proliferation medium and the cells were counted (TC10™ Automated Cell Counter, Bio-Rad Laboratories GmbH, Munich, Germany) using 0.4 % Trypan blue (Bio-Rad Laboratories GmbH), in order to select for proliferating cells. $1*10^4$ living cells where then either, for the 2D cell culture approach or the 3D cell culture approach, inoculated onto the plastic surface or the Matrigel® coated surface of one well of a 6-well culture plate and cultivated (37 °C, 5 % CO_2). For experimental set-up I, pbMEC were already treated in the first passage, whereas pbMEC used in experimental set-up II and III, were cultivated until the third passage on Matrigel® coated 6-well culture plates, and further cryopreserved in liquid nitrogen until pbMEC from all animals had been sampled. For cryopreservation $1*10^5 – 5*10^5$ pbMEC were transferred into cryopreservation medium (70 % DMEM F12-Ham, 20 % FBS, 10 % DMSO) and stored in liquid nitrogen.

3.5 Mycoplasma PCR

In order to exclude the possibility of contamination of pbMEC with mycoplasma, cell culture supernatants of pbMEC were tested. Therefore, the PCR Mycoplasma Test Kit (AppliChem GmbH, Darmstadt, Germany) was used following the manufacturer's protocol.

3.6 Immunocytochemistry

The epithelial character of the pbMEC used in the experiments, was proofed by immunocytochemistry (IC). The method has already been described in detail by Sorg et al. (2013) and Danowski et al. (2013) [38,73] and in Appendix I, II and III. In Brief, $1*10^4$ pbMEC were seeded onto wells of 8-well LabTec chamber slides (LAB-Tek, Nunc, GmbH, Langenselbold, Germany) and cultivated until confluency. The cells were fixed in ice cold methanol/acetone (1:1) for 10 min, and after several washing and blocking steps, incubated with the primary monoclonal mouse IgG anti-pan cytokeratin antibody C-11 (1:400 in PBS-T, Sigma-Aldrich). Control wells were covered with goat serum (diluted 1:10 in PBS-T). The slides were incubated over night at 4 °C in moist

atmosphere. On the next day, the slides were washed with PBS-T (3 × 5 min). Subsequently, the secondary antibody, horseradish peroxidase labeled polyclonal goat anti-mouse immunoglobulin (diluted 1:400 in PBS-T), was added (1 h, moist atmosphere). After washing the slides with PBS - T (3 × 5 min), the peroxidase was visualized by incubating the slides in PBS supplemented with 20 mg of DAB-dihydrochloride and H_2O_2 (light protected). The slides were washed with PBS - T (3 × 5 min), the cell nuclei were stained with Haemalaun after Mayer for 10 - 15 sec and rinsed with tap water for 30 – 45 sec. The slides were dehydrated with 50 %, 70 %, 96 % and 99 % ethanol and Rotihistol (Roth GmbH & Co. KG, Karlsruhe, Germany) for 2 min each and covered with a cover slip and Eukitt (Roth GmbH + Co. KG) for storage.

3.7 Cell viability test

The lipophilic membrane stain Vybrant® DIL Cell Labeling Solution (Lifetechnologies GmbH) was used to visualize the viability of pbMEC contributing to the formation of alveolar-like structures in 3D cell culture (experimental set-up I, Appendix I). The assay was performed as described in the manufacturer's instructions. Stained pbMEC were further cultivated until confluency and treated either with DMEM F12-Ham medium supplemented with 3 µg/ml PRL and 1 µg/ml HC or only with DMEM F12-Ham medium containing HC for 8 days. The medium was renewed daily and the formation of alveolar-like structures was monitored using light and fluorescence microscopy (Leica Microsystems GmbH, Wetzlar, Germany).

3.8 Cell stimulation

3.8.1 Immune stimulatory agents

For the induction of a ketotic state *in vitro* (experimental set-up III, Appendix III), the pbMEC were treated with 3 mM of the ketone body BHBA (Sigma-Aldrich, Saint Louis, USA). For the immune challenge of pbMEC samples in experimental set-up III (Appendix III), the gram-negative, heat-inactivated, *E. coli 1303* strain [38] was used in a multiplicity of infection of 30 (MOI) colony forming units per cultured cell [38]. In experimental set-up II (Appendix II) a mixture of the gram-positive, formalin inactivated, human pathogen *C. diff.* and its toxoids (IDT Biologika GmbH) was applied to the pbMEC (MOI 70).

3.8.2 Treatment schemes

The treatment of pbMEC was conducted in cell culture duplicates in all experimental set-ups mentioned below. Furthermore, 24 h prior to cell stimulation (70 % - 80 % confluency), the proliferation medium was replaced by DMEM F12-Ham medium that only contained ITS liquid

media supplement (treatment medium), in order to reduce interfering effects of FBS and the antibiotics and antimycotics.

For the induction of milk protein production (experimental set-up I), pbMEC in 2D and 3D cell culture obtained DMEM F12-Ham media with different supplements (differentiation media). The various treatment approaches are listed in Table 1. The various differentiation media were renewed daily over a period of 4 days. The cell culture supernatants were collected daily and stored at – 80 °C until further processing, while the pbMEC were lysed in Qiazol (Qiagen, Hilden, Germany) after 96 h (- 80 °C).

Table 1: Composition of the different differentiation media used for experimental set-up I

Notation		Components				
		DMEM F12-Ham	ITS	Hydrocortisone 1 µg/ml	Prolactin 3 µg/ml	Lysine 1.2 mM
2D	2D Ctr	+	+			
	2D culture	+	+	+		
3D	3D Ctr	+	+			
	3D culture	+	+	+		
	3D + PRL[1]	+	+	+	+	
	3D + LYS[2]	+	+	+		+
	3D + PRL + LYS[2]	+	+	+	+	+

[1]: PRL = Prolactin
[2]: LYS = L-Lysine
This table was copied from Appendix I: Hillreiner et al. (2017). In Vitro Cell.Dev.Biol.-Animal 2017 doi: 10.1007/s11626-017-0169-7

For experimental set-up II and III, 3 counting wells were inoculated with pbMEC from each animal. Those counting wells served to determine the amount of bacteria needed to obtain the required MOI. pbMEC were thawed, $2*10^4$ cells were reseeded onto Matrigel® coated 6-well culture plates and cultivated until confluency.

For experimental set-up II, pbMEC obtained from Brown Swiss cows, immunized against the human gram-positive pathogen *C. diff.* were stimulated with formalin-inactivated *C. diff.* (MOI 70) after 24 h of adjustment to the treatment medium. pbMEC were sampled in Qiazol (- 80 °C) directly after treatment start (0 h ctr), after 6 h, 24 h and 72 h, as we aimed to cover the immediate, intermediate and late innate immune response (Appendix II).

For experimental set-up III the pbMEC were treated with 3 mM BHBA and/or heat-inactivated *E. coli* (MOI 30, *E. coli 1303* [38]), as explicitly described in Appendix III and shown in Figure 6.

The cell culture supernatants were collected at each treatment time-point of the experiment and the pbMEC were lysed in Qiazol after 0 h, 24 h, 30 h and 54 h of treatment (storage: -80 °C).

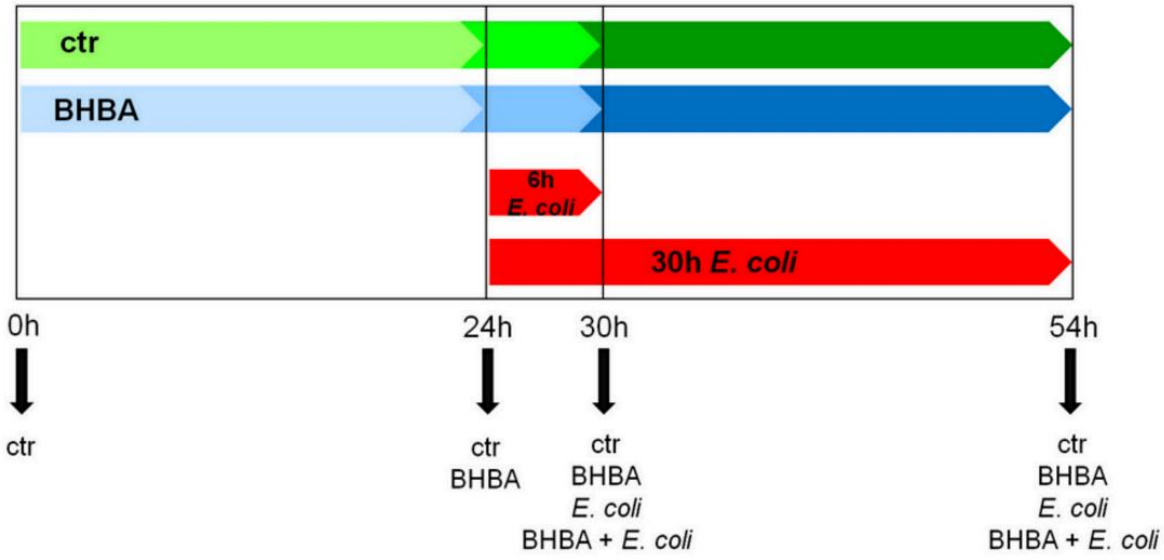


Figure 6: Treatment of pbMEC with BHBA and *E. coli*.

At each time-point (0 h, 24 h, 30 h and 54 h) untreated control samples as well as BHBA and/or *E. coli* treated samples were taken. The 30 h / 54 h BHBA and 30 h / 54 h BHBA + *E. coli* samples were adjusted to a concentration of 3 mM BHBA 24 h prior to the 6 h or 30 h *E. coli* treatment. The figure was copied from Appendix III: Hillreiner et al. (2016). PLoS ONE 11(6): e0157774. doi:10.1371/journal.pone.0157774

3.9 RNA extraction and reverse transcription

The miRNeasy Micro Kit (Qiagen) was used for the RNA extraction. The protocol was only marginally adjusted by the addition of a second incubation step of the miRNeasy Micro spin column with buffer RPE for 5 min. The yield of the extracted RNA was determined with the Nanodrop ND-1000 spectrophotometer (Peqlab, Erlangen, Germany) while its integrity was determined with the 2100 Bioanalyzer using the RNA 6000 nano Kit (Agilent Technologies, Waldbronn, Germany) following the manufacturer's instructions. The reverse transcription (RT) of RNA to cDNA in all experimental set-ups, was conducted on the T-Personal Thermocycler (Biometra, Göttingen, Germany) using 300 ng – 400 ng RNA (Annealing: 21 °C, 10 min, transcription phase: 48 °C, 50 min, degrading phase: 90 °C, 2 min) (Appendix I, II, III). On each 96-well plate (4titude, Wotton, Great Britain) that was used for the RT-PCR, non-template controls (NTC), non-reverse transcription controls (negative ctr.) and positive controls (bovine mammary gland, bovine spleen tissue) were carried along. The generated cDNA (10 ng/µl) was stored at - 20 °C.

3.10 Primer design and quality control

Bos taurus specific primer pairs were designed using Primer3web version 4.0.0 [74,75] based on published bovine nucleic acid sequences of the national Center for Biotechnology Information gene database (NCBI, National Library of Medicine, Bethesda MD, USA). All designed primer assays were commercially synthesized (Sigma-Aldrich) and tested according to their specificity using cDNA generated from bovine spleen, bovine udder parenchyma and pbMEC. Additionally, the PCR efficiencies of the qPCR assays were evaluated by individual serial dilution rows, as described in the MIQE-Guidelines [76]. Unless otherwise described, the primer pairs were designed to exhibit the optimal annealing temperature at 60 °C (Table 2). For experimental set-up I and II the CFX384™ Real-Time System (Bio-Rad Laboratories GmbH) was used, whereby the RT-qPCR for experimental set-up III, was conducted on the Biomark™ HD 96x96 system (Fluidigm, San Francisco, CA, USA). The primer pairs used for the different experimental set-ups are displayed in Table 2.

Table 2: Primer pairs for RT-qPCR measurements. Primer pairs used in the different experimental set-ups (I, II, III) are labeled in the last three columns of the table.

Gene name	NCBI reference sequence number	Primer sequence (5' -> 3') Forward Reverse	L[1] [bp]	I[2]	II[3]	III[4]
Reference genes						
18S ribosomal RNA gene (*18SrRNA*)	AF176811.1	CGGGGAGGTAGTGACGAAA CCGCTCCCAAGATCCAACTA	195		x	
H3 histone, family 3A (*H3F3A*)	NM_001014389.2	ACTTGCTACAAAAGCCGCTC ACTTGCCTCCTGCAAAGCAC	232		x	
Actin, gamma 1 (*ACTG1*)	NM_001033618	AACTCCATCATGAAGTGTGAC GATCCACATCTGCTGGAAGG	234		x	x
Glyceraldehyd-3-phosphate dehydrogenase (*GAPDH*)	NM_001034034.1	GTCTTCACTACCATGGAGAAGG TCATGGATGACCTTGGCCAG	197	x	x	x
Tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein, zeta polypeptide (*YWHAZ*)	NM_174814.2	CAGGCTGAGCGATATGATGA GACCCTCCAAGATGACCTAC	141	x	x	x

Gene name	NCBI reference sequence number	Primer sequence (5' -> 3') Forward Reverse	L[1] [bp]	I[2]	II[3]	III[4]
Cytokeratin 8 (*KRT8*)	NM_001033610	TGGTGGAGGACTTCAAGACC CGTGTCAGAAATCTGAGACTGC	215	x	x	x
Ubiquitin B (*UBB*)	NM_174133.2	AGATCCAGGATAAGGAAGGCAT GCTCCACCTCCAGGGTGAT	426		x	
TLR pathway						
Toll-like receptor 2 (*TLR2*)	NM_174197.2	CATTCCTGGCAAGTGGATTATC GGAATGGCCTTCTTGTCAATGG	201		x	
Toll-like receptor 4 (*TLR4*)	NM_174198.6	TGCTGGCTGCAAAAAGTATG TTACGGCTTTTGTGGAAACC	213		x	x
Lymphocyte antigen 96 (*LY96*)	NM_001046517.1	TGTTTCAATACGTTCTGAGCCC TCAGTGTTCCCCTCGATGG	300		x	
Lipopolysaccharide binding protein (*LBP*)	NM_001038674.2	TCCCAGTTGCTTTCCTTGCT GCGGAAGGACTTGGTGTTCT	194		x	
CD14 molecule (*CD14*)	NM_174008.1	GCAGCCTGGAACAGTTTCTC ACCAGAAGCTGAGCAGGAAC	124		x	
Myeloid differentiation primary response gene (*MYD88*)	NM_001014382.2	CTGCAAAGCAAGGAATGTGA AGGATGCTGGGGAACTCTTT	122		x	x
TCDD-inducible poly(ADP-ribose) polymerase (*TIRAP*)	NM_001206048.1	TAGTGCAGCCTCCTTCTCCT AACCCCATCAAGTGAGCCAG	176		x	
TNF receptor-associated factor 6, E3 ubiquitin protein ligase (*TRAF6*)	NM_001034661.2	GGACTGCAGCAAAAGACGAC CTTCCCGCAAAGCCATCAAG	156		x	
Interleukin-1 receptor-associated kinase 4 (*IRAK4*)	NM_001075998.1	ACAGCATCAACATACGTGCG GGTGCCCCAGTCAAACAGTA	213		x	
Interleukin-1 receptor-associated kinase 1 (*IRAK1*)	NM_001040555.1	GCCGCCCAGATCTACAAGAA TAGGAGTTCTCTTGCGGGGA	233		x	
V-rel reticuloendotheliosis viral oncogene homolog A (avian) (NF-kappa-B p65 subunit) (*RELA*)	NM_001080242.2	ACAGCTTTCAGAACCTGGGG GACGGCATTCAGGTCGTAG	140		x	x

Gene name	NCBI reference sequence number	Primer sequence (5' -> 3') Forward Reverse	L[1] [bp]	I[2]	II[3]	III[4]
Complement system						
Complement component 3 (*C3*)	NM_001040469	AAGTTCATCACCCACATCAAG CACTGTTTCTGGTTCTCCTC	191		x	
Chemokines						
Chemokine (C-C motif) ligand 2 (*CCL2*)	NM_174006.2	TCTCGCTGCAACATGAAGGT TATAGCAGCAGGCGACTTGG	121		x	x
Chemokine (C-C motif) ligand 5 (*CCL5*)	NM_175827.2	TCCATGGCAGCAGTTGTCTT TTCAGGTTCAAGGCGTCCTC	129		x	
Chemokine (C-C motif) ligand 20 (*CCL20*)	NM_174263.2	CTTGTGGGCTTCACACAGC GTTTCACCCACTTCTTCTTTGG	115		x	x
Chemokine (C-x-C motif) ligand 5 (*CXCL5*)	NM_174300.2	TTGTGAGAGAGCTGCGTTGT CCAGACAGACTTCCCTTCCA	150		x	
Interleukin 8 (*CXCL8*)	NM_173925.2	AAGAATGAGTACAGAACTTCGATGC GTTTAGGCAGACCTCGTTTCC	160		x	x
Chemokine (C-x-C motif) ligand 3 (*CXCL3*)	NM_001046513.2	TCAACCCTGAAGCTCCCATG AGTCCAGCACATCAAGTCCTT	198		x	
Chemokine (C-C motif) receptor 7 (*CCR7*)	NM_001024930.3	ATCATTGCTGTGGTCGTGGT GAAAGGGTTGACACAGCAGC	183		x	
Interleukin 13 receptor, alpha 1 (*IL13RA*)	NM_001206677.1	CAGGTTGAGGCTGGAAGACA CCCACCACTGCCATCTAAGT	193		x	
Inflammatory cytokines						
Interleukin 1, alpha (*IL1-A*)	NM_174092.1	AGAATGTGGTGATGGTGGCA ACTTTGATTGAGGGCGTCGT	224		x	
Interleukin 1, beta (*IL1-B*)	NM_174093.1	GAAGAAAGGCCCGTCTTCCT ACAGTGAAGTTCAGGCTGCA	176		x	

Gene name	NCBI reference sequence number	Primer sequence (5' -> 3') Forward Reverse	L[1] [bp]	I[2]	II[3]	III[4]
Interleukin 6 (*IL6*)	NM_173923.2	TGGTGATGACTTCTGCTTTCC AGAGCTTCGGTTTTCTCTGG	109		x	x
Interleukin 10 (*IL10*)	NM_174088.1	AGCTGTATCCACTTGCCAACC TGGGTCAACAGTAAGCTGTGC	119		x	
Tumor necrosis factor α (*TNFα*)	NM_173966.2	CCACGTTGTAGCCGACATC ACCACCAGCTGGTTGTCTTC	108		x	x
Transforming growth factor, beta 1 (*TGFβ1*)	NM_001166068.1	CCTGGACACCAACTACTGCT CCAGGACCTTGCTGTACTGT	185		x	
Acute phase proteins / danger associated molecular pattern molecules						
Serum amyloid A3 (*SAA3*)	NM_001242573.1	CACGGGCATCATTTTCTGCTT GGGCAGCGTCATAGTTTCCA	179		x	x
Haptoglobin (*HP*)	NM_001040470.1	AATGAACGATGGCTCCTCAC TTGATGAGCCCAATGTCTACC	176		x	x
S100 calcium binding protein A9 (*S100A9*)	NM_001046328.1	CTGGTGCAAAAAGAGCTGC AGCATAATGAACTCCTCGAAGC	128		x	x
S100 calcium binding protein A12 (*S100A12*)	NM_174651.2	TGGGGAGGCGCTGCTCTAGAC TCGAAATGCCCCACCCGAACG	135		x	
Antimicrobial peptides						
Lactoferrin (*LF*)	NM_180998.2	CGAAGTGTGGATGGCAAGGAA TTCAAGGTGGTCAAGTAGCGG	215		x	x
Lysozyme 1 K (*LYZ1*)	NM_001077829.1	AAGAAACTTGGATTGGATGGC ACTGCTTTTGGGGTTTTGC	185		x	x
Lactoperoxidase (*LPO*)	NM_173933.2	TGGCTGTCAACCAAGAAGC TGAGGCTCGAAAATCTCCC	134		x	
Tracheal antimicrobial peptide (*TAP*)	NM_174776.1	AGGAGTAGGAAATCCTGTAAGCTGTGT AGCATTTTACTGCCCGCCCGA	113		x	x
Lingual antimicrobial peptide (*LAP*)	NM_203435.3	AGAAATTCTCAAAGCTGCCG CAGCATTTTACTTGGGCTCC	107		x	x
Apoptosis						
Fas cell surface death receptor (*FAS*)	NM_174662.2	CGGGATCTGGGTTCACTTGT GGAGGACAAGGCTGACAACA	180		x	

Gene name	NCBI reference sequence number	Primer sequence (5' -> 3') Forward Reverse	L[1] [bp]	I[2]	II[3]	III[4]
Tumor necrosis factor receptor superfamily, member 1A (*TNFRSF1A*)	NM_174674.2	CGCCTCTGTCGTCTTAGCAT GACTGGAACTTGGGGTGGAG	170		x	
Tumor necrosis factor receptor 2 (*TNFR2*)	AF031589.1	CCAGCAGCACGGACAAGA CAATGCAGGTGACGTTGACC	153		x	
Caspase 8 (*CASP8*)	NM_001045970.2	TAGCATAGCACGGAAGCAGG GCCAGTGAAGTAAGAGGTCAG	295		x	
Caspase 3 (*CASP3*)	NM_001077840.1	TCAGTCAGTCAGTTGGGCAC GGGAGCATCTTCCACACACA	164		x	
Caspase 1 (*CASP1*)	XM_002692921	ACGTCTTGCCCTTATTATCTGC GTACTGTCAGAGGTCCGATGC	204		x	
BCL2-associated X protein (*BAX*)	NM_173894.1	AGAGGATGATCGCAGCTGTG GAAGTCCAATGTCCAGCCCA	200		x	
Anti-apoptotic regulator Bcl-xL (*Bcl-xL*)	AF245487	GGCATTCAGCGACCTGAC CCATCCAAGTTGCGATCC	203		x	
B-cell CLL/lymphoma 2 (*BCL-2*)	NM_001166486.1	ATGTGTGTGGAGAGCGTCAA GAGCAGTGCCTTCAGAGACA	195		x	
Immunoglobulin receptors						
IgG Fc receptor (*FcRN*)	AF141017.1	GAGCTGGCTCCTTGGATCTC ATACCAGGATTCCCGGAGGT	194		x	
Polymeric immunoglobulin receptor (*PIGR*)	NM_174143.1	GACACCGTGGAGAGCAAAGA GTGATTCGGAGCGTGATTGC	192		x	
Scavenger Receptor						
CD68 molecule (*CD68*)	NM_001045902.1	GGCTCCAAGGAGGCAATAG GAATGAGAGGAGCAAGTGGG	201		x	
CD40 molecule (*CD40*)	NM_001105611.2	TCGAAGGCCAACACTGTACC GCCTTTTCTCTCGCAGCTTG	197		x	
Oxidative metabolism						

Gene name	NCBI reference sequence number	Primer sequence (5' -> 3') Forward Reverse	L[1] [bp]	I[2]	II[3]	III[4]
Cytochrome P450, family 1, subfamily B, polypeptide 1 (*CYP1B1*)	NM_001192294.1	GGACTTTGACCCAACCCGAT CACTGGTGAGCAAGGATGGA	159		x	
Cytochrome P450, family 1, subfamily A, polypeptide 1 (*CYP1A1*)	AF514290.1	GGAGCCTAAAACCCACAGACA CAGCACAACTTTGGAAGGGC	177		x	
Nitric oxide synthase 2, inducible (*NOS2*)	NM_001076799.1	CATTCGATGTCAGCGGCAAG GCTGCGATTTGAGCCTCATG	174		x	
MAPK signaling						
FBJ murine osteosarcoma viral oncogene homolog (*FOS*)	NM_182786.2	ACTGCTCGCGATCATGATGT CCAGATCGGTGCAGTAGTCC	173		x	
Mitogen-activated protein kinase 8 (*MAPK8*)	NM_001192974.1	TGGAGGGGTAAAGGGCATTG AGAAACGGCCAGGAAGTGTT	156		x	
Others						
Matrix metallopeptidase 1 (*MMP1*)	NM_174112	TCTGGAGCAATGTCACACCC CCTGCACCTGGTTGAAAAGC	151		x	
Interferon regulatory factor 3 (*IRF3*)	NM_001029845.2	GCTCAACTGACGGGAAGTGG TTTGGGTTCCCATGGTCTGG	128		x	
Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse) (*MX1*)	NM_173940.2	AAGGCCACTATCCCCTGC CTCGTACTTTGGTAAACAGTCGG	277		x	
Myxovirus (influenza virus) resistance 2 (mouse) (*MX2*)	NM_173941.2	CTTCAGAGACGCCTCAGTCG TGAAGCAGCCAGGAATAGTG	232		x	x
Nucleotide-binding oligomerization domain containing 2 (*NOD2*)	NM_001002889.1	CTGGCTCCGAGGAAACACTT GTGCTCAGATGTCGTCCCAT	158		x	

Gene name	NCBI reference sequence number	Primer sequence (5' -> 3') Forward Reverse	L[1] [bp]	I[2]	II[3]	III[4]
V-akt murine thymoma viral oncogene homolog 1 (*AKT1*)	NM_173986.2	GATCACCGACTTCGGACTGT CTTCTCGTGGTCCTGGTTGT	202		x	
Wingless-type MMTV integration site family member 4 (*WNT4*)	XM_010826681.1	CGGCCTTCACAGTGACTCTT GGCCTAGGACAGTGTTTGCT	150		x	
Milk and whey protein genes						
α_{S1}-casein (*CSN1S1)*	NM_181029.2	AGCACCAAGGACTCCCTCAAGAAG CAGGTAACGCTCAGAGGGCAC	257	x		
α_{S2}-casein (*CSN1S2)*	NM_174528.2	CCCACTCTGAACAGAGAGCAGC TGGGGCAAGGCGAATTTCTGG	173	x		
β-casein (*CSN2)*[5]	NM_181008.2	GGCTATGGCTCCTAAGCACA AGTTGGAGGAAGAGGCTGGT	163	x		x
κ-casein (*CSN3)*[6]	NM_174294.1	TGCAATGATGAAGAGTTTTTTCCTAG GATTGGGATATATTTGGCTATTTTGT	151	x		x
α-lactalbumin (*LALBA)*	NM_174378.2	GGTGCAAAGACGACCAGAACCC GAGTGCTTTATGGGCCAACCAGTAG	152	x		
β-lactoglobulin (*LGB)*	X14712.1	AAGTGCCTCCTGCTTGCCCTGG TACCAAGTCCCCGCCACCTTCTGG	104	x		
JAK-STAT pathway						
Signal transducer and activator of transcription 2 (*STAT2*)	NM_001205689.1	TCCTGCTGCGCTTTAGTGAA GGATTCGCGGGTAGAGGAAG	213		x	
Runt-related transcription factor 2 (*RUNX2)*	XM_002684501.1	ACCATGGTGGAGATCATCG CCGGAGCTCAGCAGAATAA	207	x		
YY1 transcription factor (*YY1)*	NM_001098081.1	GCTTGCCCTCATAAAGGCTGCACA GCAGCCTTCGAACGTGCACTGA	192	x		
Signal transdurcer and activator of transcription 5A (*STAT5A)*	NM_001012673.1	GTGAAGCCACAGATCAAGCA TCGAATTCTCCATCCTGGTC	176	x		x

Gene name	NCBI reference sequence number	Primer sequence (5' -> 3') Forward Reverse	L[1] [bp]	I[2]	II[3]	III[4]
Janus kinase 2 (*JAK2)*	XM_865133.2	TCTGGTATCCACCCAACCATGTCT AATCATGCCGCCACTGAGCAA	201	x		
E74-like factor 5 (*ELF5)*	NM_001024569.1	ATACTGGACGAAGCGCCACGTC ACTCCTCCTGTGTCATGCCGCA	134	x		
CCAAT/enhancer binding protein, beta (*CEBPB)*	NM_176788.1	GCACAGCGACGAGTACAAGA GTTGCTCCACCTTCTTCTGG	152	x		
Prolactin receptor (*PRLR)*	NM_001039726.1	CATGGTGACCTGCATCCTC ACCCTCATGCCTCTCACATC	172	x		
mTOR pathway						
Eukaryotic translation initiation factor 4E binding protein 1 (*EIF4EBP1)*	NM_001077893.1	GAACTCACCTGTGACCAAGA CTCAAACTGTGACTCTTCACC	157	x		
Ribosomal protein S6 kinase, 70 kDa, polypeptide 1 (*RPS6KB1)*	NM_205816.1	GGCAGCCCACGAACACCTGT AGGCGTCTGCGGATTTGCCG	96	x		
v-akt murine thymoma viral oncogene homolog 1 (*AKT1)*	NM_173986.2	GATCACCGACTTCGGACTGT CTTCTCGTGGTCCTGGTTGT	202	x		

[1]Amplicon length

[2] Primers used for experimental set-up I

[3] Primers used for experimental set-up II

[4] Primers used for experimental set-up III

[5]Annealing temperature 62°C

[6]Annealing temperature 54°C

3.11 Reverse transcription quantitative PCR (RT-qPCR)

3.11.1 CFX384™ Real-Time System

RT-qPCR reactions for the evaluation of changes in the expression of selected genes, in experimental set-up I and III, were conducted with the SsoFast™ EvaGreen® Supermix on the CFX384™ Real-Time System (Bio-Rad Laboratories GmbH) (Appendix I, III). Detailed information on the 10 µl reaction mix and the cycler program are shown in Table 3 and Table 4.

Table 3: Reaction mixture for CFX384™

Ingredient	Amount per sample [µl]	Manufacturer
SsoFast™ EvaGreen® Supermix	5.00	Bio-Rad Laboratories GmbH
Forward Primer (20 µM)	0.20	Sigma-Aldrich
Reverse Primer (20 µM)	0.20	Sigma-Aldrich
VisiBlue™ qPCR mix colorant	0.07	TATAA Biocenter, Goteborg, Sweden
DEPC H_2O	3.53	5prime, Hamburg, Germany
cDNA template (10 ng/µl)	1.00	

Table 4: CFX384™ - cycler program

Step	Temperature [°C]	Time [sec]	Cycles
1. Activation of the DNA polymerase	95	30	40x
2. cDNA denaturation	95	5	
3. Primer annealing and elongation	54, 60 or 62	5	

The cycle of quantification (Cq) was automatically detected by the CFX Manager Software version 2.1 (Bio-Rad Laboratories GmbH). For the data analysis, raw Cq values, were collected by the CFX Manager Software version 2.1 (Bio-Rad Laboratories GmbH) and were transferred to GenEx 5.4.4 (MultiD, Gothenburg, Sweden) in order to identify stable expressed reference genes, suitable for the normalization of qPCR data, by application of the geNorm tool. The fold changes were calculated following the $2^{\wedge(-ddCq)}$ method described by Livak and Schmittgen (2001) [77]. Statistical calculations were conducted with SigmaPlot 12.0 or 13.0 (Systat Software GmbH, Erkrath, Germany). Briefly, delta-Cq (dCq) values were examined for normal distribution (Shapiro-Wilk) before paired t-test or a one-factorial ANOVA on repeated measurements was conducted.

P-values ≤ 0.05 were assumed as statistically significant. Graphics were created in Microsoft Excel 2013 (Microsoft Corporation, USA).

3.11.1.1 Statistical data evaluation for experimental set-up I

For the comparison of the dCq values of the 3D cell culture approaches to the dCq values of the 2D cell culture approaches, a paired t-test was used (SigmaPlot 12.0 Systat Software GmbH). Significant (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001) and distinct changes (+0.05 ≤ p ≤ 0.1) always referred to the dCq values cultivated in 2D cell culture. The second aim was to determine changes between the 3D cell culture control and the different treatment approaches in 3D cell culture. Therefore, a one-factorial ANOVA on repeated measurements was applied on the dCq values of the different treatment approaches in 3D cell culture (SigmaPlot 12.0 Systat Software GmbH). Additionally, the Tukey test method was in this case applied for the pairwise multiple comparison procedure (Appendix I).

3.11.1.2 Statistical data evaluation experimental set-up III

For the statistical evaluation (SigmaPlot 13.0, Systat Software GmbH) a paired t-test was conducted on the normally distributed data, whereby a signed rank test was used when the data was abnormally distributed (Appendix III).

3.11.2 BioMark™ HD 96x96 system

For the RT-qPCR measurements in experimental set-up II, the BioMark™ HD 96x96 system (Fluidigm) that was adapted from Sorg et al. (2013), was slightly optimized and the cDNA was generated as described in 3.9. Despite of the 18srRNA primer pair, the cDNA was pre-amplified for 16 cycles. The pre-amplification of 2 µl cDNA (10 ng/µl) was conducted on the CFX384™ Real-Time System (Bio-Rad Laboratories GmbH) using the iQ Supermix (BioRad Laboratories GmbH) and a final primer concentration of 25 nM in a total reaction volume of 15 µl (Activation of polymerase 95 °C for 3 min, 16 cycles of denaturation at 95 °C for 15 sec and 4 min of annealing and extension at 59 °C). After the pre-amplification, the cDNA was diluted 20 times and further stored at -20 °C. The efficiency of the primer assays (Table 4) was, additionally to the efficiency test on the CFX384™ Real-Time System (Bio-Rad Laboratories GmbH), tested within the first BioMark™ 96x96 Gene expression (GE) Dynamic Array chip (Fluidigm) according to the MIQE-Guidelines [76]. This second efficiency test was mandatory for the evaluation of the primer assays, as the BioMark™ system only accepts assays with an efficiency > 85 %. All in all, 4 BioMark™ 96x96 Gene expression (GE) Dynamic Array chips (Fluidigm) were used to evaluate changes in gene expression. Within each 96x96 GE dynamic array, two stably expressed samples, chosen

according to the data of the first 96x96 GE dynamic array, were carried along as interplate calibrators. Furthermore, positive controls (bovine mammary tissue, bovine spleen) and one no template control (NTC) were carried along with each 96x96 GE dynamic array. ValidPrime® (TATAA Biocenter) replaced the no reverse transcription controls, as this primer assay tested all samples for the presence of genomic DNA during the RT-qPCR run (Appendix II). The 5 µl sample premix and the 5 µl assay premix (final concentration of primers in an individual reaction: 250 nM) were prepared (Appendix II). Before both premixes were transferred to the 96x96 GE dynamic array and automatically mixed in the Fluidigm® IFC controller, the 96x96 GE dynamic array was primed. The precise methodology of the microfluidic technology can be found in a publication of Spurgeon et al. (2008) [78]. For the RT-qPCR the subsequent protocol, followed by a melting curve analysis, was applied: 98 °C, 40 sec followed by 30 cycles of 95 °C for 10 sec and 60 °C for 40 sec. The Cq values were automatically detected by the Fluidigm Real-Time PCR Analysis Software Version 4.1.2 (Fluidigm). For the data analysis, the Cq values detected by the Fluidigm Real-Time PCR Analysis Software Version 4.1.2 (Fluidigm), were also validated by means of this software. The melting curves served as quality criterion for the exclusion of primer pairs from the analysis. Additionally, primer pairs that showed too many missing data and/or bad efficiencies, were excluded from further analysis. Furthermore, based on the dynamic range of the primer assays, the cut-off Cq value was set to 26 and values larger than 26 were treated as missing data within GenEx Enterprise Version 6 (MultID Analyses AB, Gothenburg, Sweden). For those missing data points an offset of "+1" was estimated. For the calculation of fold change data the $2^{\wedge(-\Delta\Delta Cq)}$ method of Livak and Schmittgen (2001) [77] was applied. The statistical analysis was conducted as described in 3.11.1. In order to elucidate differences in gene expression, the fold change values were used to conduct a so called self-organizing tree algorithm (SOTA) analysis. Therefore, the mean of the fold changes of the whole analyzed group was calculated and subtracted from the single fold change values. Those mean centered fold changes were then analyzed with the Multi Experiment Viewer software (MeV 4.9.0, TM4) [79]. For details see Appendix II.

3.12 ELISA - Protein quantification in cell culture supernatants

The gene expression data obtained for the metabolic/immunological study with BHBA and *E. coli* (experimental set-up III, Appendix III) was validated by the determination of protein secreted into the cell culture medium. Therefore, the lactoferrin ELISA, already described by Danowski et al. (2013) [73], and the commercially available CCL2 VetSet™ ELISA Development Kit (Kingfisher Biotech, St Paul, USA) and ELISA Accessory Pack (Kingfisher Biotech) were used (Figure 7) (For details see Appendix III). The mean value of the secreted proteins, was in both cases calculated, by using the OD values of the standard curve within the linear range. The statistical analysis was

done with SigmaPlot 13.0 (Systat Software GmbH), whereby a paired t-test was used to determine statistical differences between the different treatments. Graphics were completed with Microsoft Excel 2013.

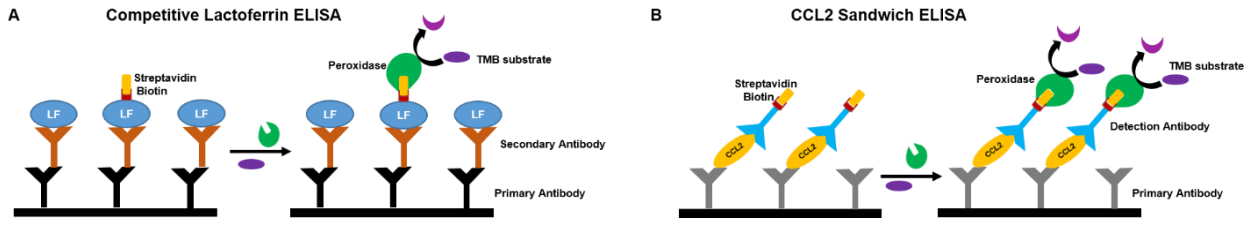


Figure 7: Principle of the competitive LF ELISA and the CCL2 Sandwich ELISA.

(A) The cell culture supernatants together with the secondary LF specific antibody were added to each well. After an incubation overnight, biotinylated LF was added, tethering to uncaptured secondary antibodies. Furthermore, streptavidin-HRP working solution was added. **(B)** The procedure of the CCL2 Sandwich ELISA was slightly different, as CCL2 was directly bound to the primary antibody, and was further captured by a secondary biotin-streptavidin bearing antibody. In both cases the HRP substrate reaction was started and the OD was measured at 450 nm.

3.13 Secretomics – LC-MS/MS measurements of cell culture supernatants

3.13.1 Sample preparation

The cell culture supernatants that were collected during experimental set-up I, were prepared prior to LC-MS/MS measurements. Potential proteases, present in the cell culture supernatants, were inhibited by the addition of a protease inhibitor (SigmaFast™ Protease Inhibitor, Sigma-Aldrich). In order to reduce the volume of the, over a time-period of 4 days, collected samples, Amicon® Ultra-2 Centrifugal Filter Devices (3 kDa, Merck Millipore, Darmstadt, Germany), were used. The protein concentration in the concentrated samples was determined photometrical (562 nm) using the bicinchoninic acid (BCA) assay. The proteins present in the cell culture supernatants were further prepared for in-solution digestion (for details see Appendix I). The digested and purified samples were further purified using a self-constructed column (Octadecyl C18 Solid Extraction Disk, 3 M Empore, 1.5 mm diameter). Prior to column loading, the column was equilibrated for four times (825 x g, 30 s). First with pure acetonitrile (25 µl), then with elution buffer (25 µl, 0.1 % formic acid, 60 % acetonitrile, Merck Millipore) and two times with desalting buffer (100 µl, 0,1 % formic acid). After sample loading (825 x g, 3 min), 50 µl desalting buffer was used to rinse the column twice. Elution took place by the addition of 50 µl elution buffer (0.1 % formic acid, 60 %

acetonitrile, Merck Millipore). The eluate was lyophilized in a speed vac (Hersteller?) and stored at – 80 °C until further analysis.

3.13.2 LC-MS/MS measurements

For the LC-MS/MS measurements an Eksigent NanoLC-Ultra $1D^+$ was coupled to an Orbitrap Velos instrument. The lyophilized eluate from 3.13.1 was dissolved in solvent A (20 µl, 0.1 % formic acid) and a volume of 10 µl was applied to the trap column (flow rate: 5 µl/min for 10 min) using the loading solvent (0.1 % formic acid in water). Afterwards, the peptides were delivered to the analytical column and separated at a flow rate of 300 nl/min and a 100 min gradient (Appendix I). A data-dependent mode that automatically switched between MS1 and MS2 was chosen for the Orbitrap Velos. Full-scan MS spectra were acquired at 360 m/z to 1300 m/z, 70,000 resolution, an automatic gain control (AGC) target value of 3×10^6 charges and maximum injection time of 100 ms for MS1. Up to 10 precursor ions were allowed for fragmentation in tandem mass spectra. MS2 spectra were acquired at 200 m/z to 2000 m/z, 17,500 resolution with AGC target value of 1×10^5 charges and maximum injection time of 50 ms. Precursor ion isolation width was set to 1.7 Th and dynamic exclusion to 20 s (Appendix I).

3.13.3 LC-MS/MS data evaluation

For the identification of peptides from raw LC-MS/MS data, the analytical software, MaxQuant [80] version 1.5.2.8 and its built-in Andromeda search engine [81] were applied. Furthermore, the UniProtKB *Bos taurus* database, version June 2015 (23870 entries) was aligned against the raw data files. Generally, oxidation of methionine and N-terminal protein acetylation were allowed as variable modifications. Additionally, carbamidomethylated cysteine was set as a fixed modification (Appendix I). Enzyme specificity was set to trypsin/P, allowing for cleavage after proline. The minimum peptide length was set to seven amino acids and a maximum of two missed-cleavages were allowed. The mass tolerance was set to 4.5 ppm for precursor ions and to 20 ppm for fragment ions. The dataset was adjusted to 1% FDR on the level of proteins and peptide spectrum matches (PSMs) (Appendix I). Microsoft Excel was applied for the qualitative analysis of the secreted proteins. Only proteins, that at least showed 2 unique spectra during LC-MS/MS analysis, were considered for further analysis. Additionally to the identification and selection of the proteins, the STRING Version 10.0 [82] database was used to generate a protein interaction network, considering only interactions with a combined score of at least 0.4, whereby unconnected nodes were concealed from the graphic. The EMBL-EBI QuickGo browser [83] was used to elucidate biological functions of the characterized proteins by gene ontology.

4 Results and Discussion

4.1 General evaluation of the pbMEC cell culture

All pbMEC cultures used within the different experimental set-ups showed the typical epithelial morphology. The cells were polygonal in shape and showed the typical cobble-stone like appearance. Furthermore, the epithelial origin was confirmed due to IC, as all pbMEC cultures were positively stained for the monoclonal mouse anti-cytokeratin pan antibody clone C-11 (Appendix I, II, III) (Figure 8). Additionally, all pbMEC cultures were tested for contaminating mycoplasma using the PCR Mycoplasma Test Kit (AppliChem GmbH). All cell cultures were mycoplasma free, so that any interfering effect on the immune response or behavior of the pbMEC due to mycoplasma, could be excluded (data not shown).

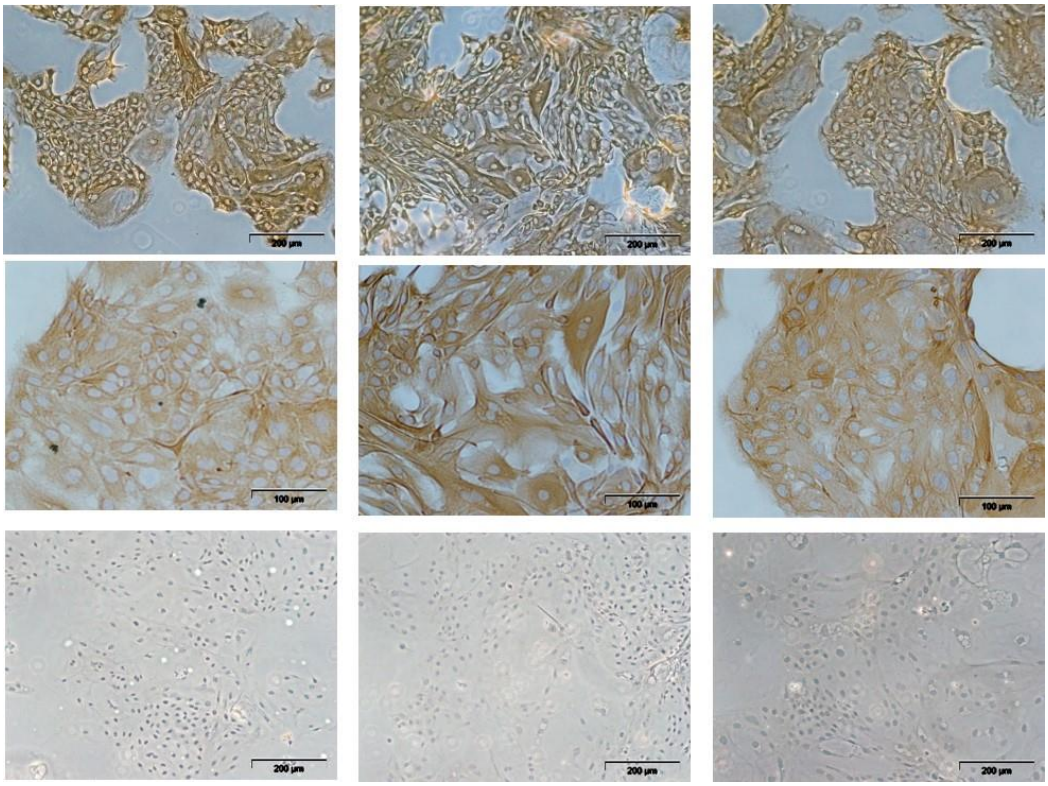


Figure 8: IC of pbMEC cultures:

All cultures were cytokeratin positive (Magnification upper row 100x, middle row 200x). In the negative control, only the stained nuclei were visible (bottom row, magnification 100x).

4.2 RNA integrity and RT-qPCR efficiency

Not only the cell culture was evaluated, but however also the quality of the RNA that was used in experimental set up I, II and III. The integrity of the RNA was very good, as an average RIN of 9.72 ± 0.073 (n=82) could be determined when 82 randomly collected RNA samples from all experimental set-ups, were analyzed on the 2100 Bioanalyzer with the 6000 Nano chips. Furthermore, all primer assays used within experimental set-up II were proofed concerning their efficiency on the BioMark™ 96x96 GE dynamic array, resulting in an average r^2 - value of 0.97 ± 0.01 (n=61). As the efficiency of the primer assays on the BioMark™ system were between 1.0 – 1.8, and as some of the primers for the RT-qPCR were adopted from Sorg et al. (2013), only a few of the adopted primer assays and all new designed primer assays that were used for experimental set-up I and III, were tested for the CFX384™ Real-Time System, resulting in an overall r^2 - value of 0.9984 ± 0.00029 (n=42).

4.3 Establishment of a physiological functional 3D cell culture model of non-invasively isolated pbMEC

We showed that due to the cultivation of pbMEC on Matrigel®, and the supplementation of the cell culture medium with lactogenic hormones over a time period of 8 days (experimental set-up I, Appendix I), alveolar-like structures were built (Figure 9). The viability of the cells, contributing to those alveolar-like structures was proofed by the fluorescence dye Vybrant® DIL Cell Labeling Solution (Life Technologies GmbH) (Figure 9). Our findings are in accordance with several published studies [24,55,59,84], which also showed the importance of an ECM-like scaffold for the maintenance of *in vivo*-like characteristics of the pbMEC *in vitro*. The differentiation of pbMEC, cultured in 3D cell culture, into polarized cells, that are hence able to build tubular and alveolar-like structures, has also been described in several studies [24,54,85,86]. However, most of the research done until now was conducted with stable cell lines or pbMEC extracted from tissue of slaughtered animals and not, as in this study, with non-invasively extracted primary cells.

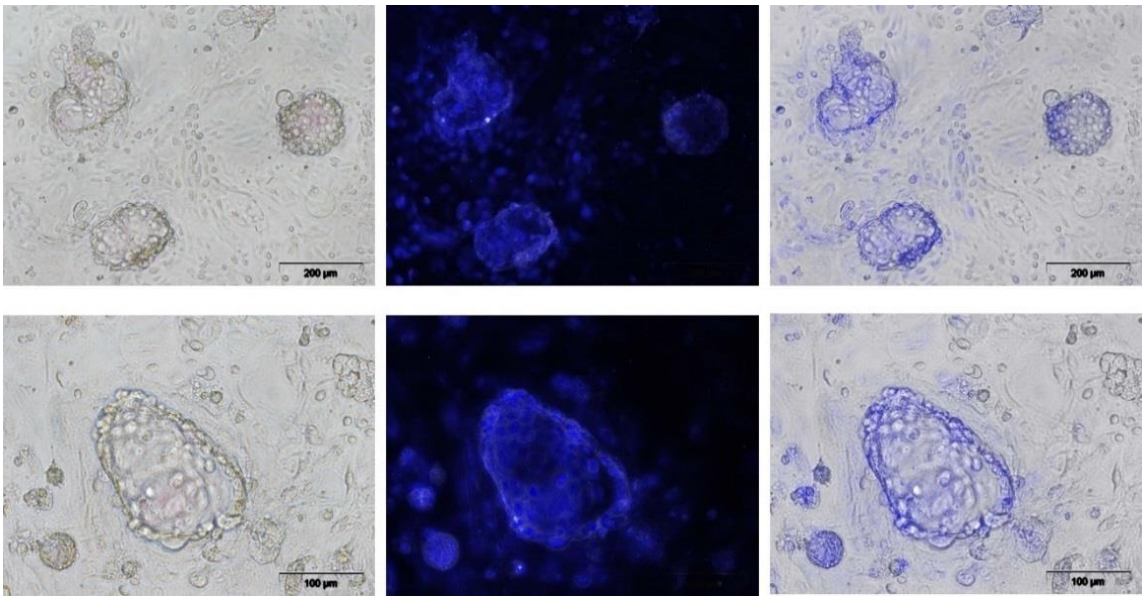
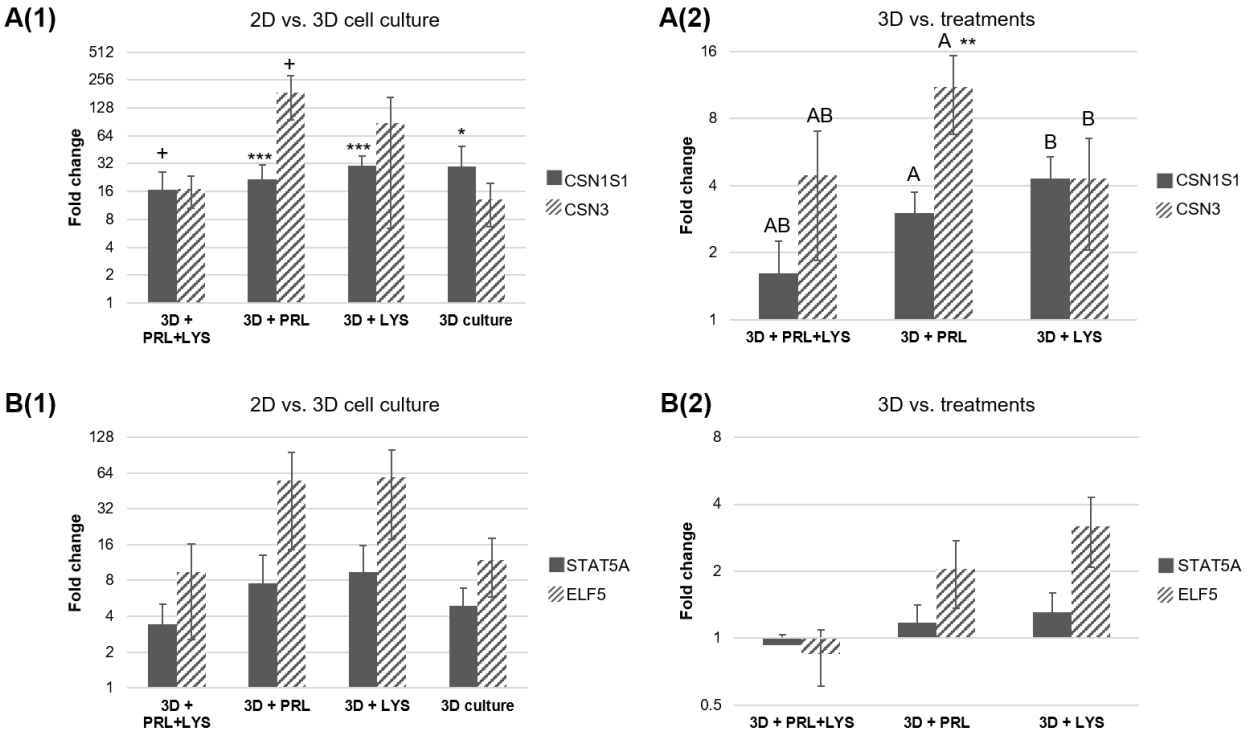


Figure 9: Formation of alveolar-like structures of pbMEC cultured on Matrigel®.

pbMEC cultured on Matrigel® and supplemented with PRL and HC over a time-period of 8 days, formed alveolar-like-structures (left, magnification 200x). The pbMEC contributing to those so called mammospheres, incorporated the lipophilic fluorescence dye Vybrant® DIL Cell Labeling Solution (Life Technologies GmbH) into their cell membrane, whereby their viability was confirmed (middle and right, magnification 200x).

As the pbMEC cultivated in 3D cell culture, were able to recapitulate a tissue like organization, we further proofed whether the pbMEC cultivated *in vitro* were still functional and whether a difference in the cellular functionality between cells cultured in 2D cell culture, 3D cell culture and between the different treatment approaches in 3D cell culture, can be determined (Table 1). We showed, that the 3D cell culture itself, as well as the treatment with lactogenic substances, had a positive influence on the gene expression profile of pbMEC (Appendix I: Figure 3). The expression of genes coding for milk proteins, like CSN1S1, CSN3 as well as for components of the intracellular signaling pathways (JAK-STAT: STAT5A, ELF5, CEBPB, YY1; mTOR: RPS6KB1, AKT1), promoting the transcriptional activation of milk protein synthesis, was distinctly as well as significantly altered, especially due to the cultivation of pbMEC on Matrigel® (Figure 10 A(1), B(1), C(1), D(1)). The superior effect of the ECM on the milk protein gene expression shown here, is in accordance with studies of Riley et al. (2010), Kozlowski et al. (2011) and Schmidhauser et al. (1992) [24,26,87]. Only a slight or even no effect of the lactogenic hormone PRL or the essential amino acid LYS was determined when compared to the pure 3D cell culture approach (Figure 10 A(2), B(2), C(2), D(2)). This was contradictory to a study of Lu et al. (2012) [25], who showed that supplementation of cell culture medium with LYS resulted in an increased CSN2 gene expression. We however detected a slight effect of the essential amino acid LYS on the expression of the gene

coding for CEBPB (Figure 10 C(2)). Furthermore, in our experiments CSN3 and AKT1 showed a significant induction of the gene expression when additionally to the 3D cell culture approach, the lactogenic hormone PRL was supplemented in the cell culture medium (Figure 10 A(2), D(2)). The same trend for the low responsiveness of milk protein gene expression towards PRL, has already been described earlier by Kozlowski et al. (2011) and Riley et al. (2010) [24,26].



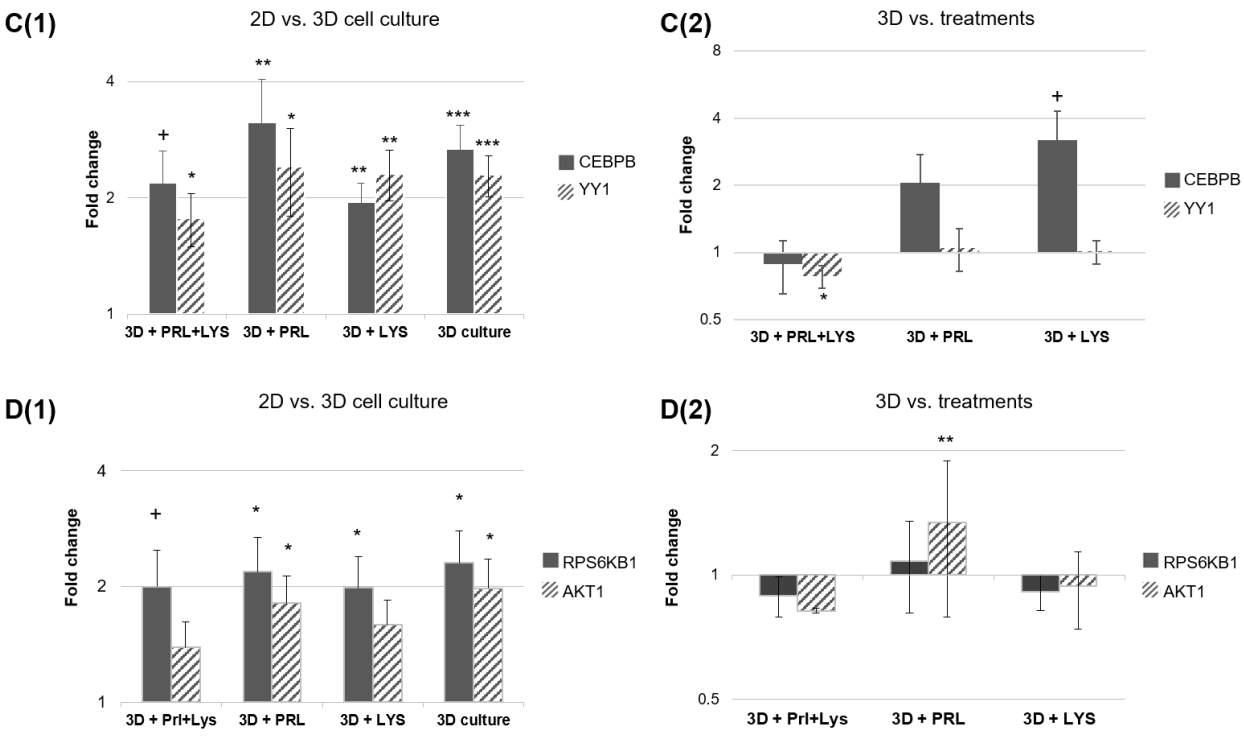


Figure 10: Fold change of genes coding for milk proteins and pathway components during different 2D and 3D cell culture approaches:

(A) The fold changes of the genes coding for the milk proteins CSN1S1 and CSN3 are shown **(A1)** relative to the 2D cell culture control and **(A2)** relative to the 3D cell culture control. **(B, C)** The fold changes of genes coding for components of the JAK-STAT signaling pathway are shown **(B1/C1)** relative to the 2D cell culture control and **(B2/C2)** relative to the 3D cell culture control. **(D)** The fold changes of genes coding for components of the mTOR signaling pathway are shown **(D1)** relative to the 2D cell culture control and **(D2)** relative to the 3D cell culture control. **(A1, B1, C1, D1)** Distinct ($^{+}$ $0.05 \leq p \leq 0.10$) and significant changes indicated in the graphs (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$) always referred to the dCq values of pbMEC cultivated in 2D cell culture. **(A2, B2, C2, D2)** Distinct ($^{+}$ $0.05 \leq p \leq 0.10$) and significant changes indicated in the graphs (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$) always referred to the dCq values of pbMEC cultivated in 3D cell culture and additionally, superscript letters, display significant differences in the gene expression between the different treatment approaches in 3D cell culture.

The functionality of the pbMEC cultured in 3D cell culture and the onset of milk and whey protein production and secretion, was further proven by LC-MS/MS measurements in cell culture supernatants. All in all, in the 3D cell culture supernatants, 56 proteins, that showed more than 2 unique spectra, were identified (Figure 11, Appendix I: Supplementary Material Table S1). As the milk an whey proteins CSN1S1, CSN2, LALBA and BLG and furthermore, several other proteins, were detected (FASN, SFN (14-3-3 σ), LGALS3, TGFB1) that are discussed, not only to be involved in cell proliferation and differentiation, but also in the induction and promotion of lactation [88–95], we were able to confirm our RT-qPCR results for the milk protein gene expression. Furthermore, we were able to detect proteins involved in actin filament organization and

cytoskeletal reconstruction (ACTN1, CNN2, FSCN1, CFL1) (Figure 11, Appendix I: Supplementary Material Table S1).

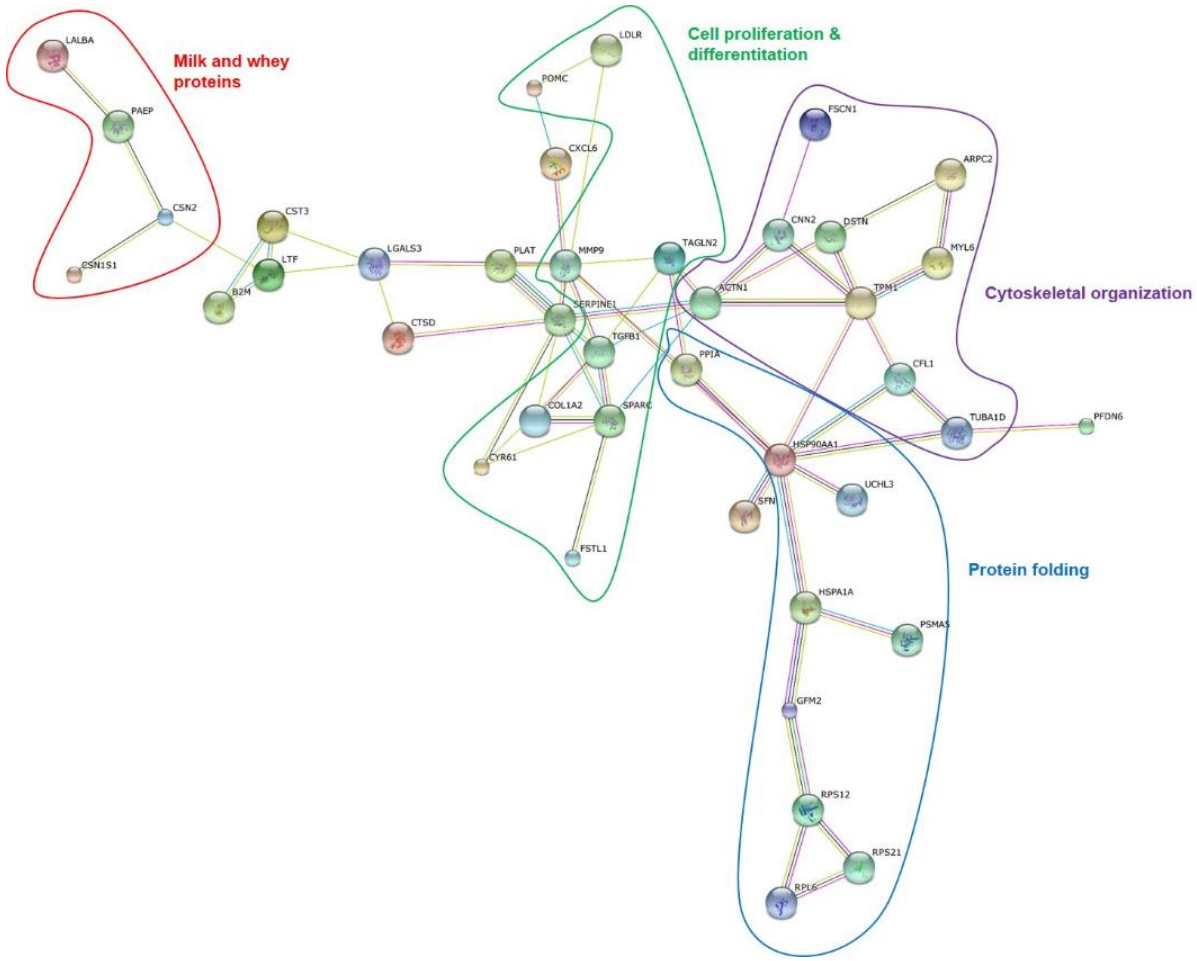


Figure 11: Protein network of proteins secreted from pbMEC cultivated in 3D cell culture.

Secreted proteins clustered together concerning their characteristics. Therefore, milk and whey proteins (red), proteins involved in cell proliferation and differentiation (green), proteins responsible for cytoskeletal organization (purple) and proteins promoting the protein folding process (blue), could be identified in cell culture supernatants of pbMEC, using LC-MS/MS measurements. The protein network was generated with String 10 [82] and proteins with similar biological functions were identified using the EMBL-EBI, QuickGO browser. Disconnected proteins were concealed. This figure was copied from Appendix I: Hillreiner et al. (2017). In Vitro Cell.Dev.Biol.-Animal 2017 doi: 10.1007/s11626-017-0169-7

As a functional cytoskeleton is of great importance for the general promotion of intracellular and intercellular signaling processes and for mammary epithelial cell differentiation and hence regulation of milk protein production [96–99], we postulated the successful induction of lactogenesis in the 3D cell culture model of pbMEC. However, not only proteins involved in

cytoskeletal organization and milk protein gene expression were detected, but also proteins involved in immune regulatory processes. Among them were proteins with antimicrobial and immunomodulatory functions [100,101], like LF, and chemotactic molecules (CXCL3, CXCL6, S100A8) that are responsible for the recruitment of leucocytes to the site of infection (Appendix I: Supplementary Material Table S1).

Summarizing this first experimental set-up, we successfully established a functional 3D cell culture model of pbMEC that favored the organization into polarized, multicellular structures, resulting in a more faithful representation of the intact mammary gland. Based on this functional cell culture model, it should in the future be possible to better and more precisely investigate physiological processes like the immune response and metabolic processes. Therefore, the before established 3D cell culture model of pbMEC was further used for the RT-qPCR experiments (experimental set-up II) and the evaluation of the effect of ketosis on the innate immune response of pbMEC (experimental set-up III).

4.4 RT-qPCR for the elucidation of molecular biomarkers in the innate immune system

pbMEC are also attributed to the first cellular barrier against invading pathogens, and hence play an important role in the induction of the innate immune response of the bovine mammary gland. Deficits in the recognition of possible pathogens, via the TLR, as well as deficits in the downstream signaling pathways could be the reason for the occurrence of so called low responder animals [19]. The so called high and low responder animals show a quite diverse overall health status as well as immune reaction upon vaccination. The in general healthier high responder animals are characterized by a good and fast immune response, resulting in higher immunoglobulin levels in milk, and a lower susceptibility to inflammatory diseases, increasing the profitability of the dairy cow [42–44]. The selection for high responder animals, based on molecular biomarkers, is therefore not only promising for the dairy industry itself but also for the medical sector, aiming to produce medical dietary supplements enriched with specific immunoglobulins against various diseases [41]. We think that not only the adaptive immune system, and hence the lymphocytes, should be considered for the search of molecular biomarkers, but also and especially those cells that induce the innate immune response and hence activate the adaptive immunity. The animals used within our experiment were triggered to produce and secrete specific immunoglobulins against *C. diff.*, due to an immunization against this human pathogen. This pathogen was chosen, as the vaccination of cows against this gram-positive pathogen is promising in case of the

production of immune milk that could prevent the development of *C. diff.* associated diarrhea in humans [45]. The animals were divided into the low (n=4) and high responder (n=5) group, based on the *C. diff.* specific IgA content in milk, whereby the threshold was set to 8 µg/ml (Appendix II: Figure 1). For the search for molecular biomarkers on gene level, the RT-qPCR on the BioMark™ HD 96x96 system (Fluidigm) was chosen. The differential gene expression of 61 innate immune genes, of pbMEC challenged with the formalin inactivated pathogen *C. diff.,* was evaluated (Table 2). The gene expression profile of the low and high responder animals was compared and it was apparent that during all treatment time-points (6 h, 24 h, 72 h), the pbMEC of the high responder group were better capable to induce the gene expression of various innate immune genes (Appendix II: Additional File 1, Table S2). The treatment with *C. diff.* in the high responder group, resulted in 18 significantly induced genes and 4 distinctly up-regulated genes. In the low responder group only 14 genes were significantly up-regulated and 2 genes were distinctly up-regulated through the stimulation of pbMEC with the gram-positive pathogen *C. diff.* (Appendix II: Additional File 1, Table S2). Despite of the, for gram-positive germs expected small changes in gene expression [17,19–21], significant differences in the gene expression of several innate immune components could be revealed between the high and low responder cows, using a normal t-test (Appendix II: Table 1). Additionally, a SOTA analysis was conducted, to visualize the differences in gene expression between the high and low responder group. Therefore, the mean value of all fold changes in the high and low responder group was calculated for each gene. The mean value of the whole group, was then subtracted from the fold changes of the high and low responder group. Values smaller than the mean value of the whole group were displayed in red, whereas mean values of the groups that were above the mean value of the whole group, were displayed in green. This SOTA analysis (Figure 12 and Appendix II: Figure 4, 5, 6) revealed the time-dependent activation of different classes of immune genes. Nineteen of the 61 genes were immediately (6 h) regulated after treatment start in cell culture, whereas 11 genes were immediately (6 h) as well as intermediately (24 h) induced and 21 genes only showed a differential gene expression 72 h after treatment start (Figure 12).

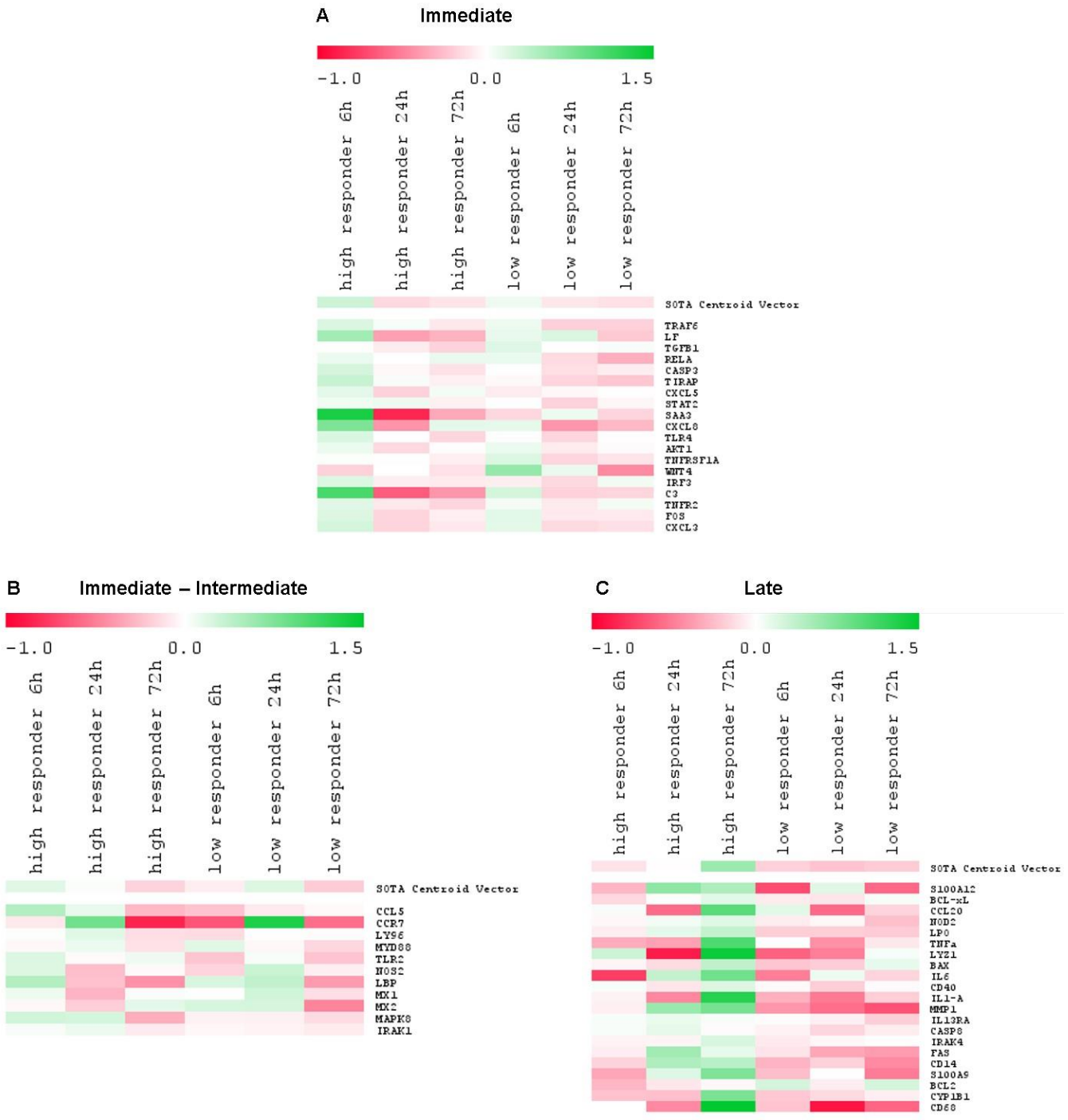


Figure 12: SOTA dendogram - time-dependent activation of different immune genes in the high and low responder group.

(A) 19 genes were immediately induced after treatment start, especially in the high responder group. **(B)** 11 genes were induced 6 h as well as 24 h after treatment start. **(C)** Especially in the high responder group around 21 genes were induced 72 h post induction.

A distinctly or even a statistically significant higher induction of the gene expression within the high responder group when compared to the low responder group, could be revealed for the genes coding for the TLR-pathway components LY96, CD14, TIRAP and RELA, for the chemokines CXCL8, CCL5, CXCL5, the inflammatory cytokines IL6 and IL1-A, the antimicrobial peptides LYZ1, LPO, apoptosis related factors (Bax, FAS, CASP8, CASP3) and the danger associated molecular pattern molecules S100A9 and S100A12, (Appendix II: Table 1). As nearly at all time-points a differential expression of the genes coding for LY96, CD14, TIRAP, RELA, CXCL8, CCL5, CXCL5, IL6, IL1-A, LYZ1, LPO, S100A9 and S100A12 was detected between the high and low responder animals, those genes could be favorable as potential molecular biomarkers (Figure 13). The differences in gene expression were not always statistically significant, but however distinctly different, as the inter-animal variations between the 4 to 5 individual biological replicates, were too divers (Figure 13).

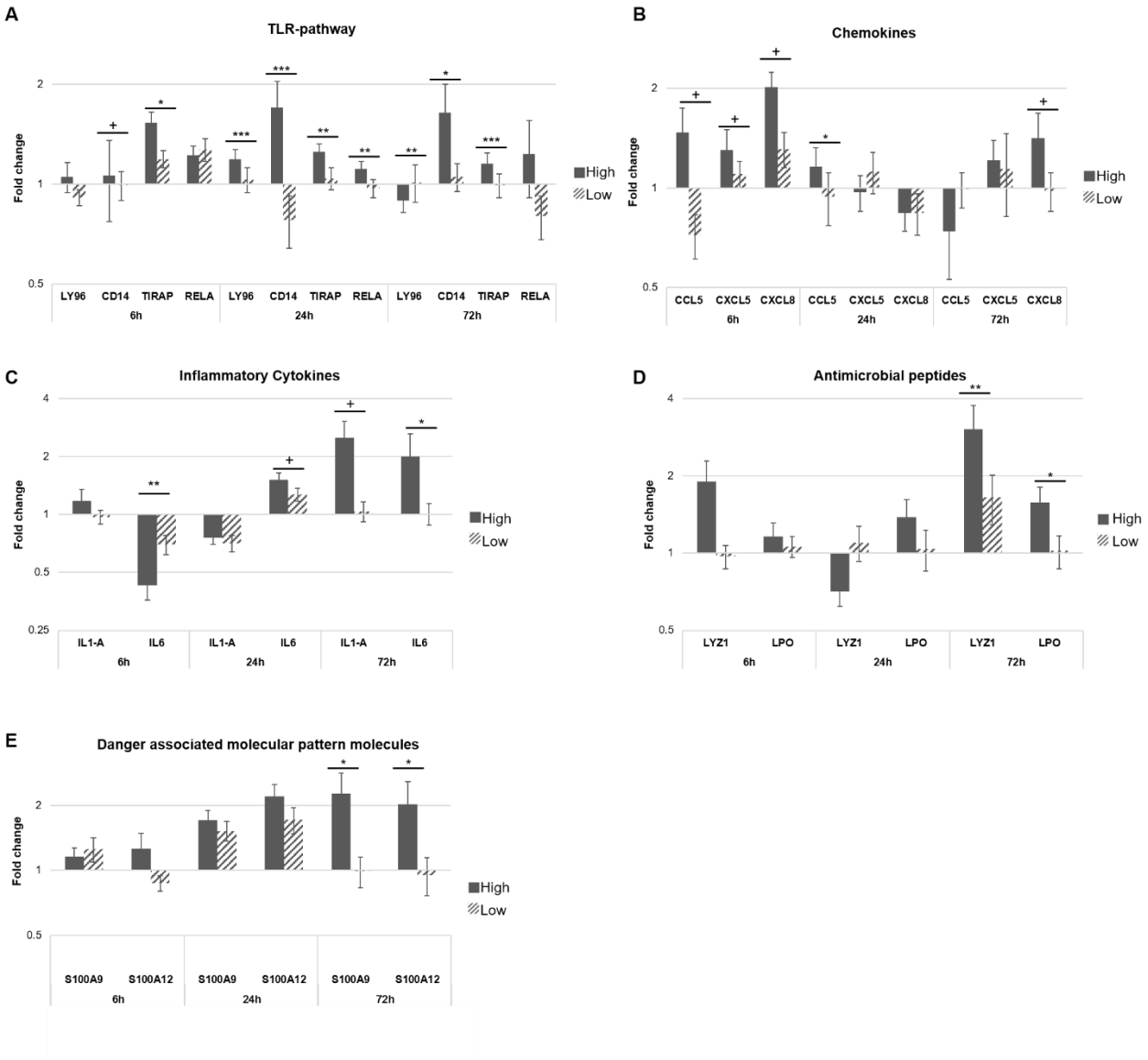


Figure 13: Differential gene expression between high and low responder animals – innate immune genes as potential molecular biomarkers:

The genes that could be utilized as potential molecular biomarkers are members of different innate immune families. Differential regulated genes coding for components of the **(A)** TLR-pathway, **(B)** for chemokines, **(C)** for inflammatory cytokines, **(D)** for acute phase proteins and **(E)** for danger associated molecular pattern molecules are shown. The distinct ($0.01 \leq p < 0.05$) or significant differences between the dCq values of the high and low responder animals, were evaluated with a t-test (* $p \leq 0.05$, ** $p \leq 0.01$, ***$p \leq 0.001$).

Like Strandberg et al. (2005), we also found that not the TLR receptors itself, but hence the availability and signaling capacity of the downstream effector molecules seems to be responsible for the proper activation of NFkB, the key transcription factor of the innate immune response [19]. The significantly higher gene expression of genes coding for key elements in the TLR pathway in the high responder group, seems to be accompanied with a faster and more efficient activation of genes coding for innate immune effector molecules, like chemokines, inflammatory cytokines,

antimicrobial peptides and danger associated molecular pattern molecules. The potential molecular biomarkers mentioned above, are discussed to be major initiators of the inflammatory response [14,19,102,103], leading to the activation of a positive feedback-loop in innate immunity. It is already known that for example CXCL8 recruits polymorphonuclear leukocytes (PMN) to the site of infection [21,104], whereas CCL5 mostly recruits memory T-helper cells, eosinophils and dendritic cells [21,105]. S100A9 and S100A12 that are known to be induced through the presence of gram-positive pathogens [14,103] are hence discussed to increase the transcription of cytokines and chemokines and therefore, to promote the enhanced activation of leukocytes [106,107]. The interplay of all those molecules within the high responder animals is likely to lead towards an immediate induction of the adaptive immune system and hence effective production and secretion of specific immunoglobulins.

4.5 The ketone body BHBA interferes with the innate immune response of pbMEC

As it is also of great interest to understand the complex interaction between innate immune system and external bacterial factors and/or internal metabolites, we evaluated the effect of the ketone body BHBA on the innate immune response of pbMEC against the mastitis pathogen *E. coli*. The more immunogenic gram-negative mastitis pathogen *E. coli* that was used as bacterial stimuli, strongly induced the gene expression of a variety of innate immune effector molecules. Amongst the most significantly induced genes were genes coding for CCL2, CCL20, CXCL8, IL6, TNFα, LF, LYZ1, TAP, SAA3, S100A9 and C3 (Figure 14). This was in accordance with several other studies [17,19,73] that also showed the predominant effect of *E. coli* on the expression of innate immune genes, whereby TLR4 and the TLR pathway components were mainly unaffected due to the bacterial stimuli (Appendix III: Table 2).

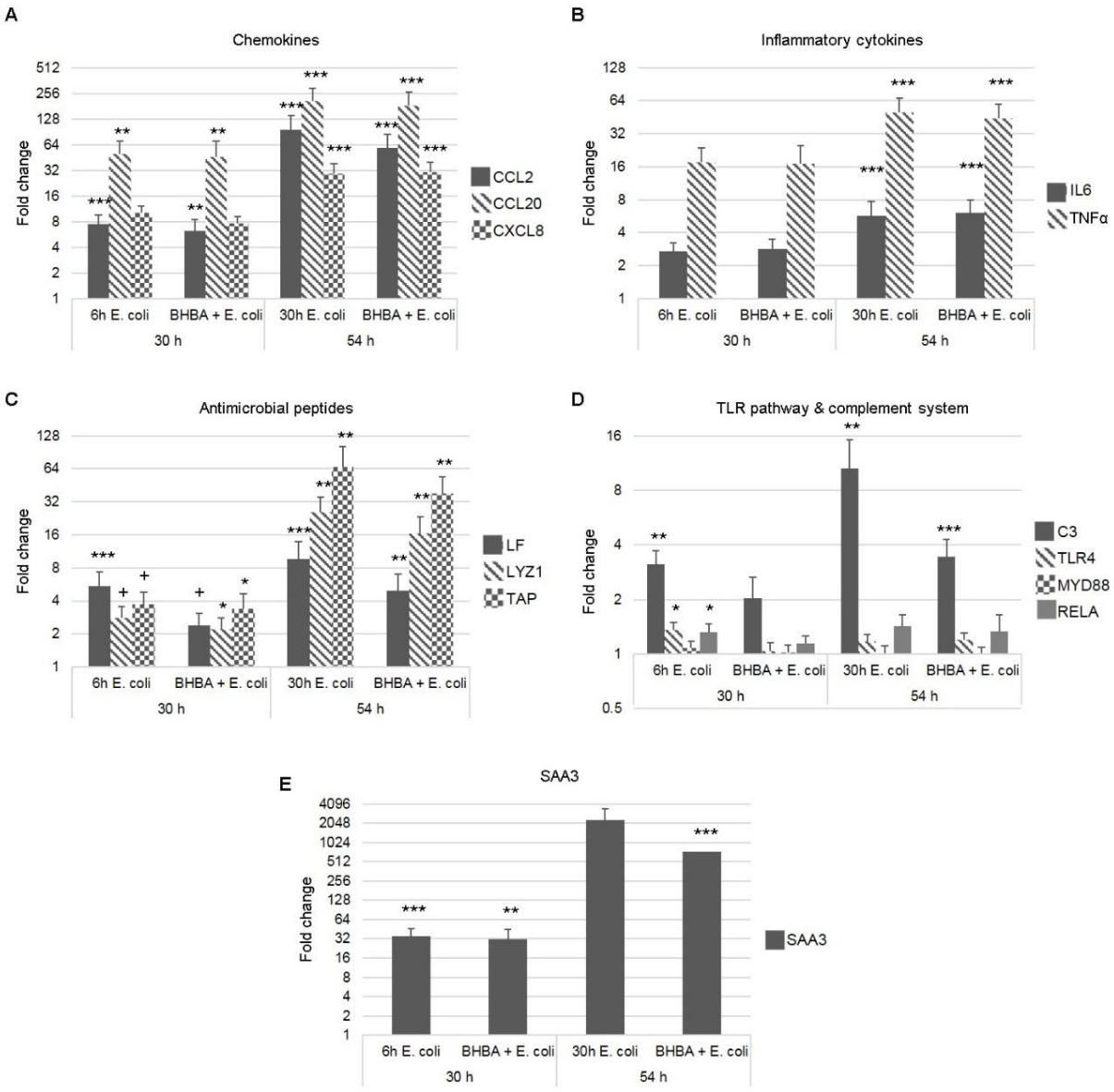


Figure 14: Effect of *E. coli* on the gene expression of selected innate immune genes.

(A) The genes coding for the chemokines CCL2, CCL20 and CXCL8 were highly significant induced upon stimulation with *E. coli*. **(B)** The inflammatory cytokines IL6 and TNFα, were significantly altered after 30 h of immune treatment, whereas **(C)** the genes coding for the antimicrobial peptides as well as **(E)** the acute phase protein SAA3, were affected during all treatment time-points**. (D)** The genes coding for TLR4 and components of the TLR pathway were only slightly induced, however, the gene coding for the complement component C3 was significantly induced upon stimulation with *E.coli* (* p ≤ 0.05, ** p≤ 0.01, ***p≤ 0.001).

As the direct effect of the, in ketosis, predominant ketone body BHBA on the innate immune response of pbMEC, has not been explicitly studied until now, we wanted to know how this ketone body could be responsible for the often coinstantaneous appearance of ketosis and mastitis in dairy cows. It is presumed that elevated levels of BHBA are responsible for a reduced activity of immune cells and that they have a negative effect on the chemotactic function of leukocytes

[2,108]. Therefore, it could be possible, that BHBA already negatively acts on different innate immune genes which hence leads to a lower production of chemotactic factors and in consequence to a declined immune response. The short term (30 h) and long term (54 h) effect of 3 mM BHBA on the gene expression of innate immune genes was evaluated, whereby only a very weak induction of immune gene expression could be determined (Appendix III: Table 2). Based on these findings, the metabolite BHBA alone seemed not to have strong immunogenic properties. However, the co-stimulation of pbMEC with *E. coli* and 3 mM BHBA resulted in distinctly and significantly declined gene expression profiles of CCL2, SAA3, LF and C3 (Figure 15). Especially long-term (54 h) supplementation with 3 mM BHBA tended to affect the innate immune response capability of pbMEC during the co-stimulation. Despite of the significant differences in the gene expression of CCL2, SAA3, LF and C3, nearly all genes coding for chemokines, inflammatory cytokines, antimicrobial peptides, acute phase proteins and the complement components, were likely to be attenuated when BHBA and *E. coli* were present in the culture medium (Appendix III: Table 2). The lack of statistical significance in those cases could be due to the diverse immune response of the pbMEC obtained from 6 different animals (6 biological replicates). However, the statistically significant attenuation of the above mentioned genes, was further confirmed on protein level, applying cell culture supernatants of all samples in a competitive LF-ELISA and a commercially available CCL2 sandwich ELISA (Figure 15 A, B). The ELISA measurements confirmed the RT-qPCR results, as it could be clearly seen that the protein production and secretion of LF and CCL2 was increased in the same way as the gene expression of both genes. Both the gene expression and the amount of secreted protein increased, when the treatment period with *E. coli* was prolonged from 6 h to 30 h, indicating the proper functionality of the innate immune defense in pbMEC. Additionally, both the gene expression and the amount of secreted protein significantly declined when pbMEC were co-stimulated with 3 mM BHBA and *E. coli* during both treatment time points (Figure 15 A, B).

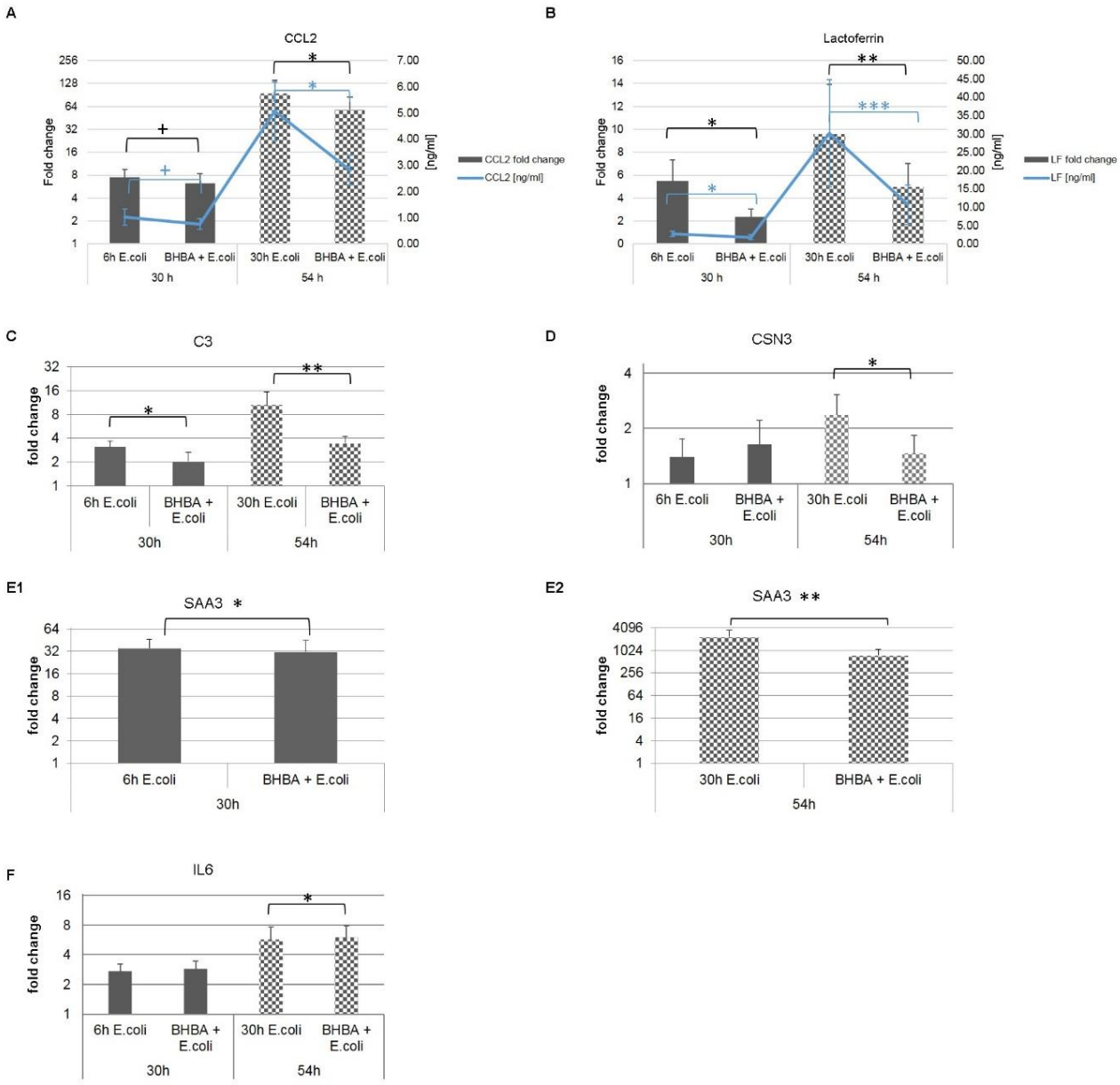


Figure 15: Co-stimulation with *E. coli* and BHBA attenuates the gene expression as well as the protein biosynthesis. (A, B) The RT-qPCR data of LF and CCL2 was confirmed with ELISA measurements.

(A) Bar graphs indicate the fold change of LF gene expression due to the different treatment approaches. Line graphs indicate the amount of LF that was secreted into the cell culture supernatant upon stimulation with *E. coli* (6 h, 30 h) or with both *E. coli* and 3 mM BHBA. **(B)** Amounts of secreted CCL2 are indicated with line graphs, whereas the differential gene expression of CCL2 is displayed with bar graphs. Fold changes of the **(C)** complement component C3, **(D)** the milk protein CSN3, **(E1-2**) the acute phase protein SAA3 and **(F)** the inflammatory cytokine IL6, are displayed in bar graphs. Significant changes in gene expression (black) as well as in the protein content (blue) are indicated by stars (*p ≤ 0.05: **p ≤ 0.01, ***p ≤ 0.001).

We therefore showed that genes of different innate immune families were affected by the metabolite BHBA. Especially, the chemokine CCL2 [14,109] is known to be fundamental for the development of a chemotactic gradient [16,18] and hence the recruitment of innate leukocytes that are not directly responsive to pathogens [110,111]. Interestingly, also the acute phase protein SAA3 that is discussed to be a potential biomarker for the inflammatory disease mastitis, was significantly altered due to BHBA, indicating the suppressive effect of this metabolite. The attenuation of gene expression and hence protein biosynthesis of those chemotactic molecules emphasized the findings of Suriyasathaporn et al. (1999, 2000) [2,108], that BHBA negatively affects the chemotactic function of leukocytes. Therefore, it might be possible that BHBA, that within our experiments clearly affected the gene expression of humoral defense molecules, as well as of chemotactic effector molecules, might be responsible for the elevated mastitis susceptibility in cows, suffering from long-term NEB.

5 Conclusion

We showed that the method of the non-invasive isolation of pbMEC from fresh cow milk, which was recently established by Sorg et al. (2012), is also suitable for the generation of a 3D cell culture system. The pbMEC cultivated on the ECM-like scaffold Matrigel® recapitulated various features they normally only have *in vivo*. Due to the scaffold, they maintained their polarized phenotype and built alveolar-like structures. Especially, due to the Matrigel® matrix, the pbMEC were able to express milk protein genes and hence secrete the produced proteins into the cell culture supernatant. This indicated that a functional 3D cell culture system of pbMEC was successfully established. This 3D cell culture system could in the future be used to obtain more faithful and transferable data, as the 3D cell culture approach represents a more *in vivo*-like model of the functional bovine mammary gland.

Additionally, this new cell culture approach served to elucidate the genes coding for the innate immune components, LY96, CD14, TIRAP, RELA, CXCL8, CCL5, CXCL5, IL6, IL1-A, LYZ1, LPO, S100A9 and S100A12 as potential molecular biomarkers. The potential molecular biomarkers are promising in case of the selection for high responder animals, that are able to immediately respond to external bacterial stimuli and hence produce higher amounts of immunoglobulins in milk. Those innate immune components are discussed to be crucial for the proper activation of the transcription factor NFκB and the formation of a chemotactic gradient, resulting in the recruitment of leukocytes and hence the induction of the adaptive immune response and immunoglobulin production.

Furthermore, we determined that long term exposure of pbMEC to the ketone body BHBA clearly attenuated the innate immune response competence of pbMEC against the predominant mastitis pathogen *E. coli*. Not only the gene expression (CCL2, LF, C3, SAA3, CSN3, IL6), but also the protein biosynthesis of innate immune genes like CCL2 and LF was altered in pbMEC, due to the metabolite. Therefore, we postulate that it is likely that the ketone body BHBA negatively affects the innate immune response capacity of pbMEC, and hence leads to a higher mastitis susceptibility in ketotic cows.

6 Perspectives

As the 3D cell culture approach of pbMEC seems to be promising for the investigation of molecular mechanisms that take place in the bovine mammary gland, the model should be further optimized in the future. To obtain even more faithful in *vivo-like* data, the pbMEC could also be cultured in the ECM-like scaffold Matrigel® and not only on top of the scaffold. It could be, that due to the cultivation in the scaffold, a branched network of connected alveoli is formed, even better representing the glandular tissue of the udder. However, this approach needs further improvements as it has to be evaluated whether the supplementation with oxygen and nutrients is sufficient when pbMEC are incorporated into the Matrigel® matrix. Additionally, the polarization and differentiation process of the pbMEC in 3D cell culture could be further visualized by fluorescence staining of basal surface receptors (integrin-1), adherence junction proteins (E-cadherin) and tight junction proteins, as described by Kozlowski et al (2011) [24]. The cultivation period and the treatment with lactogenic substances could be optimized and prolonged from 4 - 8 days up to 16 – 20 days, providing more time for the pbMEC to organize in tissue-like structures and produce and secrete milk and whey proteins. Furthermore, the LC-MS/MS measurements need to be further optimized as we were only able to qualitatively but not quantitatively determine the secreted proteins. Perhaps, preliminary experiments with stable cell lines, could be advantageous to determine the required cell density, cultivation period and amount of cell culture supernatant required for the LC-MS/MS measurements. Concerning the search for molecular biomarkers in gene expression that can in the future perhaps be used for the selection of fast immune responder animals, the results obtained with the pbMEC should of course be contemplated in conjunction with the gene expression data of blood lymphocytes. Furthermore, the experimental set-up should be repeated with pathogens, like *S. aureus* and *E. coli* that contribute to the most prevalent inflammatory disease affecting the animal's welfare and profitability. In general, it could be advantageous to focus on the miRNAs to obtain new insights in the regulation of the gene expression in the bovine mammary gland. miRNAs, associated with the post-transcriptional regulation of genes of the TLR signaling pathway (bta-miR146a, bta-miR-181a) as well as of genes coding for chemokines (bta-miR-16a, bta-miR23a, bta-miR-365-3p) and others, could be of interest to find a pathogen and perhaps also a high responder directed microRNA expression profile. In order to investigate the special set of microRNAs assigned to high responder animals, different metabolic disorders or pathogens, an advanced high-throughput sequencing method, like the next-generation sequencing approach, should be applied.

7 References

1. Seegers H, Fourichon C, Beaudeau F. Production effects related to mastitis and mastitis economics in dairy cattle herds. Vet Res. 2003; 34: 475–491. doi: 10.1051/vetres:2003027.
2. Suriyasathaporn W, Heuer C, Noordhuizen-Stassen EN, Schukken YH. Hyperketonemia and the impairment of udder defense: a review. Vet. Res. 2000; 31: 397–412. doi: 10.1051/vetres:2000128.
3. Dirksen G, Baumgartner W. Innere Medizin und Chirurgie des Rindes. 5th ed. Stuttgart: Parey; 2006.
4. Andersson L. Subclinical ketosis in dairy cows. Vet Clin North Am Food Anim Pract. 1988; 4: 233–251.
5. Duffield TF, Lissemore KD, McBride BW, Leslie KE. Impact of hyperketonemia in early lactation dairy cows on health and production. J. Dairy Sci. 2009; 92: 571–580. doi: 10.3168/jds.2008-1507.
6. Wellnitz O, Zbinden C, Huang X, Bruckmaier RM. Short communication: Differential loss of bovine mammary epithelial barrier integrity in response to lipopolysaccharide and lipoteichoic acid. J. Dairy Sci. 2016. doi: 10.3168/jds.2016-10927.
7. Nguyen DA, Neville MC. Tight junction regulation in the mammary gland. J Mammary Gland Biol Neoplasia. . 1998; 3: 233–246.
8. Schukken YH, Günther J, Fitzpatrick J, Fontaine MC, Goetze L, Holst O, et al. Host-response patterns of intramammary infections in dairy cows. Vet Immunol Immunopathol. . 2011; 144: 270–289. doi: 10.1016/j.vetimm.2011.08.022.
9. Akers RM. Lactation and the mammary gland. 1st ed. Ames, Iowa: Iowa State University Press; 2002.
10. Loeffler K, Gäbel G. Anatomie und Physiologie der Haustiere. 33 Tabellen. 13th ed. Stuttgart: Ulmer; 2013.
11. Sordillo LM, Streicher KL. Mammary gland immunity and mastitis susceptibility. J Mammary Gland Biol Neoplasia. 2002; 7: 135–146.
12. Hibbitt KG, Cole CB, Reiter B. Antimicrobial proteins isolated from the teat canal of the cow. J Gen Microbiol. 1969; 56: 365–371. doi: 10.1099/00221287-56-3-365.
13. Treece JM, Morse GE, Levy C. Lipid analyses of bovine teat canal keratin. J. Dairy Sci. 1966; 49: 1240-&.
14. Günther J,. Koczan D, Yang W, Nürnberg G, Repsilber D, Schubert HJ, Park Z, Maqbool N, Molenaar A, Seyfert HM. Assessment of the immune capacity of mammary epithelial cells: comparison with mammary tissue after challenge with Escherichia coli. Vet. Res. 2009; 40: 31. doi: 10.1051/vetres/2009014.
15. Pareek R, Wellnitz O, Van Dorp R, Burton J, Kerr D. Immunorelevant gene expression in LPS-challenged bovine mammary epithelial cells. J Appl Genet. 2005: 171–177.
16. Rainard P, Riollet C. Innate immunity of the bovine mammary gland. Vet. Res. 2006; 37: 369–400. doi: 10.1051/vetres:2006007.
17. Sorg D, Danowski K, Korenkova V, Rusnakova V, Küffner R, Zimmer R, Meyer HHD, Kliem H. Microfluidic high-throughput RT-qPCR measurements of the immune response of primary bovine mammary epithelial cells cultured from milk to mastitis pathogens. Animal. 2013; 7: 799–805. doi: 10.1017/S1751731112002315.
18. Hunziker W and Kraehenbuhl JP. Epithelial transcytosis of immunoglobulins. J Mammary Gland Biol Neoplasia. 1998: 287–302.

19. Strandberg Y, Gray C, Vuocolo T, Donaldson L, Broadway M, Tellam R. Lipopolysaccharide and lipoteichoic acid induce different innate immune responses in bovine mammary epithelial cells. Cytokine. 2005; 31: 72–86. doi: 10.1016/j.cyto.2005.02.010.
20. Griesbeck-Zilch B, Meyer H, Kühn C, Schwerin M, Wellnitz O. Staphylococcus aureus and Escherichia coli cause deviating expression profiles of cytokines and lactoferrin messenger ribonucleic acid in mammary epithelial cells. J. Dairy Sci. 2008; 91: 2215–2224. doi: 10.3168/jds.2007-0752.
21. Günther J, Koy M, Berthold A, Schuberth HJ, Seyfert HM. Comparison of the pathogen species-specific immune response in udder derived cell types and their models. Vet Res. 2016; 47: 22. doi: 10.1186/s13567-016-0307-3.
22. Stelwagen K (2011). Mammary gland, milk biosynthesis and secretion | milk protein. Encyclopedia of Dairy Sciences, Academic Press,New Yorkpp: pp. 359–366.
23. Rosen JM, Wyszomierski SL, Hadsell D. Regulation of milk protein gene expression. Annu Rev Nutr. 1999; 19: 407–436. doi: 10.1146/annurev.nutr.19.1.407.
24. Kozłowski M, Wilczak J, Motyl T, Gajewska M. Role of extracellular matrix and prolactin in functional differentiation of bovine BME-UV1 mammary epithelial cells. Pol J Vet Sci. 2011; 14: 433–442.
25. Lu LM, Li QZ, Huang JG, Gao XJ. Proteomic and functional analyses reveal MAPK1 regulates milk protein synthesis. Molecules. 2012; 18: 263–275. doi: 10.3390/molecules18010263.
26. Riley LG, Gardiner-Garden M, Thomson PC, Wynn PC, Williamson P, Raadsma HW, et al. The influence of extracellular matrix and prolactin on global gene expression profiles of primary bovine mammary epithelial cells in vitro. Anim Genet. 2010; 41: 55–63. doi: 10.1111/j.1365-2052.2009.01964.x.
27. Capuco AV, Akers RM (2011). Lactation | galactopoiesis, effects of hormones and growth factors. Encyclopedia of dairy sciences. Academic Press, New York pp 26‑31. doi: 10.1016/B978-0-12-374407-4.00252-1
28. Akers RM. Major advances associated with hormone and growth factor regulation of mammary growth and lactation in dairy cows. J. Dairy Sci. 2006; 89(4): 1222-34
29. Rezaei R, Wu Z, Hou Y, Bazer FW, Wu G. Amino acids and mammary gland development: nutritional implications for milk production and neonatal growth. J Anim Sci Biotechnol. 2016; 7: 20. doi: 10.1186/s40104-016-0078-8.
30. Bionaz M, Loor JJ. Gene networks driving bovine mammary protein synthesis during the lactation cycle. Bioinform Biol Insights. 2011; 5: 83–98. doi: 10.4137/BBI.S7003.
31. Sigl T, Meyer HHD, Wiedemann S. Gene expression analysis of protein synthesis pathways in bovine mammary epithelial cells purified from milk during lactation and short-term restricted feeding. J Anim Physiol Anim Nutr (Berl). 2014; 98: 84–95. doi: 10.1111/jpn.12039.
32. Choi YS, Chakrabarti R, Escamilla-Hernandez R, Sinha S. Elf5 conditional knockout mice reveal its role as a master regulator in mammary alveolar development: failure of Stat5 activation and functional differentiation in the absence of Elf5. Developmental Biology. 2009; 329: 227–241. doi: 10.1016/j.ydbio.2009.02.032.
33. Harris J, Stanford PM, Sutherland K, Oakes SR, Naylor MJ, Robertson FG, et al. Socs2 and elf5 mediate prolactin-induced mammary gland development. Mol Endocrinol. 2006; 20: 1177–1187. doi: 10.1210/me.2005-0473.
34 Yang X, Yang C, Farberman A, Rideout TC, de Lange, CFM, France J, et al. The mammalian target of rapamycin-signaling pathway in regulating metabolism and growth. J. Anim. Sci. 2008; 86: E36-50. doi: 10.2527/jas.2007-0567.
35. Bishop JD, Nien WL, Dauphinee SM, Too, Catherine KL. Prolactin activates mammalian target-of-rapamycin through phosphatidylinositol 3-kinase and stimulates phosphorylation of

p70S6K and 4E-binding protein-1 in lymphoma cells. J Endocrinol. 2006; 190: 307–312. doi: 10.1677/joe.1.06368.
36. Hayashi AA, Proud CG. The rapid activation of protein synthesis by growth hormone requires signaling through mTOR. Am J Physiol Endocrinol Metab. 2007; 292: E1647-55. doi: 10.1152/ajpendo.00674.2006.
37. Gilbert FB, Cunha P, Jensen K, Glass EJ, Foucras G, Robert-Granié C, et al. Differential response of bovine mammary epithelial cells to Staphylococcus aureus or Escherichia coli agonists of the innate immune system. Vet Res. 2013; 44: 40. doi: 10.1186/1297-9716-44-40.
38. Sorg D, Fandrey E, Frölich K, Meyer H, Kliem H. Mammary immunity of White Park and Highland cattle compared with Brown Swiss and Red Holstein. Anim. Genet. Resour. 2013; 52: 91–104. doi: 10.1017/S2078633612000781.
39. Janeway CA, Murphy K, Travers P, Walport M. Janeway's immunobiology. 7th ed. New York, London: Garland Science Taylor and Francis Group; op. 2008.
40. Aderem A, Ulevitch RJ. Toll-like receptors in the induction of the innate immune response. Nature. 2000; 406: 782–787. doi: 10.1038/35021228.
41. Hurley WL, Theil PK. Perspectives on immunoglobulins in colostrum and milk. nutrients. 2011; 3: 442–474. doi: 10.3390/nu3040442.
42. Thompson-Crispi K, Atalla H, Miglior F, Mallard BA. Bovine mastitis: frontiers in immunogenetics. Front Immunol. 2014; 5: 493. doi: 10.3389/fimmu.2014.00493.
43. Mallard BA, Emam M, Paibomesai M, Thompson-Crispi K, Wagter-Lesperance L. Genetic selection of cattle for improved immunity and health. Jpn J Vet Res. 2015; 63 Suppl 1: S37-44.
44. De Vliegher S, Fox LK, Piepers S, McDougall S, Barkema HW. Invited review: Mastitis in dairy heifers: nature of the disease, potential impact, prevention, and control. J. Dairy Sci. 2012; 95: 1025–1040. doi: 10.3168/jds.2010-4074.
45. Van Dissel, Jaap T, De Groot N, Hensgens CM, Numan S, Kuijper EJ, Veldkamp P, et al. Bovine antibody-enriched whey to aid in the prevention of a relapse of Clostridium difficile-associated diarrhoea: preclinical and preliminary clinical data. J Med Microbiol. . 2005; 54: 197–205. doi: 10.1099/jmm.0.45773-0.
46. Korhonen H, Marnila P, Gill HS. Bovine milk antibodies for health. Br J Nutr. 2000; 84 Suppl 1: S135-46.
47. Takemura K, Hogan JS, Lin J, Smith KL. Efficacy of immunization with ferric citrate receptor FecA from Escherichia coli on induced coliform mastitis. J. Dairy Sci. 2002; 85: 774–781. doi: 10.3168/jds.S0022-0302(02)74135-0.
48. Korhonen H, Marnila P, Gill HS. Milk immunoglobulins and complement factors. Br J Nutr. 2000; 84 Suppl 1: S75-80.
49. Mehra R, Marnila P, Korhonen H. Milk immunoglobulins for health promotion. International Dairy Journal. 2006; 16: 1262–1271. doi: 10.1016/j.idairyj.2006.06.003.
50. Reilly RM, Domingo R, Sandhu J. Oral delivery of antibodies. Future pharmacokinetic trends. Clin Pharmacokinet. 1997; 32: 313–323. doi: 10.2165/00003088-199732040-00004.
51. Pampaloni F, Reynaud EG, Stelzer EHK. The third dimension bridges the gap between cell culture and live tissue. Nat Rev Mol Cell Biol. 2007; 8: 839–845. doi: 10.1038/nrm2236.
52. Kozlowski M, Gajewska M, Majewska A, Jank M, Motyl T. Differences in growth and transcriptomic profile of bovine mammary epithelial monolayer and three-dimensional cell cultures. J. Physiol. Pharmacol. 2009; 60 Suppl 1: 5–14.
53. Baker BM, Chen CS. Deconstructing the third dimension: how 3D culture microenvironments alter cellular cues. J Cell Sci. 2012; 125: 3015–3024. doi: 10.1242/jcs.079509.

54. Debnath J, Muthuswamy SK, Brugge JS. Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. Methods. 2003; 30: 256–268.
55. Kleinman HK, Philp D, Hoffman MP. Role of the extracellular matrix in morphogenesis. Curr Opin Biotechnol. . 2003; 14: 526–532. doi: 10.1016/j.copbio.2003.08.002.
56. Von Der Mark K, Gauss V, Von Der Mark H, Müller P. Relationship between cell shape and type of collagen synthesised as chondrocytes lose their cartilage phenotype in culture. Nature. 1977; 267: 531–532. doi: 10.1038/267531a0.
57. Petersen OW, Rønnov-Jessen L, Howlett AR, Bissell MJ. Interaction with basement membrane serves to rapidly distinguish growth and differentiation pattern of normal and malignant human breast epithelial cells. Proc. Natl. Acad. Sci. U.S.A. 1992; 89: 9064–9068.
58. Bissell MJ, Rizki A, Mian IS. Tissue architecture: the ultimate regulator of breast epithelial function. Curr. Opin. Cell Biol. 2003; 15: 753–762.
59. Monzani PS, Bressan FF, Mesquita LG, Sangalli JR, Meirelles FV. β-casein gene expression by in vitro cultured bovine mammary epithelial cells derived from developing mammary glands. Genet. Mol. Res. 2011; 10: 604–614. doi: 10.4238/vol10-2gmr1034.
60. O'Brien LE, Jou TS, Pollack AL, Zhang Q, Hansen SH, Yurchenco P, et al. Rac1 orientates epithelial apical polarity through effects on basolateral laminin assembly. Nat Cell Biol. 2001; 3: 831–838. doi: 10.1038/ncb0901-831.
61. Pelham RJ, Wang Yl. Cell locomotion and focal adhesions are regulated by substrate flexibility. Proc Natl Acad Sci U S A. 1997; 94: 13661–13665.
62. Zegers MM, Le O'Brien, Yu W, Datta A, Mostov KE. Epithelial polarity and tubulogenesis in vitro. Trends In Cell Biology. 2003; 13: 169–176. doi: 10.1016/S0962-8924(03)00036-9.
63. Lutolf MP, Hubbell JA. Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. Nature Biotechnology. 2005; 23: 47–55. doi: 10.1038/nbt1055.
64. Antoni D, Burckel H, Josset E, Noel G. Three-dimensional cell culture: a breakthrough in vivo. Int J Mol Sci. 2015; 16: 5517–5527. doi: 10.3390/ijms16035517.
65. Griffith LG, Swartz MA. Capturing complex 3D tissue physiology in vitro. Nat Rev Mol Cell Biol. 2006; 7: 211–224. doi: 10.1038/nrm1858.
66. Bornstein P, Sage EH. Matricellular proteins: extracellular modulators of cell function. Curr Opin Cell Biol. 2002; 14: 608–616.
67. Paralkar VM, Vukicevic S, Reddi AH. Transforming growth factor β type 1 binds to collagen IV of basement membrane matrix: Implications for development. Developmental Biology. 1991; 143: 303–308. doi: 10.1016/0012-1606(91)90081-D.
68. Tschumperlin DJ, Dai G, Maly IV, Kikuchi T, Laiho LH, McVittie AK, et al. Mechanotransduction through growth-factor shedding into the extracellular space. Nature. 2004; 429: 83–86. doi: 10.1038/nature02543.
69. Cukierman E, Pankov R, Yamada KM. Cell interactions with three-dimensional matrices. Curr Opin Cell Biol. 2002; 14: 633–639.
70. Schwartz MA. Integrin signaling revisited. Trends In Cell Biology. 2001; 11: 466–470.
71. Geiger B, Bershadsky A, Pankov R, Yamada KM. Transmembrane crosstalk between the extracellular matrix--cytoskeleton crosstalk. Nat Rev Mol Cell Biol. 2001; 2: 793–805. doi: 10.1038/35099066.
72. Buehring GC. Culture of Mammary Epithelial Cells from Bovine Milk. J. Dairy Sci. 1990; 73: 956–963. doi: 10.3168/jds.S0022-0302(90)78752-8.
73. Danowski K, Gross JJ, Meyer HHD, Kliem H. Effects of induced energy deficiency on lactoferrin concentration in milk and the lactoferrin reaction of primary bovine mammary

epithelial cells in vitro. J Anim Physiol Anim Nutr (Berl). 2013; 97: 647–655. doi: 10.1111/j.1439-0396.2012.01305.x.
74. Koressaar T, Remm M. Enhancements and modifications of primer design program Primer3. Bioinformatics. 2007; 23: 1289–1291. doi: 10.1093/bioinformatics/btm091.
75. Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, et al. Primer3--new capabilities and interfaces. Nucleic Acids Res. 2012; 40: e115. doi: 10.1093/nar/gks596.
76. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE Guidelines: Minimum information for publication of quantitative real-time PCR experiments. Clinical Chemistry. 2009; 55: 611–622. doi: 10.1373/clinchem.2008.112797.
77. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods. 2001; 25: 402–408. doi: 10.1006/meth.2001.1262.
78. Spurgeon SL, Jones RC, Ramakrishnan R. High throughput gene expression measurement with real time PCR in a microfluidic dynamic array. PLoS ONE. 2008; 3: e1662. doi: 10.1371/journal.pone.0001662.
79. Saeed AI, Sharov V, White J, Li J, Liang W, Bhagabati N, et al. TM4: a free, open-source system for microarray data management and analysis. BioTechniques. 2003; 34: 374–378.
80. Cox J, Mann M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nature Biotechnology. 2008; 26: 1367–1372. doi: 10.1038/nbt.1511.
81. Cox J, Neuhauser N, Michalski A, Scheltema RA, Olsen JV, Mann M. Andromeda: a peptide search engine integrated into the MaxQuant environment. J Proteome Res. 2011; 10: 1794–1805. doi: 10.1021/pr101065j.
82. Jensen LJ, Kuhn M, Stark M, Chaffron S, Creevey C, Muller J, et al. STRING 8--a global view on proteins and their functional interactions in 630 organisms. Nucleic Acids Res. 2009; 37: D412-6. doi: 10.1093/nar/gkn760.
83. Binns D, Dimmer E, Huntley R, Barrell D, O'Donovan C, Apweiler R. QuickGO: a web-based tool for Gene Ontology searching. Bioinformatics. 2009; 25: 3045–3046. doi: 10.1093/bioinformatics/btp536.
84. Rose MT, Aso H, Yonekura S, Komatsu T, Hagino A, Ozutsumi K, et al. In vitro differentiation of a cloned bovine mammary epithelial cell. J Dairy Res. 2002; 69: 345–355.
85. Barcellos-Hoff MH, Aggeler J, Ram TG, Bissell MJ. Functional differentiation and alveolar morphogenesis of primary mammary cultures on reconstituted basement membrane. Development. 1989; 105: 223–235.
86. Weaver VM, Lelièvre S, Lakins JN, Chrenek MA, Jones JCR, Giancotti F, Werb Z, Bissell MJ. beta4 integrin-dependent formation of polarized three-dimensional architecture confers resistance to apoptosis in normal and malignant mammary epithelium. Cancer Cell. 2002; 2: 205–216.
87. Schmidhauser C, Casperson GF, Myers CA, Sanzo KT, Bolten S, Bissell MJ. A novel transcriptional enhancer is involved in the prolactin- and extracellular matrix-dependent regulation of beta-casein gene expression. Mol Biol Cell. 1992; 3: 699–709.
88. Bionaz M, Loor JJ. Gene networks driving bovine milk fat synthesis during the lactation cycle. BMC Genomics. 2008; 9. doi: 10.1186/1471-2164-9-366.
89. Aitken A. 14-3-3 and its possible role in co-ordinating multiple signalling pathways. Trends In Cell Biology. 1996; 6: 341–347.
90. Morrison D. 14-3-3: modulators of signaling proteins. Science. 1994; 266: 56–57.
91. Pawson T, Scott JD. Signaling through scaffold, anchoring, and adaptor proteins. Science. 1997; 278: 2075–2080.

92. Beck T, Hall MN. The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors. Nature. 1999; 402: 689–692.
93. Mori H, Inoue M, Yano M, Wakabayashi H, Kido H. 14-3-3tau associates with a translational control factor FKBP12-rapamycin-associated protein in T-cells after stimulation by pervanadate. FEBS Lett. 2000; 467: 61–64.
94. Vries LD, De Casey T, Dover H, Van de Haar MJ, Plaut K. Effects of transforming growth factor-β on mammary remodeling during the dry period of dairy cows. J. Dairy Sci. 2011; 94: 6036–6046. doi: 10.3168/jds.2011-4590.
95. Musters S, Coughlan K, McFadden T, Maple R, Mulvey T, Plaut K. Exogenous TGF-beta1 promotes stromal development in the heifer mammary gland. J. Dairy Sci. 2004; 87: 896–904. doi: 10.3168/jds.S0022-0302(04)73234-8.
96. Zoubiane GS, Valentijn A, Lowe ET, Akhtar N, Bagley S, Gilmore AP, et al. A role for the cytoskeleton in prolactin-dependent mammary epithelial cell differentiation. J Cell Sci. 2004; 117: 271–280. doi: 10.1242/jcs.00855.
97. Aplin AE, Juliano RL. Integrin and cytoskeletal regulation of growth factor signaling to the MAP kinase pathway. J Cell Sci. 1999; 112 (Pt 5): 695–706.
98. Assoian RK, Schwartz MA. Coordinate signaling by integrins and receptor tyrosine kinases in the regulation of G1 phase cell-cycle progression. Curr Opin Genet Dev. 2001; 11: 48–53.
99. Van der Flier A, Sonnenberg A. Function and interactions of integrins. Cell Tissue Res. 2001; 305: 285–298.
100. Van Hooijdonk AC, Kussendrager KD, Steijns JM. In vivo antimicrobial and antiviral activity of components in bovine milk and colostrum involved in non-specific defence. Br J Nutr. 2000; 84 Suppl 1: S127-34.
101. Caccavo D, Pellegrino NM, Altamura M, Rigon A, Amati L, Amoroso A, et al. Antimicrobial and immunoregulatory functions of lactoferrin and its potential therapeutic application. J Endotoxin Res. 2002; 8: 403–417. doi: 10.1179/096805102125001000.
102. Boudjellab N, Chan-Tang HS, Zhao X. Bovine interleukin-1 expression by cultured mammary epithelial cells (MAC-T) and its involvement in the release of MAC-T derived interleukin-8. Comp Biochem Physiol , Part A Mol Integr Physiol. 2000; 127: 191–199.
103. Lutzow Y,Strandberg C, Donaldson L, Gray CP, Vuocolo T, Pearson RD, Reverter A, et al. Identification of immune genes and proteins involved in the response of bovine mammary tissue to Staphylococcus aureus infection. BMC Vet Res. 2008; 4: 18. doi: 10.1186/1746-6148-4-18.
104. Griffith JW, Sokol CL, Luster AD. Chemokines and chemokine receptors: positioning cells for host defense and immunity. Annu Rev Immunol. 2014; 32: 659–702. doi: 10.1146/annurev-immunol-032713-120145.
105. Marques RE, Guabiraba R, Russo RC, Teixeira MM. Targeting CCL5 in inflammation. Expert Opin Ther Targets. 2013; 17: 1439–1460. doi: 10.1517/14728222.2013.837886.
106. Yang Z, Tao T, Raftery MJ, Youssef P, Di Girolamo N, Geczy CL. Proinflammatory properties of the human S100 protein S100A12. J Leukoc Biol. 2001; 69: 986–994.
107. Ryckman C, Vandal K, Rouleau P, Talbot M, Tessier PA. Proinflammatory activities of S100: proteins S100A8, S100A9, and S100A8/A9 induce neutrophil chemotaxis and adhesion. J Immunol. . 2003; 170: 3233–3242. doi: 10.4049/jimmunol.170.6.3233.
108. Suriyasathaporn W, Daemen AJ, Noordhuizen-Stassen EN, Dieleman SJ, Nielen M, Schukken YH. Beta-hydroxybutyrate levels in peripheral blood and ketone bodies supplemented in culture media affect the in vitro chemotaxis of bovine leukocytes. Vet. Immunol. Immunopathol. 1999; 68: 177–186.

109. Molenaar AJ, Harris DP, Rajan GH, Pearson ML, Callaghan MR, Sommer L, et al. The acute-phase protein serum amyloid A3 is expressed in the bovine mammary gland and plays a role in host defence. Biomarkers. 2009; 14: 26–37. doi: 10.1080/13547500902730714.
110. Gray GD, Knight KA, Nelson RD, Herron MJ. Chemotactic requirements of bovine leukocytes. Am J Vet Res. 1982; 43: 757–759.
111. Carroll EJ, Mueller R, Panico L. Chemotactic factors for bovine leukocytes. Am J Vet Res. 1982; 43: 1661–1664.

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9 Scientific communications

Original peer-reviewed scientific publications

Hillreiner M, Flinspach C, Pfaffl MW, Kliem H (2016) Effect of the Ketone Body Beta-Hydroxybutyrate on the Innate Defense Capability of Primary Bovine Mammary Epithelial Cells. PLoS ONE 11(6): e0157774. doi:10.1371/journal.pone.0157774

Hillreiner M, Müller NI, Koch HM, Schmautz C, Küster B, Pfaffl MW, Kliem H (2017) Establishment of a 3D cell culture model of primary bovine mammary epithelial cells extracted from fresh milk. In Vitro Cell.Dev.Biol.-Animal 2017. doi:10.1007/s11626-017-0169-7

Hillreiner M, Schmautz C, Ballweg I, Korenkova V, Pfaffl MW, Kliem H
Gene expression profiling in pbMEC– in search of molecular biomarkers to predict immunoglobulin production in bovine milk.
BMC Vet Res. 2017 Nov 29;13(1):369. doi: 10.1186/s12917-017-1293-z

Abstracts and posters presented at scientific conferences

Christiane Schmautz; Maria Hillreiner, Michael W. Pfaffl; Heike Kliem 2014, 3D Cell culture of bovine mammary epithelial cells from milk - Induction of lactogenesis and further use of the 3D cell culture model*. 3D cell culture 2014, Advanced Model Systems, Applications & Enabling Technologies, Freiburg, Germany* (24 -27 June 2014, presenting author: Maria Hillreiner)

Maria Hillreiner; Christiane Schmautz; Milica Maier; Michael W. Pfaffl; Heike Kliem 2015, Evaluation and quantification of the lactogenesis of primary bovine epithelial cells *in vitro* - 3D cell culture model of the bovine mammary gland – *6*^{7h} *international qPCR and NGS 2015 Event, Freising, Germany* (23 – 27 March 2015)

Scientific Talks

M. Sc. Maria Hillreiner, Dr. Heike Kliem, Prof. Dr. Michael Pfaffl, Lehrstuhl für Physiologie, TU München, „3D-Zellkulturmodell des bovinen Euters - Erforschung der Euterphysiologie und speziell der Euterimmunantwort in vitro unter den neuen Gesichtspunkten der 3-dimensionalen Zellkultur, Weihenstephaner Milchwirtschaftliche Herbsttagung, Freising, Germany (8-9 Octoberr 2015)

10 Curriculum Vitae

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11 Appendix

Appendix I:

Hillreiner M, Müller NI, Koch HM, Schmautz C, Küster B, Pfaffl MW, Kliem H

Establishment of a 3D cell culture model of primary bovine mammary epithelial cells extracted from fresh milk.

In Vitro Cellular & Developmental Biology – Animal 2017: doi:10.1007/s11626-017-0169-7

Appendix II:

Hillreiner M, Schmautz C, Ballweg I, Korenkova V, Pfaffl MW, Kliem H

Gene expression profiling in pbMEC– in search of molecular biomarkers to predict immunoglobulin production in bovine milk.

BMC Vet Res. 2017 Nov 29;13(1):369. doi: 10.1186/s12917-017-1293-z

Appendix III:

Hillreiner M, Flinspach C, Pfaffl MW, Kliem H

Effect of the Ketone Body Beta-Hydroxybutyrate on the Innate Defense Capability of Primary Bovine Mammary Epithelial Cells.

PLoS ONE 11(6): e0157774. doi:10.1371/journal.pone.0157774

Appendix I

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Establishment of a 3D cell culture model of primary bovine mammary epithelial cells extracted from fresh milk

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Abstract For the investigation of molecular processes underlying diseases of the bovine mammary gland, primary bovine mammary epithelial cells (pbMEC) are used. They are known to contribute to the innate immune system of the bovine mammary gland. The functionality of pbMEC depends on the maintenance of in vivo characteristics. So far, the optimization of pbMEC culture conditions was intended in a variety of experiments. For this purpose, most of the studies used stable cell lines or primary cells obtained from udder biopsies of slaughtered animals. By contrast, within our study, pbMEC of healthy and first lactating Brown Swiss cows were non-invasively isolated from fresh milk. The non-invasively isolated pbMEC were cultivated on the extracellular matrix-like scaffold Matrigel®. Further, they were challenged with different compositions of proliferation media, containing lactogenic hormones and/or the essential amino acid L-lysine. Changes in expression levels of genes coding for milk proteins and for components of the janus kinase/signal transducers and activators of transcription (JAK-STAT) and mTOR pathways were analyzed by RT-qPCR. The secreted proteins were analyzed by LC-MS/MS measurements. We showed for the first time the establishment of a physiologically functional 3D cell culture model of pbMEC isolated from fresh milk. This represents a primary cell culture model system, based on non-invasive cell collection, that can be used to unravel physiological processes in an unbiased manner.

Keywords Primary bovine mammary epithelial cells · 3D cell culture · Lactogenesis · Proteomics · RT-qPCR · Extracellular matrix

Introduction

Primary bovine mammary epithelial cells are located in the glandular tissue of the bovine mammary gland and are lining the alveoli, the milk building subunits of the gland. Therefore, the main task of primary bovine mammary epithelial cells (pbMEC) is the synthesis and secretion of milk and whey proteins (Rainard and Riollet 2006). However, they also contribute to the innate immune system of the bovine mammary gland, as they are able to recognize pathogens and furthermore secrete effector molecules, like chemokines and inflammatory cytokines, in order to attract immune cells to the site of infection (Rainard and Riollet 2006; Sorg et al. 2013). A lot of research has already been done to elucidate the mechanisms that occur when mastitis pathogens managed to cross the blood-mammary gland barrier. It is therefore very important to study those immune mechanisms in an in vivo-like environment where the pbMEC behave natively, so that the immune response obtained in vitro is more comparable to the immune response in vivo. In order to establish a 3D cell culture model, cells are cultivated on an extracellular matrix (ECM)-like scaffold. It has been shown that cells in 3D cell culture recapitulate a tissue-like organization and show higher cell

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proliferation levels compared to the 2D monolayer cell culture (Kozlowski et al. 2009). Therefore, the morphology and behavior of the cells in 3D architecture are very similar to the in vivo characteristics (Pampaloni et al. 2007). The first successful 2D cell culture of pbMEC extracted from milk was established by Buehring (1990). The isolation of pbMEC extracted from cow milk is a non-invasive and highly reproducible method, which allows the sampling of larger cohorts (Sorg et al. 2012; Buehring 1990) and reduces the cross-contamination of pbMEC cultures with fibroblasts. However, the tissue culture method and the use of stable cell lines are still predominant in this research field (Sorg et al. 2012). Therefore, we wanted to show that the establishment of a fully functional 3D cell culture model of pbMEC extracted from fresh milk is possible and hence able to compete with models generated with stable cell lines or cells from tissue biopsies. Additionally, we wanted to show that pbMEC cultured in 3D cell culture are capable to induce milk protein gene expression and subsequently secrete the produced proteins into the cell culture supernatant, using RT-qPCR and LC-MS/MS measurements as conformational methods.

Materials and Methods

3D cell culture of pbMEC extracted from milk The pbMEC used within this study were isolated non-invasively from fresh milk of five healthy Brown Swiss cows in mid-lactation (days 100–200) according to the method first described from Buehring (1990), further established and developed by Sorg et al. (2012) and Danowski et al. (2013) with slight optimizations in the washing procedure. Briefly, 1-L fresh cow milk was used to obtain pbMEC for one well of a six-well tissue culture plate. Six liters of fresh milk was taken each day for a time period of 2–3 wk. The sampling intervals and quantity of 1 L/one-well batches depended on the cell quality and total amount in the milk. Therefore, the sampling period and number of pbMEC isolations varied between the different animals. For the treatment, only one of the daily samples per animal was used, and the samples were not pooled. The milk was defatted (4°C, 1850×*g*, 10 min), and the remaining cell pellet was washed for several times with 1× Hanks balanced salt solution (HBSS buffer, Sigma-Aldrich, Saint Louis, MO). The 1× HBSS buffer was further supplemented with antibiotics and antimycotics to reduce the possibility of unintended contaminations in cell culture (Sorg et al. 2012). The washing procedure was optimized due to the insertion of a second filtration step, using two filter devices with different pore sizes (100 and 40 μm, Greiner Bio-One GmbH, Frickenhausen, Germany). pbMEC obtained from 1-L fresh milk were either for the 3D cell culture experiments, seeded onto six-well culture plates, coated with 2.4 mg/ml Matrigel® (growth factor reduced, Corning Inc., Corning, NY), or for the 2D cell culture controls, seeded on the plastic surface of six-well culture plate. The pbMEC were further cultivated (5% CO_2, 37°C) in proliferation media consisting of DMEM F12-Ham medium (Sigma-Aldrich) supplemented with 1 μg/ml of the lactogenic hormone hydrocortisone (Sigma-Aldrich), FBS (gibco® Life Technologies GmbH, Darmstadt, Germany), antibiotics, antimycotics, and insulin-transferrin-selenite (ITS) liquid media supplement (Sigma-Aldrich) (Sorg et al. 2013) until confluency. ITS liquid media supplement consists of insulin, transferrin, and selenite and is routinely used as supplement for bovine mammary epithelial cells (Danowski et al. 2013; Sorg et al. 2013; Jedrzejczak and Szatkowska 2014). After 2 wk, the pbMEC were detached with 0.25% trypsin/EDTA solution (Sigma-Aldrich) and 1×10^4 pbMEC were subcultivated either onto six-well culture plates coated with 2.4 mg/ml Matrigel® or onto normal six-well culture plates. For each of the five individual cows used in this experimental setup, cell culture duplicates for every experimental condition (Table 1) were obtained. After reaching confluency (1–2 wk) of the primary cell cultures, duplicates of pbMEC cultured on Matrigel® and duplicates of pbMEC cultured on plastic were treated with different proliferation media that were refreshed every day over a 4-d treatment interval. The different treatment approaches and the composition of the media are listed in Table 1. As the differentiation media were renewed daily, the cell culture supernatants were also collected daily and stored at −80°C until further analysis.

Immunocytochemistry For all pbMEC used within this experiment, 1×10^4 cells per cow were seeded into eight-well LabTec chamber slides (LAB-Tek, Nunc, GmbH, Langenselbold, Germany) in order to confirm the epithelial character by immunocytochemistry. For the immunocytochemistry (IC), pbMEC were taken from the same sample that was also used for further treatment. The IC was conducted according to a protocol described in Danowski et al. (2013) and Sorg et al. (2013) using the anti-cytokeratin pan antibody clone C-11 (1:400 in PBST; Sigma-Aldrich).

Microscopic cell viability test During the induction of lactogenesis, the differentiation of pbMEC was monitored using light microscopy (Leica Microsystems GmbH, Wetzlar, Germany). In order to verify the viability of the pbMEC involved in mammosphere formation, the pbMEC were stained with Vybrant® DIL Cell Labeling Solution (Life Technologies GmbH) according to the manufacturer's instructions. The stained pbMEC were then cultured and treated with 3 μg/ml prolactin (PRL) and 1 μg/ml hydrocortisone (HC) (corresponds to 3D + PRL, Table 1) for respectively 4 days to potentiate the number of alveolar-like structures present in the cell culture dishes.

Table 1. Composition of the different differentiation media used for the induction of lactogenesis

	Notation	Components				
		DMEM F12-Ham	ITS	Hydrocortisone 1 μg/ml	Prolactin 3 μg/ml	L-lysine 1.2 mM
2D	2D Ctr	+	+			
	2D culture	+	+	+		
3D	3D Ctr	+	+			
	3D culture	+	+	+		
	3D + PRL	+	+	+	+	
	3D + LYS	+	+	+		+
	3D + PRL + LYS	+	+	+	+	+

Ctr control, *PRL* prolactin, *LYS* L-lysine, *ITS* ITS liquid media supplement, consists of insulin, transferrin and selenite

Proteomics and secretomics-Preparation of the cell culture supernatants for protein quantification with LC-MS/MS The collected cell culture supernatants of the treated samples were directly supplemented with SigmaFast™ Protease Inhibitor (Sigma-Aldrich). Cell culture supernatants were concentrated by filtration using Amicon® Ultra-2 Centrifugal Filter Devices (3 kDa, Merck Millipore, Darmstadt, Germany). In brief, the filter unit was pre-rinsed with ddH_2O (3000×*g*, 15 min, 4°C) to remove remaining glycine from cellular membranes. The cell culture supernatants of all four treatment d were pooled, added to the filter device, and centrifuged several times (3000×*g*, 20–25 min, 4°C). To recover the concentrated proteins, the filter unit was inverted and centrifuged (1000×*g*, 2 min, 4°C). The total protein content was determined with the bicinchoninic acid (BCA) assay. The absorbance was measured photometrically at 562 nm using a microwell plate reader (Tecan Group AG, Männedorf, Switzerland) and the protein concentration of the sample was quantified using a protein standard of known concentration (Smith et al. 1985). The collected cell culture concentrate was stored at −80°C.

In-solution digestion of the protein concentrate For the in-solution digestion, 100 μg protein was added to 500 μl lysis buffer (40 mM Tris-HCl, pH 8.0 (Merck Millipore), 8 M urea (Roth GmbH & Co. KG, Karlsruhe, Germany). Disulfide bonds were reduced for 45 min at 56°C by adding DTT to a final concentration of 10 mM (AppliChem GmbH, Darmstadt, Germany). For the alkylation of the proteins, 50 mM chloracetamide (Sigma-Aldrich) was added and incubated for 60 min at room temperature. The sample was diluted with 5 vol. of 40 mM Tris pH 8.0 (Roth GmbH & Co. KG) to dilute the urea concentration below 1.5 μM. Trypsin (Promega, Mannheim, Germany) was added in a protease/protein ratio of 1:100 (*w*/*w*). Then, pre-digestion was performed at 37°C for 2 h. Afterwards, trypsin was added again in a protease/protein ratio of 1:100 and digestion was performed at 37°C overnight. For the inactivation of trypsin, the samples were acidified with formic acid (Merck Millipore) to pH 4.0 and subsequently stored at −80°C.

Affinity purification For the LC-MS/MS analysis, the digested samples were purified using a self-made column of five Octadecyl C18 Solid Phase Extraction Disks (3M Empore, Saint Paul, MN, 1.5 mm diameter, Sigma-Aldrich) that were squeezed into a 200-μl pipette tip. The column was first equilibrated with 25 μl pure acetonitrile (825×*g*, 30 s) and afterwards with 25 μl elution buffer (0.1% formic acid, 60% acetonitrile, Merck Millipore) (825×*g*, 30 s). Those equilibration steps were followed by two further equilibrations with 100-μl desalting buffer (0.1% formic acid). The samples were loaded onto the prepared column for several times (825×*g*, 3 min, reverse transcription (RT)) to assure proper binding. Afterwards, the flow through was discarded. The column was rinsed two times with 50 μl desalting buffer before elution was performed with 50 μl elution buffer (0.1% formic acid, 60% acetonitrile, Merck Millipore). The eluate was dried in a speed vac and the lyophilisate was stored at −80°C.

Secretome measurements by LC-MS/MS Lyophilized samples were reconstituted in 20 μl of solvent A (0.1% formic acid). LC-MS/MS measurements were performed by coupling an Eksigent NanoLC-Ultra $1D^+$ to an Orbitrap Velos instrument. Ten microliters of dissolved peptides was delivered to the trap column at a flow rate of 5 μl/min in loading solvent (0.1% formic acid in water) for 10 min. Peptides were delivered to the analytical column and were separated at a flow rate of 300 nl/min and a 110 min gradient. A gradient from 0 to 34% of solvent B was used for the separation (A = 0.1% formic acid, 5% DMSO in H_2O; B = 0.1% formic acid, 5% DMSO in acetonitrile): 0–2 min = 0% of solvent B; 2–100 min = 0–27% of solvent B; 100–101 min = 34–80% of solvent B; 101–105 min = 80% of solvent B; 105–106 min = 80–0% of solvent B; and 106–110 min = 0% of solvent B. The Orbitrap Velos was operated in data-dependent

mode, automatically switching between MS1 and MS2. Full-scan MS spectra were acquired at 360 *m/z* to 1300 *m/z*, 70,000 resolution, an automatic gain control (AGC) target value of 3×10^6 charges, and maximum injection time of 100 ms for MS1. Up to 10 precursor ions were allowed for fragmentation in tandem mass spectra. MS2 spectra were acquired at 200 *m/z* to 2000 *m/z*, 17,500 resolution with AGC target value of 1×10^5 charges, and maximum injection time of 50 ms. Precursor ion isolation width was set to 1.7 Th and dynamic exclusion to 20 s.

Proteomic data analysis The quantitative proteomics software package MaxQuant (Cox and Mann 2008) version 1.5.2.8 and its built-in Andromeda search engine (Cox et al. 2011) were used for the identification of peptides from raw LC-MS/MS data. Raw files were searched against the UniProtKB *Bos taurus* database, version June 2015 (23,870 entries). Carbamidomethylated cysteine was set as a fixed modification and oxidation of methionine and N-terminal protein acetylation were allowed as variable modifications. Enzyme specificity was set to trypsin/P, allowing for cleavage after proline. The minimum peptide length was set to seven amino acids and a maximum of two missed cleavages were allowed. The mass tolerance was set to 4.5 ppm for precursor ions and to 20 ppm for fragment ions. The dataset was adjusted to 1% FDR on the level of proteins and peptide spectrum matches (PSMs). The secreted proteins were qualitatively evaluated using Microsoft Excel (Microsoft Inc., Redmond, WA), and only proteins detected with at least two unique spectra were reported. Furthermore, a protein interaction network was built using STRING Version 10.0 (Jensen et al. 2009) including the following parameters: String interactions were considered if they had a combined score of at least 0.4. Unconnected nodes were displayed for the visual representation. The identified proteins were grouped according to their biological function, according to gene ontology (GO) terms using the UniProt-GOA database (Huntley et al. 2015) within the EMBL-EBI QuickGo browser (Binns et al. 2009).

Gene expression analysis-RNA extraction and reverse transcription After the treatment of the pbMEC in 2D and 3D cell culture with the respective media for 4 d, pbMEC were washed with PBS and lysed in Qiazol (Qiagen, Hilden, Germany). The RNA was extracted using the miRNeasy Micro Kit (Qiagen), following the manufacturer's instructions, with an additional incubation step for 5 min with buffer RPE after the first washing step with buffer RPE. Three hundred nanograms of RNA was reverse transcribed into cDNA, using the M-MLV (H-) Point Mutant Enzyme (Promega), 10 mM dNTPs, and 50 μM random hexamer primers (Invitrogen Life Technologies, Darmstadt, Germany). The RT reaction was conducted on the T-Personal Thermocycler (Biometra, Göttingen, Germany) according to the following protocol: annealing, 21°C, 10 min; transcription phase, 48°C, 50 min; and degrading phase, 90°C, 2 min. cDNA was stored at −20°C.

Primer design and RT-qPCR measurements Specific primer pairs (Table 2, Sigma-Aldrich) targeting the genes coding for milk proteins and components of the JAK-STAT and mTOR signaling pathway were designed with Primer3web version 4.0.0 (Koressaar and Remm 2007; Untergasser et al. 2012), based on published bovine nucleic acid sequences of the National Center for Biotechnology Information gene database (NCBI, National Library of Medicine, Bethesda, MD), and used for RT-qPCR measurements on the CFX384™ Real-Time System (Bio-Rad Laboratories GmbH, München, Germany). For the RT-qPCR, the SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories GmbH), VisiBlue™ qPCR mix colorant (TATAA Biocenter, Gothenburg, Sweden), RNase-free water, 20 μM of each primer (Sigma-Aldrich), and 10 ng/μl cDNA were used. (Activation of the DNA polymerase: 95°C, 30 s; 40 cycles of cDNA denaturation: 95°C, 5 s; specific primer annealing and elongation: 54°C/60°C/62°C, 5 s) (Table 2). The MIQE guidelines were considered for the setup and implementation of the RT-qPCR experiments (Bustin et al. 2009).

Data processing and evaluation of relative gene expression data In RT-qPCR, the cycle of quantification (Cq) was recorded. This is the PCR cycle at which the baseline-corrected amplification curve crosses the threshold value. The Cq values of the analyzed genes were normalized to a validated set of non-regulated reference genes (evaluated with Genex, Multid, Gothenburg, Sweden), consisting of YWHAZ, GAPDH, and KRT8 (Table 2). To obtain the dCq value, the raw Cq value was subtracted from the average of the reference genes' Cq. The dCq of the treated sample was subtracted from the dCq of the control sample to result in the ddCq value. Fold changes of gene expression were determined according to the 2^(−ddCq) method for each biological sample (Livak and Schmittgen 2001). Subsequently, the arithmetic mean of the replicates of each experimental group was calculated. The standard error of the mean (SEM) was determined by dividing the standard deviation of the samples by the square root of the sample size. Two different calculations were conducted. First of all, in order to obtain the differences between the 2D and the 3D cell culture approaches, the dCq value of pbMEC cultivated in 2D cell culture was in this case used to calculate the ddCq [ddCq = dCq(2D) − dCq(3D variations)]. This resulted in ddCq values that were used for the determination of the fold changes. For the statistical analysis, a paired *t* test was used, and the dCq values of the 3D cell culture approaches were compared to the dCq values of the 2D cell culture (Sigma-Plot

Table 2. Primer pairs for RT-qPCR measurements

Gene name	NCBI reference sequence number	Primer sequence (5′–3′) Forward Reverse	L [bp][a]
Reference genes			
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta polypeptide (*YWHAZ*)	NM_174814.2	CAGGCTGAGCGATATGATGA GACCCTCCAAGATGACCTAC	141
Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)	NM_001034034.1	GTCTTCACTACCATGGAGAAGG TCATGGATGACCTTGGCCAG	197
Keratin 8 (*KRT8*)	NM_001033610	TGGTGGAGGACTTCAAGACC CGTGTCAGAAATCTGAGACTGC	215
Milk protein genes			
α_{S1}-casein (*CSN1S1*)	NM_181029.2	AGCACCAAGGACTCCCTCAAGAAG CAGGTAACGCTCAGAGGGCAC	257
β-casein (*CSN2*)[b]	NM_181008.2	GGCTATGGCTCCTAAGCACA AGTTGGAGGAAGAGGCTGGT	163
κ-casein (*CSN3*)[c]	NM_174294.1	TGCAATGATGAAGAGTTTTTTCCTAG GATTGGGATATATTTGGCTATTTTGT	151
JAK-STAT pathway			
Runt-related transcription factor 2 (*RUNX2*)	XM_002684501.1	ACCATGGTGGAGATCATCG CCGGAGCTCAGCAGAATAA	207
YY1 transcription factor (*YY1*)	NM_001098081.1	GCTTGCCCTCATAAAGGCTGCACA GCAGCCTTCGAACGTGCACTGA	192
Signal transducer and activator of transcription 5A (*STAT5A*)	NM_001012673.1	GTGAAGCCACAGATCAAGCA TCGAATTCTCCATCCTGGTC	176
Janus kinase 2 (*JAK2*)	XM_865133.2	TCTGGTATCCACCCAACCATGTCT AATCATGCCGCCACTGAGCAA	201
E74-like factor 5 (*ELF5*)	NM_001024569.1	ATACTGGACGAAGCGCCACGTC ACTCCTCCTGTGTCATGCCGCA	134
CCAAT/enhancer binding protein, beta (*CEBPB*)	NM_176788.1	GCACAGCGACGAGTACAAGA GTTGCTCCACCTTCTTCTGG	152
Prolactin receptor (*PRLR*)	NM_001039726.1	CATGGTGACCTGCATCCTC ACCCTCATGCCTCTCACATC	172
mTOR pathway			
Eukaryotic translation initiation factor 4E binding protein 1 (*EIF4EBP1*)	NM_001077893.1	GAACTCACCTGTGACCAAGA CTCAAACTGTGACTCTTCACC	157
Ribosomal protein S6 kinase, 70 kDa, polypeptide 1 (*RPS6KB1*)	NM_205816.1	GGCAGCCCACGAACACCTGT AGGCGTCTGCGGATTTGCCG	96
v-akt murine thymoma viral oncogene homolog 1 (*AKT1*)	NM_173986.2	GATCACCGACTTCGGACTGT CTTCTCGTGGTCCTGGTTGT	202

[a] Amplicon length

[b] Annealing temperature 62°C

[c] Annealing temperature 54°C

12.0, Systat Software GmbH, Erkrath, Germany). Therefore, distinct ($^{+}0.05 \leq p \leq 0.10$) and significant changes ($^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$) always referred to the dCq values of pbMEC cultivated in 2D cell culture. The second calculation was conducted in order to determine changes in gene expression between the 3D cell culture control and the different treatment approaches in 3D cell culture. In this case, the dCq value of pbMEC cultivated in 3D cell culture was used to calculate the ddCq values for the 3D treatment approaches [ddCq = dCq(3D control) − dCq(3D treatments)]. For the statistical analysis, a one-factorial ANOVA on repeated measurements was used for the dCq values of the different treatment approaches in 3D cell culture (Sigma-Plot 12.0, Systat Software GmbH). Furthermore, the Tukey test method was applied for the pairwise multiple comparison procedure.

Results

Evaluation of the epithelial character Because of the low abundance of pbMEC in milk and the therefore limited disposability of pbMEC within our experiments, we decided to verify only the overall epithelial character of our cells using the monoclonal mouse anti-cytokeratin pan antibody clone C-11 (1:400 in PBST, Sigma-Aldrich). All pbMEC cultures were positively stained for the monoclonal mouse anti-cytokeratin

pan antibody clone C-11 following the method described in Danowski et al. (2013) and Sorg et al. (2013) (Fig. 1).

Mammosphere development and cell viability test The development of mammospheres was observed from day 1 till day 4 (Fig. 2*B*), when pbMEC were cultured in 3D cell culture on Matrigel® coated six-well plates and treated with HC and PRL. By contrast, pbMEC that were cultured on the plastic surface of the tissue culture plates only formed a cell monolayer, although the pbMEC received the same treatment (Fig. 2*A*). Due to the Vybrant® DIL Cell Labeling approach, it was shown that the observed alveolar-like structures, which further grew during 4 d of induction, consisted of proliferating, functional cells as a fluorescent signal of each cell, contributing to the alveolar-like structure, was determined via microscopy (Fig. 2*C*, *D*).

Effect of 3D cell culture and the treatment with lactogenic substances on the gene expression profile of pbMEC-Induction of milk protein gene expression We showed that the expression of genes coding for milk proteins was induced due to the cultivation of pbMEC on Matrigel®. When compared to the 2D cell culture, the expression of the gene coding for the milk protein αS1-casein (CSN1S1) was significantly induced and up-regulated in 3D cell culture (Fig. 3*A*). There was also a significant difference in CSN1S1 gene expression when the different treatment approaches in 3D cell culture were compared. Especially the treatment with only PRL (3D + PRL) and only L-lysine (3D + LYS) resulted in an induced expression of the gene coding for CSN1S1 when compared to the Matrigel® control (Table 3). Furthermore, the expression of the genes coding for κ-casein (CSN3) and β-casein (CSN2) was strongly affected by the cultivation of pbMEC in 3D cell culture; however, the induction was not statistically significant (Fig. 3*A*) due to high variations in gene expression from different animals. Additionally, it was shown that the expression of the gene coding for CSN3 was responsive to PRL, when compared to the Matrigel® control (Table 3).

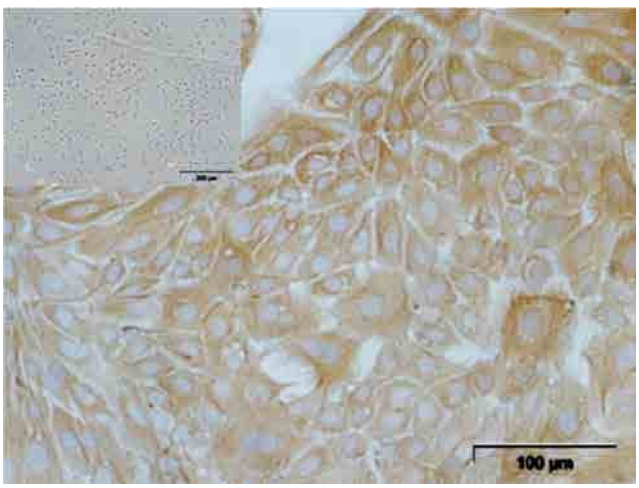

Figure 1. Anti-pan cytokeratin positively stained pbMEC (magnification ×200). The insert shows the negative control (magnification ×100). The IC was successfully conducted, as the cobblestone-like morphology and the epithelial character were confirmed

Induction of the JAK-STAT signaling pathway Furthermore, the effect of 3D cell culture and lactogenic hormones on components of the janus kinase/signal transducers and activators of transcription (JAK-STAT) signaling pathway was evaluated. The genes coding for the prolactin receptor (PRLR), JAK2, and RUNX2 were not differentially expressed in our experiment (Table 3). We showed that the expression of the gene coding for STAT5A was strongly induced through the cultivation of pbMEC in 3D cell culture; however, no significant changes were determined (Fig. 3*B*) due to high variations in gene expression of the five independent biological replicates. The additional treatment of pbMEC cultured on Matrigel® with lactogenic hormones did not result in any differential gene expression when compared to the Matrigel® control (Table 3). The gene coding for the transcription factor ELF5 showed a similar expression pattern to the gene coding for STAT5A with a generally higher but also not statistically significant up-regulation of the gene expression due to the cultivation on Matrigel® (Fig. 3*B*). CEBPB gene expression was significantly induced in pbMEC cultured on Matrigel® when compared to 2D cell culture (Fig. 3*C*). However, no effect on CEBPB gene expression was determined due to the addition of PRL and/or LYS. The gene expression of the transcription factor YY1 was significantly up-regulated in all treatment groups in pbMEC cultivated in 3D cell culture when compared to 2D cell culture (Fig. 3*C*).

Induction of the mTOR signaling pathway As the mTOR signaling pathway also contributes to the induction of milk protein gene expression, the expression of genes coding for the pathway components RPS6KB1, EIF4EBP1, and AKT1 was evaluated. EIF4EBP1 gene expression was induced due to the cultivation of pbMEC on Matrigel®. However, no significant induction of the gene expression was demonstrated (Fig. 3*D*). By contrast, the genes coding for RPS6KB1and AKT1 were significantly altered by the 3D cell culture approach (Fig. 3*D*). The expression of both genes was significantly induced due to the cultivation of pbMEC on Matrigel® (Fig. 3*D*). A differential gene expression for AKT1 was detected when the different treatment approaches in 3D cell culture were compared using a one-factorial ANOVA on repeated measurements (Table 3).

Detection of secreted proteins through LC-MS/MS analysis of cell culture supernatants The cell culture supernatants of pbMEC cultured on Matrigel® were collected and the secretome (secreted proteins) was analyzed using a qualitative LC-MS/MS measurement approach. It was possible to clearly

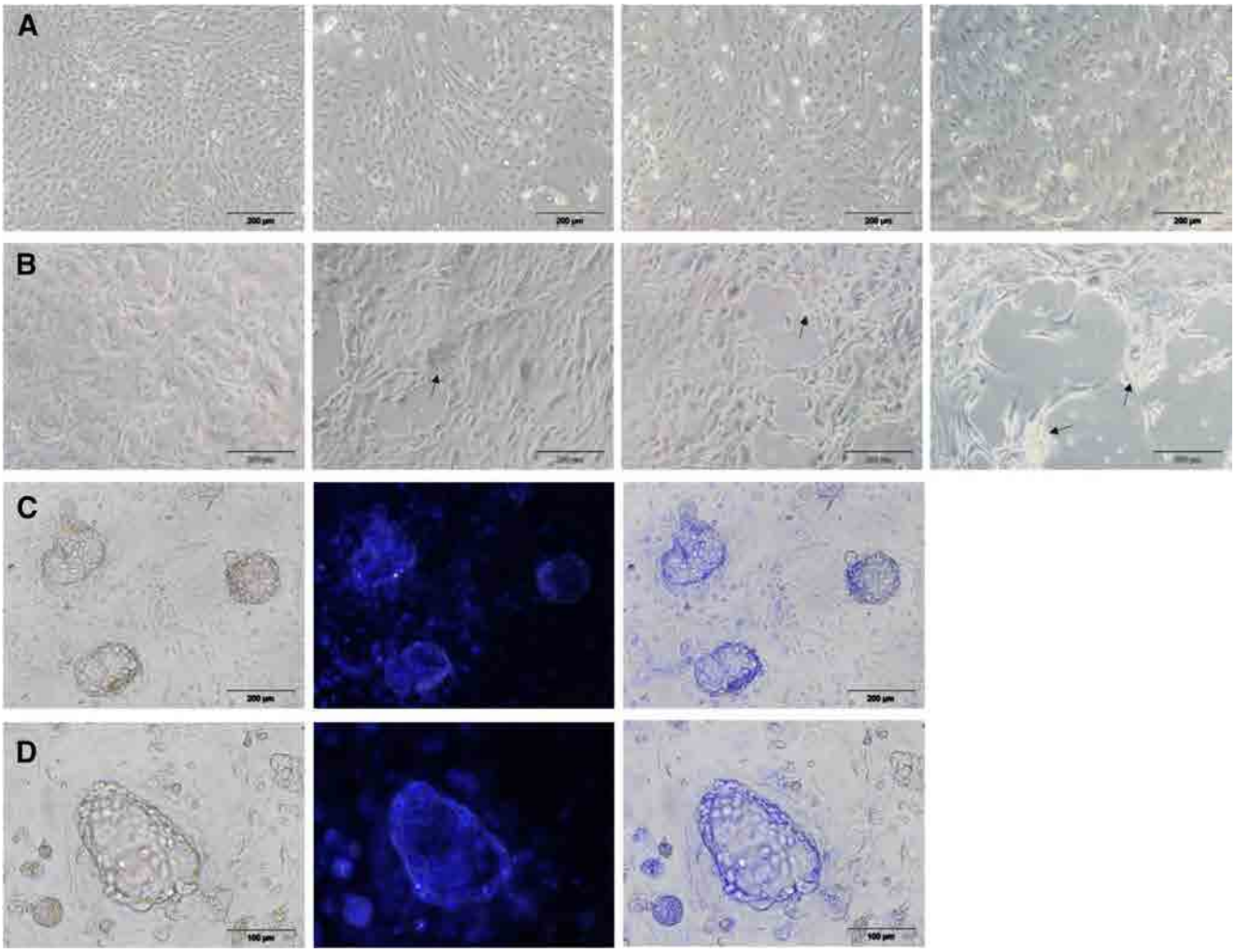

Figure 2. Development of mammospheres in 3D cell culture. pbMEC were cultured on 2.4 mg/ml Matrigel® (Corning Inc.) and treated with differentiation medium containing hydrocortisone and prolactin for up to 4 d. (*A*) pbMEC cultivated on the plastic surface of six-well tissue culture plates only formed a monolayer culture of pbMEC during the treatment period. (*B*) Whereby, pbMEC cultured on 2.4 mg/ml Matrigel® (Corning Inc.) further differentiated when treated with HC and PRL. This resulted in the development of alveolar-like structures over a period of 4 d. *Arrows* indicate the areas where alveolar-like structures were formed. (*C*), (*D*) Confirmation of the viability of pbMEC within the alveolar-like structures using a live staining with the fluorescent dye Vybrant® DIL Cell Labeling Solution (Life Technologies GmbH) after 3 and 4 d of differentiation. The strong fluorescence signal of pbMEC, contributing to the alveolar-like structures, confirmed the active proliferation of pbMEC contributing to the formation of mammospheres (magnification: *A*–*C*: ×100; *D*: ×200)

identify 56 proteins with more than 2 unique spectra (Tables S1 and 4 and Fig. 4). Those proteins were only present in cell culture supernatants of pbMEC cultures, as no unique spectra were determined in the medium control (Matrigel® + medium, without cells) for the multiple biological replicates. Among the secreted proteins, the milk and whey proteins αS1-casein (CSN1S1), β-casein (CSN2), α-lactalbumin (LALBA), and β-lactoglobulin (BLG) were found as well as proteins involved in cell proliferation and differentiation like 14-3-3 protein sigma (SFN), transgelin-2 (TAGLN2), galectin (LGALS3), colony stimulating factor 1 (CSF1), and transforming growth factor beta-induced protein ig-h3 (TGFB1). Additionally, proteins involved in cytoskeletal organization and reconstruction (alpha-actinin-1 (ACTN1), calponin-2 (CNN2), fascin actin-bundling protein 1 (FSCN1), Cofilin-1 (CFL1)) and immune response (beta-2-microglobulin (B2M), lactotransferrin (LTF), C-X-C motif chemokine 3 (CXCL3), C-X-C motif chemokine 6 (CXCL6), secretogranin-2 (SCG2), protein S100-A8 (S100A8)) were identified. In Table S1, further identified proteins of the before mentioned subcategories as well as proteins involved in protein folding, proteolysis, and translation are listed (Appendix, Table S1).

Discussion

This is one of the first studies dealing with the establishment of a functional 3D cell culture model of pbMEC isolated from

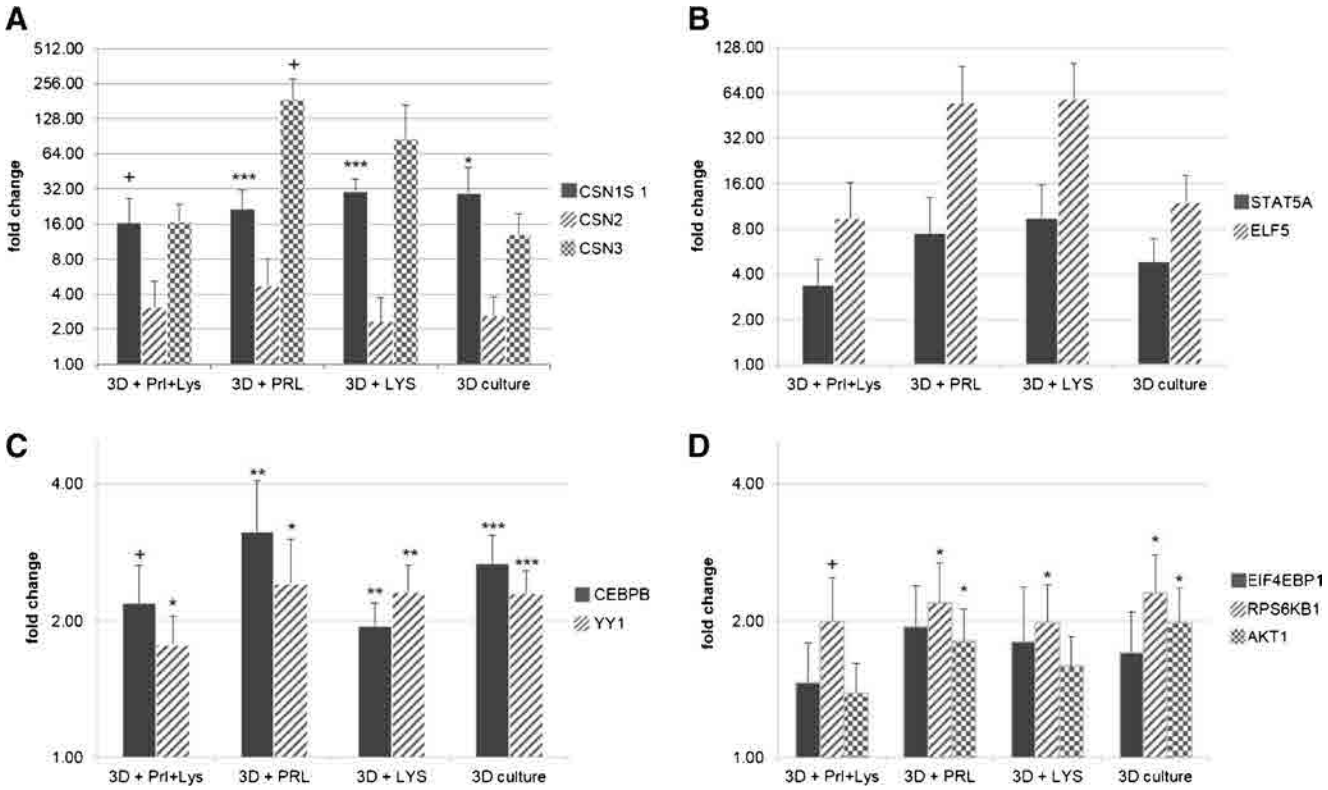

Figure 3. Fold changes of milk protein gene expression (*A*) and genes coding for components of the JAK-STAT pathway (*B*, *C*). Gene expression values were calculated based on the $2^{\wedge(-\mathrm{ddCq})}$ method of Livak and Schmittgen (2001), whereby the dCq value of pbMEC cultivated in 2D cell culture was in this case used to calculate the ddCq values that were used for the determination of the fold changes. Therefore, distinct ($^{+}0.05 \leq p \leq 0.10$) and significant changes indicated in the graphs ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$) always referred to the dCq values of pbMEC cultivated in 2D cell culture. (*A*) The gene expression of CSNS1 (αS1-casein) was distinctly and significantly altered due to the 3D cell culture of pbMEC. CSN3 (κ-casein) gene expression was also induced through the cultivation on Matrigel®; however, no significant changes could be calculated in the paired *t* test. (*B*) The genes coding for STAT5A and ELF5 components of the JAK/STAT pathway showed an up-regulation of the gene expression, which however was not statistically significant. (*C*) The expression of the genes coding for CEBPB and YY1, also components of the JAK/STAT pathway, were both significantly induced due to the cultivation of pbMEC on Matrigel®. (*D*) The expression of genes coding for the mTOR pathway components RPS6KB1 and AKT1 was also differentially regulated due to the cultivation of pbMEC on Matrigel®

fresh cow milk, sampled non-invasively. This study explicitly describes the reaction of in vivo-like primary cells, on the presence of an extracellular matrix-like scaffold in vitro, and the supplementation with the lactogenic hormone PRL and/or the amino acid LYS. The effect described here is not comparable with the effect of lactogenic hormones on stable cell lines. It is already known that those stable cell lines have lost most of their in vivo characteristics during the transformation process in vitro (Jedrzejczak and Szatkowska 2014). Furthermore, within experiments with stable cell lines, no effect of inter-animal variation has to be expected on the experimental outcome. By contrast, we worked with primary cells, obtained from five different animals. As every animal is fairly different also, the primary cells obtained for the experiments show a diverse response towards the different treatment approaches. This is likely to lead to high standard deviations and therefore less significant changes in gene expression. The results are nevertheless more likely to be better transferable when compared to results obtained with stable cell lines. One main task of pbMEC in vivo is the production and secretion of milk and whey proteins. The maintenance of this ability in vitro is a great evidence of the viability and functionality of these mammary epithelial cells. We therefore tried to maintain this in vivo architecture and characteristic of pbMEC. The concentration of the supplements (PRL = 3 μg/ml, HC = 1 μg/ml, and L-LYS = 1.2 mM) was selected according to previous studies of Riley et al. (2010), Kozłowski et al. (2011), and Lu et al. (2012).

The advantages of the milk cell extraction method The first successful 2D cell culture of pbMEC extracted from fresh milk was established by Buehring (1990). Despite of working with primary cells from tissue biopsies or with stable cell lines, Buehring (1990) demonstrated that the milk cell extraction method is a good alternative to other more invasive methods, especially, when primary cells are favored for the experimental work (BuehringÇ). It has been shown that pbMEC that are physiologically shed into the milk during

Table 3. Fold changes of pbMEC cultured in 3D cell culture compared to the untreated 3D cell culture control

Genes		3D + Prl[a]		3D + LYS[b]		3D + Prl + LYS[c]	
Milk protein genes							
CSN1S1	Fold	3.01	A	4.30	B	1.62	AB
	SEM	0.73		1.07		0.63	
CSN2	Fold	3.33		0.84		3.83	
	SEM	1.19		0.20		2.88	
CSN3	Fold	11.09	A**	4.29	B	4.45	AB
	SEM	4.28		2.23		2.60	
JAK-STAT pathway							
PRLR	Fold	3.32		3.15		3.45	
	SEM	1.48		2.03		1.40	
JAK2	Fold	1.34		1.19		1.10	
	SEM	0.35		0.23		0.22	
STAT5A	Fold	1.17		1.31		0.83	
	SEM	0.24		0.29		0.11	
ELF5	Fold	2.05		3.18		0.85	
	SEM	0.69		1.10		0.24	
CEBPB	Fold	2.05		3.18	–+	0.89	
	SEM	0.69		1.10		0.24	
RUNX2	Fold	1.10		1.20		1.02	
	SEM	0.46		0.25		0.27	
YY1	Fold	1.05		1.01		0.78	–*
	SEM	0.23		0.12		0.09	
mTOR pathway							
EIF4EBP1	Fold	1.42		1.05		0.91	
	SEM	0.46		0.20		0.12	
RPS6KB1	Fold	1.08		0.91		0.89	
	SEM	0.27		0.09		0.10	
AKT1	Fold	1.34	–**	0.94		0.82	
	SEM	0.55		0.20		0.12	

The effect of the different treatment approaches was investigated in 3D cell culture. For the statistical analysis of the different treatment approaches, a one-factorial ANOVA on repeated measurements with a post hoc pairwise comparison including the Tukey test for an adjustment of the p values for multiple testing was conducted. Uppercase letters indicate significant differences between the different treatment approaches in 3D cell culture. Differences between the 3D cell culture control and the different treatment approaches are indicated as stars

$^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{+}0.05 \leq p \leq 0.10$

[a] 3D + PRL = 3D cell culture on 2.4 mg/ml Matrigel®, DMEM-F12 Ham + ITS + hydrocortisone + prolactin

[b] 3D + LYS = 3D cell culture on 2.4 mg/ml Matrigel®, DMEM-F12 Ham + ITS + hydrocortisone + L-lysine

[c] 3D + PRL + LYS = 3D cell culture on 2.4 mg/ml Matrigel®, DMEM-F12 Ham + ITS + hydrocortisone + prolactin + L-lysine

the lactation process do have important advantages when compared to cells obtained from tissue biopsies (Sorg et al. 2012). The animal welfare as well as the practicability of the cell isolation strategy are in the foreground. Therefore, no additional stressor is added to the daily routine of the animal. Furthermore, there is the possibility to obtain pbMEC from one animal throughout the whole lactation period and during different treatment approaches. Additionally, it has been shown that the severe problem of cross-contamination with fibroblasts can be strictly avoided (Sorg et al. 2012). They are an important source for the investigation of cell metabolism, cell morphology, or immunological responses (Boutinaud et al. 2008). Sorg et al. (2012) showed that pbMEC extracted from milk maintained the ability to secrete cholesterol in vitro. Furthermore, the pbMEC were capable to better and longer maintain the in vivo-like gene expression profile, when compared to tissue-derived pbMEC (Sorg et al. 2012).

Maintenance of a polarized pbMEC phenotype in 3D cell culture Until now, most of the cell culture experiments with pbMEC were conducted in 2D cell culture approaches, where pbMEC were cultured on the plastic surfaces of tissue culture plates (Kozlowski et al. 2009). However, pbMEC cultured under these conditions only form monolayers. It is very difficult to transfer results, obtained under those simplified conditions, to a whole organ or organism (Pampaloni et al. 2007; Kozlowski et al. 2009). As the cells are separated from their cellular environment, they lose their tissue-specific architecture. This results in the loss of several important in vivo characteristics, as for example a coordinated cell-cell communication (Pampaloni et al. 2007; Kozlowski et al. 2009). The cultivation of cells in 3D cell culture however attempts to close the gap between 2D cell culture and the physiological tissue (Pampaloni et al. 2007). Therefore, cells are cultivated in or on top of scaffolds that mimic the composition of the extracellular matrix. The commercially available extracellular matrix-like scaffold Matrigel®, for example, consists of laminin (60%), collagen IV (30%), entactin (8%), heparin sulfate proteoglycan, and several growth factors (Corning Inc.). It has already been shown that cells cultivated in 3D cell culture characteristically changed their behavior and showed more natural and normal physiological properties (Pampaloni et al. 2007). The stress response declined and cells with a tissue-specific polarized phenotype were obtained (Pampaloni et al. 2007; Bissell et al. 2003). Furthermore, a tissue-like organization and a significantly induced cell proliferation level were detected within those 3D cell culture approaches, when compared to 2D cell culture (Kleinman et al. 2003; Kozlowski et al. 2009). For the cultivation of pbMEC in 3D cell culture, it was shown that they were able to recapitulate several morphological characteristics they normally only exhibit in vivo (Rose et al. 2002; Kozłowski et al. 2011; Monzani et al. 2011). The reticular-like structure of the polymerized Matrigel® enables a 3D orientation of the pbMEC resulting in a polarized phenotype, so that cells with an apico-basal polarity can be obtained in vitro (Barcellos-Hoff et al. 1989). The pbMEC are hence able to reorganize in

Table 4. Secreted proteins—evaluated with LC-MS/MS measurements

Identified protein	Uniprot ID	Gen
Onset of lactation		
Alpha-S1-casein	P02662	CSN1S1
Beta-casein	P02666	CSN2
Alpha-lactalbumin	P00711	LALBA
Beta-lactoglobulin	P02754	PAEP
Fatty acid synthase	F1N647	FASN
14-3-3 protein sigma	Q0VC36	SFN
Cell proliferation and differentiation		
Transgelin-2	Q5E9F5	TAGLN2
Galectin	A6QLZ0	LGALS3
Transforming growth factor beta-induced protein ig-h3	F1MBS3	TGFB1
Cytoskeletal organization		
Alpha-actinin-1	Q3B7N2	ACTN1
Calponin-2	Q3SYU6	CNN2
Fascin actin-bundling protein 1	Q3MHK9	FSCN1
Cofilin-1	Q5E9F7	CFL1
Immune system		
Beta-2-microglobulin	P01888	B2M
Lactotransferrin	P24627	LTF
C-X-C motif chemokine 3	F1MD23	CXCL3
C-X-C motif chemokine 6	P80221	CXCL6
Secretogranin-2	P20616	SCG2
Protein S100-A8	P28782	S100A8

tubular- and alveolar-like structures (Weaver et al. 2002; Debnath et al. 2003; Kozłowski et al. 2011). In our study, we also showed that pbMEC formed so-called mammospheres upon cultivation on Matrigel® and under treatment with lactogenic hormones. Due to a cell viability staining with the fluorescent cell labeling dye Vybrant® DIL, we further demonstrated that pbMEC participating in the formation of those alveolar-like structures were proliferating and physiologically functional. For our 3D cell culture approach, it was necessary to supplement the glucocorticoid hydrocortisone in all treatment approaches. This glucocorticoid is required in the bovine mammary gland to induce the differentiation of the alveolar system (Tucker 2000).

3D cell culture of pbMEC maintains milk protein gene expression and activity of underlying gene expression pathways-Milk protein gene expression pbMEC are known to contribute to the bovine milk production and hence composition. Caseins and whey proteins are the main proteins present in bovine milk (Cerbulis and Farrell 1975; Rimbach et al. 2010; Stelwagen 2011). In general, the composition of milk depends on the cow's breed, breeding season, stage of lactation, feeding, and external influences and therefore often varies (Rimbach et al. 2010; Månsson 2008). The caseins are classified as αS1-, αS2-, β-, and κ-caseins (Stelwagen 2011). In our study, αS1-, β-, and κ-casein were evaluated using RT-qPCR. The milk protein gene expression was in our study mainly induced due to the cultivation of the pbMEC on the ECM-like scaffold Matrigel®. Additionally, the different treatment approaches (PRL and/or LYS) did also alter the expression of all milk protein genes. The gene coding for αS1-casein (CSN1S1) was responsive to PRL and to LYS. The combination of both factors however did not result in any differential gene expression. This trend has also been shown, by Riley et al. (2010). They were also able to show that an appropriate ECM-like scaffold, like Matrigel®, is essential for the formation of mammospheres and that PRL induced the expression of the gene coding for αS1-casein. The strong but however not statistically significant induction of β- and κ-casein gene expression was in accordance with studies of Riley et al. (2010) and Kozłowski et al. (2011). Kozłowski et al. (2011) showed that the BME-UV1 cell line, when cultivated on Matrigel®, produced high amounts of β-casein even in the absence of PRL. They showed evidence that the expression of β-casein on post-transcriptional level did not significantly differ between cells grown on Matrigel® in the presence or absence of PRL. Furthermore, Schmidhauser et al. (1992) revealed that milk protein gene expression mainly depends on cell interactions with the basement membrane, as an ECM response element exists on the promotor region of the β-casein gene. Our results are therefore in agreement with previous studies already published in this research field. We also showed the stimulating effect of the ECM and the lactogenic hormone PRL on the induction of milk protein gene expression and hence, supposedly, milk protein production.

JAK/STAT signaling pathway Due to hormone receptors, the mammary epithelium is able to directly respond to PRL. As those receptors are members of the cytokine receptor superfamily and associate with the Janus kinase (JAK) family of tyrosine kinases (Capuco and Akers 2011), the effect of 3D cell culture and the treatment with lactogenic hormones on the gene expression of components of the JAK-STAT pathway was examined. Our results were in accordance with a study of Bionaz and Loor (2011). We also showed that the expression of the gene coding for the prolactin receptor (PRLR) and the kinase JAK2 were not affected due to 3D cell culture and the addition of lactogenic hormones (Bionaz and Loor 2011). The transcription factor STAT5A is an essential regulator of mammary gland development and responsible for prolactin signaling, alveolar development, and milk protein gene expression (Bionaz and Loor 2011; Liu et al. 2012). Therefore, a pronounced effect of the 3D cell culture as well as the treatment with lactogenic hormones was expected. We indeed

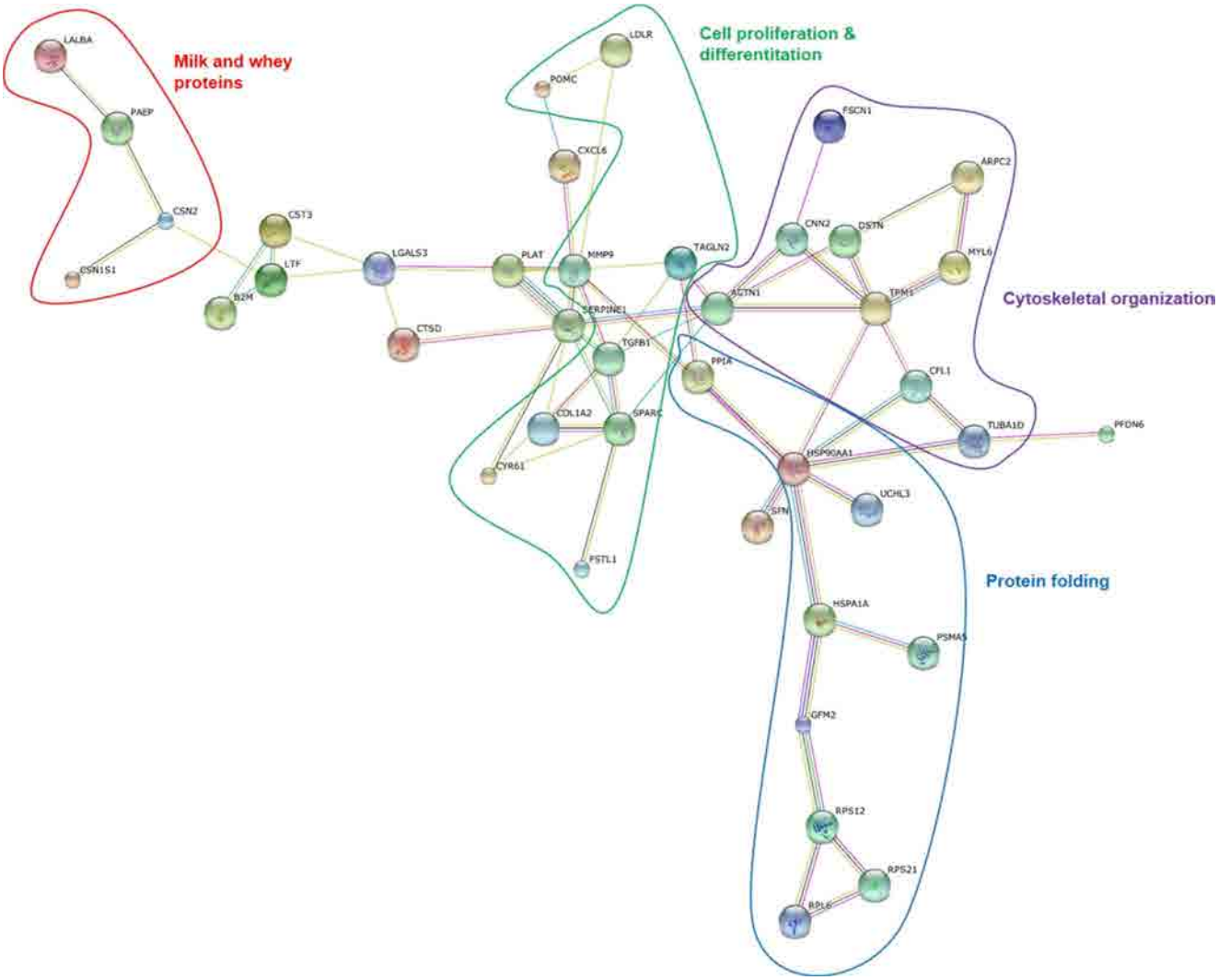

Figure 4. Secreted proteins by pbMEC cultured in 3D cell culture: The protein association network was generated with String 10 (Jensen et al. 2009), whereby disconnected nodes are not shown. Proteins with similar biological functions (EMBL-EBI, QuickGO browser, gene ontology) were clustered together. Among the secreted proteins, milk and whey proteins (*red*), proteins that promote cell proliferation and differentiation (*green*), proteins that induce cytoskeletal organization (*purple*), and proteins involved in protein folding were identified among others, using LC-MS/MS measurements.

detected a slightly increased expression of the gene coding for STAT5A due to the cultivation of pbMEC on Matrigel®. This matched with the increased expression of genes coding for CSN1S1, CSN2, and CSN3. In order to form a stable activation complex on the β-casein promotor to induce transcriptional activation, protein-protein interactions between STAT5A and CEBPB are required (Rosen et al. 1999). Therefore, it is already known that CEBPB plays a fundamental role in the regulation of CSN2 gene expression (Doppler et al. 1995). The gene expression of CSN2 was induced through the cultivation on Matrigel® as well as through the stimulation with lactogenic hormones and/or LYS in our study. Therefore, it was expected that the gene expression of CEBPB was also induced due to these cultivation and treatment approaches. We demonstrated that CEBPB gene expression was only affected due to the cultivation of pbMEC on Matrigel® but was not influenced by the addition of the lactogenic hormone PRL. This finding can be compared to a study of Doppler et al. (1995). They showed that the CEBPB expression in mouse mammary epithelial cells was not altered on post-transcriptional level by the presence of lactogenic hormones. However, they were not able to give evidence about the effect of 3D cell culture, as they only utilized a 2D cell culture approach. The multifunctional transcription factor YY1 is known to either activate or repress transcription of milk proteins (Rosen et al. 1999). The CSN2 promotor therefore contains a negative regulatory region, where YY1 is able to bind in the absence of lactogenic hormones (Rosen et al. 1999). We demonstrated that YY1 gene expression was significantly up-regulated in all treatment

groups when pbMEC cultured in 3D cell culture were compared to pbMEC cultured in 2D cell culture. Remarkably, the challenge with PRL and LYS together induced a significant down-regulation of the gene expression of YY1 when compared to the Matrigel® control. The treatments with only PRL or LYS did not show any effect on YY1 gene expression when compared to the Matrigel® control. Sigl et al. (2014) demonstrated that the gene expression of YY1 did not differ significantly during lactation and concluded that YY1 seems to have only little importance in the milk protein gene expression.

mTOR pathway The mTOR pathway and its components play an essential role in the promotion of cellular metabolism and tissue growth. Components of the pathway act as regulators of protein synthesis at transcriptional and translational level (Bionaz and Loor 2011). The mTOR pathway can be activated due to the presence of essential amino acids, like LYS, and other extracellular signals, like insulin (Bionaz and Loor 2011). We investigated several components of the mTOR pathway concerning their responsiveness to 3D cell culture and the treatment with the lactogenic hormone PRL and the essential amino acid LYS. The most responsive gene was the gene coding for RPS6KB1. This kinase is known to phosphorylate the ribosomal protein S6 and therefore promotes the initiation of protein biosynthesis (Bishop et al. 2006; Yang et al. 2008; Bionaz and Loor 2011). In our study, the differential expression of RPS6KB1 was induced due to the cultivation of pbMEC in 3D cell culture and not through the different treatment approaches. It has already been discussed that an increased phosphorylation of S6 through RPS6KB1 correlates with an increased milk protein synthesis (Hayashi et al. 2009) and hence the onset of lactation (Toerien and Cant 2007). The gene expression of AKT1, another initiator kinase of the mTOR pathway, was expected to be up-regulated through the lactogenesis-inducing treatments. The study of Burgos et al. (2010) demonstrated that the treatment of bovine mammary acini with hydrocortisone, prolactin, and insulin increased the phosphorylation of AKT1 and therefore induced milk protein synthesis. However, the expression of the gene coding for AKT1 was only significantly up-regulated upon cultivation of the pbMEC in 3D cell culture. No differential gene expression due to the different treatment approaches was detected when compared to the Matrigel® control. The same trend was demonstrated for the expression of the gene coding for the downstream effector molecule EIF4EBP1. The phoshporylation of this downstream effector molecule leads to the formation of the eIF4E complex which furthermore results in an enhanced milk protein synthesis in pbMEC (Bionaz and Loor 2011; Hayashi and Proud 2007; Yang et al. 2008). Other studies that used 2D culture approaches and explant cultures described an induction of EIF4EBP1 activity upon stimulation of the mammary gland with lactogenic hormones (Toerien and Cant 2007) and essential amino acids (Burgos et al. 2010). By contrast within our experiments, an increased EIF4EBP1 gene expression was only detected due to the 3D cell culture approach. We assume, that in our case, the effect of the ECM-like scaffold surpassed the effect of the lactogenic hormones.

Secretomics The RT-qPCR measurements demonstrated the onset of gene expression of several genes related to lactation. We however wanted to additionally evaluate whether an increased gene expression also resulted in the secretion of proteins involved in lactogenesis and cell differentiation. In order to further verify the functionality of pbMEC in vitro, we evaluated the secretome of pbMEC cultured in 3D cell culture using a LC-MS/MS approach. Only the secretome of pbMEC cultured on Matrigel® was qualitatively evaluated. A quantitative evaluation of differences between the 2D cell culture and 3D cell culture approach and the different treatments in 3D cell culture was not possible, as the inter-animal variations were too prominent. In order to explicitly and quantitatively discuss the differences in the secretome of pbMEC from 3D cell culture and 2D cell culture, an experimental setup with stable cell lines should possibly be used. This could guarantee high reproducibility between the samples and hence the LC-MS/MS measurements. However, we successfully confirmed that pbMEC in 3D cell culture were able to secrete various proteins. Those proteins are known to be involved in lactation, cell proliferation, cell differentiation, immunity, protein folding, and proteolysis indicating that they were fully functional when cultivated on Matrigel®. This in fact means that the 3D cell cultures of pbMEC were functional in terms of the production of certain proteins. However, on the basis of the protein expression data, we were not able to demonstrate that the 3D cell cultures were more functional than the 2D cell cultures of pbMEC.

Onset of lactation The main milk (CSN1S1, CSN2) and whey proteins (LALB, LGB) were detected in the cell culture supernatants by LC-MS/MS. Therefore, the onset of CSN1S1, CSN2, and CSN3 gene expression, which had been evaluated with RT-qPCR measurement, was validated on the protein level. Furthermore, we detected the secretion of the fatty acid synthase FASN into the cell culture medium. It is already known that lactogenesis is characterized by a dramatic up-regulation in expression of genes associated with fatty acid uptake (Bionaz and Loor 2008). Accordingly, also the expression of genes involved in fatty acid de novo synthesis, like FASN, is up-regulated in the early phase of lactation (Bionaz and Loor 2008). The secreted SFN (14-3-3 σ) protein could also be important for the induction of lactogenesis and milk protein production. SFN is an isoform of the 14-3-3 protein family (Aitken 1996). It is known that those proteins are involved in several biological processes including cell growth,

cell cycle, and signal transduction (Morrison 1994; Aitken 1996; Pawson and Scott 1997). Furthermore, Beck and Hall (1999) and Mori et al. (2000) showed that 14-3-3 proteins positively regulate the mTOR pathway and therefore promote the production of milk proteins. Additionally, it has been shown that the 14-3-3 σ isoform is only expressed in epithelial cells (Fu et al. 2000). Therefore, we postulate that the production of SFN in pbMEC and the secretion in the surrounding cell culture medium may have a positive impact on the initiation of lactation.

Cell proliferation and differentiation It was also possible to detect proteins involved in epithelial cell differentiation and polarization. We detected galectin (LGALS3) that is known to be important during mammary gland remodeling in the mouse alveolar system (Mengwasser et al. 2002). Furthermore, TGFB1 that is known to be involved in cell adhesion and ECM organization during mammary remodeling was identified (De Vries et al. 2011). De Vries et al. (2011) and Musters et al. (2004) showed that TGFB1 is required to induce the remodeling of the mammary gland of dairy cows, as a coordinated change in stromal and epithelial tissue is required during the remodeling process. TGFB1 regulates the expression of ECM proteins and proteases and is known to have cell-type dependent effects on proliferation. Therefore, Musters et al. (2004) concluded that TGFB1 selectively acts on the stromal compartment of the bovine mammary gland, resulting in cell proliferation and conversion of fibroblasts into myofibroblasts, promoting the formation of the alveolar system.

Cytoskeletal organization It is known that adhesion to the ECM is crucial for the regulation of the differentiated state of epithelial cells in the mammary gland (Roskelley et al. 1995; Streuli and Edwards 1998). In vivo, the mammary epithelial cells are connected to a specialized ECM, the so-called basement membrane (Prince et al. 2002). It has already been shown that the ECM protein laminin is of great importance in the case of pbMEC differentiation and the induction of milk protein gene expression (Streuli et al. 1995). One of the main components of Matrigel® that was used as ECM-like scaffold within our experiments is laminin (60%). The pbMEC were therefore able to interact with the laminin present in the scaffold, like in vivo, via integrins in the epithelial cell membrane. It has already been proved that integrins actively influence the cellular differentiation via the activation of enzymatic signaling molecules (Aplin and Juliano 1999; Assoian and Schwartz 2001; van der Flier and Sonnenberg 2001). Alternatively, it is discussed that the adhesion of pbMEC to the ECM through the actin cytoskeleton is another possibility to control the cell phenotype indirectly through a receptor-mediated signaling via the cytoskeleton (Aplin and Juliano 1999; Zoubiane et al. 2004). Zoubiane et al. (2004) further showed that an intact cytoskeleton is crucial for mammary epithelial cell differentiation and the regulation of milk protein gene expression, as the cytoskeleton is responsible for the delivery of the transcription factor STAT5 to the nucleus. We were able to detect several proteins involved in cytoskeletal and especially in actin filament organization (cofilin-1, calponin-2, alpha-actinin-1), cytoskeletal anchoring to the plasma membrane (dystroglycan), and establishment of apical-basal polarity (FSCN1), indicating the presence of fully differentiated and functional pbMEC in vitro.

Immune system It is already known that pbMEC, besides their main task to produce milk and whey proteins, also contribute to the innate immune system. We ascertained the production and secretion of the humoral immune factors B2M and LTF. LTF is an iron binding glycoprotein that is mainly produced by glandular epithelial cells and is found in many physiological fluids including milk (Van Hooijdonk et al. 2000). The protein is known for its effective antimicrobial, antiviral, and immunomodulatory activities (Caccavo et al. 2002). Wellnitz et al. (2006), Griesbeck-Zilch et al. (2008), and Sorg et al. (2013) already showed that the expression of the gene coding for LTF is responsive to the stimulation with gram-negative bacteria. Our findings that pbMEC are capable to permanently produce and secrete LTF are in accordance with Sorg et al. (2013), who showed that LTF can also be detected in the cell culture supernatant using ELISA measurements. We further showed evidence for the presence of chemotactic C-X-C motif chemokines, like CXCL3 and CXCL6, in pbMEC cell culture supernatants. These chemokines are responsible for the recruitment of leukocytes and macrophages in case of inflammation. The secretion of proteins involved in inflammatory response, which was monitored with LC-MS/MS measurements, is an additional indicator for the functionality of the pbMEC cultured in 3D cell culture.

Conclusion

To our knowledge, this is one of the first studies that demonstrated the suitability of pbMEC, which were non-invasively isolated from fresh cow milk, for the establishment of a physiologically functional primary 3D cell culture model. This model is promising to unravel the basic molecular mechanisms underlying normal, unbiased, and physiological processes like milk protein production, cell differentiation, immune response, and in future metabolic disorders. We exclusively showed that the cellular behavior is mainly influenced by the presence of the ECM-like scaffold Matrigel® and not primarily by lactogenic hormones itself.

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References

Aitken A (1996) 14-3-3 and its possible role in co-ordinating multiple signalling pathways. Trends Cell Biol 6(9):341–347

Aplin AE, Juliano RL (1999) Integrin and cytoskeletal regulation of growth factor signaling to the MAP kinase pathway. J Cell Sci 112(Pt 5):695–706

Assoian RK, Schwartz MA (2001) Coordinate signaling by integrins and receptor tyrosine kinases in the regulation of G1 phase cell-cycle progression. Curr Opin Genet Dev 11(1):48–53

Barcellos-Hoff MH, Aggeler J, Ram TG, Bissell MJ (1989) Functional differentiation and alveolar morphogenesis of primary mammary cultures on reconstituted basement membrane. Development 105(2):223–235

Beck T, Hall MN (1999) The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors. Nature 402(6762):689–692

Binns D, Dimmer E, Huntley R, Barrell D, O'Donovan C, Apweiler R (2009) QuickGO: a web-based tool for Gene Ontology searching. Bioinformatics 25(22):3045–3046. doi:10.1093/bioinformatics/btp536

Bionaz M, Loor JJ (2008) Gene networks driving bovine milk fat synthesis during the lactation cycle. BMC Genomics 9. doi: 10.1186/1471-2164-9-366

Bionaz M, Loor JJ (2011) Gene networks driving bovine mammary protein synthesis during the lactation cycle. Bioinform Biol Insights 5: 83–98. doi:10.4137/BBI.S7003

Bishop JD, Nien WL, Dauphinee SM, Too CKL (2006) Prolactin activates mammalian target-of-rapamycin through phosphatidylinositol 3-kinase and stimulates phosphorylation of p70S6K and 4E-binding protein-1 in lymphoma cells. J Endocrinol 190(2):307–312. doi:10.1677/joe.1.06368

Bissell MJ, Rizki A, Mian IS (2003) Tissue architecture: the ultimate regulator of breast epithelial function. Curr Opin Cell Biol 15(6): 753–762

Boutinaud M, Ben Chedly MH, Delamaire E, Guinard-Flament J (2008) Milking and feed restriction regulate transcripts of mammary epithelial cells purified from milk. J Dairy Sci 91(3):988–998. doi:10.3168/jds.2007-0587

Buehring GC (1990) Culture of mammary epithelial cells from bovine milk. J Dairy Sci 73(4):956–963. doi:10.3168/jds.S0022-0302(90)78752-8

Burgos SA, Dai M, Cant JP (2010) Nutrient availability and lactogenic hormones regulate mammary protein synthesis through the mammalian target of rapamycin signaling pathway. J Dairy Sci 93(1):153–161. doi:10.3168/jds.2009-2444

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55(4):611–622. doi:10.1373/clinchem.2008.112797

Caccavo D, Pellegrino NM, Altamura M, Rigon A, Amati L, Amoroso A, Jirillo E (2002) Antimicrobial and immunoregulatory functions of lactoferrin and its potential therapeutic application. J Endotoxin Res 8(6):403–417. doi:10.1179/096805102125001000

Capuco AV, Akers RM (2011) Lactation | galactopoiesis, effects of hormones and growth factors. Encyclopedia of dairy sciences. Academic Press, New York pp 26–31. doi: 10.1016/B978-0-12-374407-4.00252-1

Cerbulis J, Farrell HM (1975) Composition of milks of dairy cattle. I. Protein, lactose, and fat contents and distribution of protein fraction. J Dairy Sci 58(6):817–827. doi:10.3168/jds.S0022-0302(75)84644-3

Cox J, Mann M (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat Biotechnol 26(12):1367–1372. doi:10.1038/nbt.1511

Cox J, Neuhauser N, Michalski A, Scheltema RA, Olsen JV, Mann M (2011) Andromeda: a peptide search engine integrated into the MaxQuant environment. J Proteome Res 10(4):1794–1805. doi: 10.1021/pr101065j

Danowski K, Gross JJ, Meyer HHD, Kliem H (2013) Effects of induced energy deficiency on lactoferrin concentration in milk and the lactoferrin reaction of primary bovine mammary epithelial cells in vitro. J Anim Physiol Anim Nutr (Berl) 97(4):647–655. doi:10.1111/j.1439-0396.2012.01305.x

De Vries LD, Casey T, Dover H, Van de Haar MJ, Plaut K (2011) Effects of transforming growth factor-β on mammary remodeling during the dry period of dairy cows. J Dairy Sci 94(12):6036–6046. doi: 10.3168/jds.2011-4590

Debnath J, Muthuswamy SK, Brugge JS (2003) Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. Methods 30(3):256–268

Doppler W, Welte T, Philipp S (1995) CCAAT/enhancer-binding protein isoforms beta and delta are expressed in mammary epithelial cells and bind to multiple sites in the beta-casein gene promoter. J Biol Chem 270(30):17962–17969

Fu H, Subramanian RR, Masters SC (2000) 14-3-3 proteins: structure, function, and regulation. Annu Rev Pharmacol Toxicol 40:617–647. doi:10.1146/annurev.pharmtox.40.1.617

Hayashi AA, Proud CG (2007) The rapid activation of protein synthesis by growth hormone requires signaling through mTOR. Am J Physiol Endocrinol Metab 292(6):E1647–E1655. doi:10.1152/ajpendo.00674.2006

Hayashi AA, Nones K, Roy NC, McNabb WC, Mackenzie DS, Pacheco D, McCoard S (2009) Initiation and elongation steps of mRNA translation are involved in the increase in milk protein yield caused by growth hormone administration during lactation. J Dairy Sci 92(5):1889–1899. doi:10.3168/jds.2008-1334

Huntley RP, Sawford T, Mutowo-Meullenet P, Shypitsyna A, Bonilla C, Martin MJ, O'Donovan C (2015) The GOA database: gene ontology annotation updates for 2015. Nucleic Acids Res 43(Database issue): D1057–D1063. doi:10.1093/nar/gku1113

Jedrzejczak M, Szatkowska I (2014) Bovine mammary epithelial cell cultures for the study of mammary gland functions. In Vitro Cell Dev Biol Anim 50(5):389–398. doi:10.1007/s11626-013-9711-4

Jensen LJ, Kuhn M, Stark M, Chaffron S, Creevey C, Muller J, Doerks T, Julien P, Roth A, Simonovic M, Bork P, Mering C von (2009) STRING 8—a global view on proteins and their functional interactions in 630 organisms. Nucleic Acids Res 37(Database issue): D412-D416. doi: 10.1093/nar/gkn760

Kleinman HK, Philp D, Hoffman MP (2003) Role of the extracellular matrix in morphogenesis. Curr Opin Biotechnol 14(5):526–532. doi:10.1016/j.copbio.2003.08.002

Koressaar T, Remm M (2007) Enhancements and modifications of primer design program Primer3. Bioinformatics 23(10):1289–1291. doi:10.1093/bioinformatics/btm091

Kozlowski M, Gajewska M, Majewska A, Jank M, Motyl T (2009) Differences in growth and transcriptomic profile of bovine mammary epithelial monolayer and three-dimensional cell cultures. J Physiol Pharmacol 60(Suppl 1):5–14

Kozłowski M, Wilczak J, Motyl T, Gajewska M (2011) Role of extracellular matrix and prolactin in functional differentiation of bovine BME-UV1 mammary epithelial cells. Pol J Vet Sci 14(3):433–442

Liu XF, Li M, Li QZ, Lu LM, Tong HL, Gao XJ (2012) Stat5a increases lactation of dairy cow mammary gland epithelial cells cultured in vitro. In Vitro Cell Dev Biol Anim 48(9):554–561. doi:10.1007/s11626-012-9545-5

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods 25(4):402–408. doi:10.1006/meth.2001.1262

Lu LM, Li QZ, Huang JG, Gao XJ (2012) Proteomic and functional analyses reveal MAPK1 regulates milk protein synthesis. Molecules 18(1):263–275. doi:10.3390/molecules18010263

Månsson HL (2008) Fatty acids in bovine milk fat. Food Nutr Res 52. doi: 10.3402/fnr.v52i0.1821

Mengwasser J, Liu FT, Sleeman JP (2002) Galectin-3 is strongly upregulated in nonapoptosing mammary epithelial cells during rat mammary gland involution. Glycobiology 12(2):129–134

Monzani PS, Bressan FF, Mesquita LG, Sangalli JR, Meirelles FV (2011) β-casein gene expression by in vitro cultured bovine mammary epithelial cells derived from developing mammary glands. Genet Mol Res 10(2):604–614. doi:10.4238/vol10-2gmr1034

Mori H, Inoue M, Yano M, Wakabayashi H, Kido H (2000) 14-3-3tau associates with a translational control factor FKBP12-rapamycin-associated protein in T-cells after stimulation by pervanadate. FEBS Lett 467(1):61–64

Morrison D (1994) 14-3-3: modulators of signaling proteins? Science 266(5182):56–57

Musters S, Coughlan K, McFadden T, Maple R, Mulvey T, Plaut K (2004) Exogenous TGF-beta1 promotes stromal development in the heifer mammary gland. J Dairy Sci 87(4):896–904. doi:10.3168/jds.S0022-0302(04)73234-8

Pampaloni F, Reynaud EG, Stelzer EHK (2007) The third dimension bridges the gap between cell culture and live tissue. Nat Rev Mol Cell Biol 8(10):839–845. doi:10.1038/nrm2236

Pawson T, Scott JD (1997) Signaling through scaffold, anchoring, and adaptor proteins. Science 278(5346):2075–2080

Prince JM, Klinowska TCM, Marshman E, Lowe ET, Mayer U, Miner J, Aberdam D, Vestweber D, Gusterson B, Streuli CH (2002) Cell-matrix interactions during development and apoptosis of the mouse mammary gland in vivo. Dev Dyn 223(4):497–516. doi:10.1002/dvdy.10070

Rainard P, Riollet C (2006) Innate immunity of the bovine mammary gland. Vet Res 37(3):369–400. doi:10.1051/vetres:2006007

Riley LG, Gardiner-Garden M, Thomson PC, Wynn PC, Williamson P, Raadsma HW, Sheehy PA (2010) The influence of extracellular matrix and prolactin on global gene expression profiles of primary bovine mammary epithelial cells in vitro. Anim Genet 41(1):55–63. doi:10.1111/j.1365-2052.2009.01964.x

Rimbach G, Möhring JF, Erbersdobler H (2010) Milch, Lebensmittel-Warenkunde für Einsteiger, Part of the series Springer-Lehrbuch pp 1–22. doi: 10.1007/978-3-642-04486-1_1

Rose MT, Aso H, Yonekura S, Komatsu T, Hagino A, Ozutsumi K, Obara Y (2002) In vitro differentiation of a cloned bovine mammary epithelial cell. J Dairy Res 69(3):345–355

Rosen JM, Wyszomierski SL, Hadsell D (1999) Regulation of milk protein gene expression. Annu Rev Nutr 19:407–436. doi:10.1146/annurev.nutr.19.1.407

Roskelley CD, Srebrow A, Bissell MJ (1995) A hierarchy of ECM-mediated signalling regulates tissue-specific gene expression. Curr Opin Cell Biol 7(5):736–747

Schmidhauser C, Casperson GF, Myers CA, Sanzo KT, Bolten S, Bissell MJ (1992) A novel transcriptional enhancer is involved in the prolactin- and extracellular matrix-dependent regulation of beta-casein gene expression. Mol Biol Cell 3(6):699–709

Sigl T, Meyer HHD, Wiedemann S (2014) Gene expression analysis of protein synthesis pathways in bovine mammary epithelial cells purified from milk during lactation and short-term restricted feeding. J Anim Physiol Anim Nutr (Berl) 98(1):84–95. doi:10.1111/jpn.12039

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC (1985) Measurement of protein using bicinchoninic acid. Anal Biochem 150(1):76–85

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 milk and tissue. In Vitro Cell Dev Biol-Animal 48(9):550–553. doi:10.1007/s11626-012-9544-6

Sorg D, Fandrey E, Frölich K, Meyer H, Kliem H (2013) Mammary immunity of White Park and Highland cattle compared with Brown Swiss and Red Holstein. Anim Genet Resour 52:91–104. doi:10.1017/S2078633612000781

Stelwagen K (2011) Mammary gland, milk biosynthesis and secretion | milk protein. Encyclopedia of Dairy Sciences, Academic Press, New Yorkpp:359–366

Streuli CH, Edwards GM (1998) Control of normal mammary epithelial phenotype by integrins. J Mammary Gland Biol Neoplasia 3(2):151–163

Streuli CH, Schmidhauser C, Bailey N, Yurchenco P, Skubitz AP, Roskelley C, Bissell MJ (1995) Laminin mediates tissue-specific gene expression in mammary epithelia. J Cell Biol 129(3):591–603

Toerien CA, Cant JP (2007) Abundance and phosphorylation state of translation initiation factors in mammary glands of lactating and nonlactating dairy cows. J Dairy Sci 90(6):2726–2734. doi:10.3168/jds.2006-778

Tucker HA (2000) Hormones, mammary growth, and lactation: a 41-year perspective. J Dairy Sci 83(4):874–884. doi:10.3168/jds.S0022-0302(00)74951-4

Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG (2012) Primer3—new capabilities and interfaces. Nucleic Acids Res 40(15):e115. doi:10.1093/nar/gks596

Van der Flier A, Sonnenberg A (2001) Function and interactions of integrins. Cell Tissue Res 305(3):285–298

Van Hooijdonk AC, Kussendrager KD, Steijns JM (2000) In vivo antimicrobial and antiviral activity of components in bovine milk and colostrum involved in non-specific defence. Br J Nutr 84(Suppl 1):S127–S134

Weaver VM, Lelièvre S, Lakins JN, Chrenek MA, Jones JCR, Giancotti F, Werb Z, Bissell MJ (2002) beta4 integrin-dependent formation of polarized three-dimensional architecture confers resistance to apoptosis in normal and malignant mammary epithelium. Cancer Cell 2(3):205–216

Wellnitz O, Reith P, Haas SC, Meyer HHD (2006) Immune relevant gene expression of mammary epithelial cells and their influence on leukocyte chemotaxis in response to different mastitis pathogens. Vet Med (Praha) 51:125–132

Yang X, Yang C, Farberman A, Rideout TC, de Lange CFM, France J, Fan MZ (2008) The mammalian target of rapamycin-signaling pathway in regulating metabolism and growth. J Anim Sci 86(14 Suppl):E36–E50. doi:10.2527/jas.2007-0567

Zoubiane GS, Valentijn A, Lowe ET, Akhtar N, Bagley S, Gilmore AP, Streuli CH (2004) A role for the cytoskeleton in prolactin-dependent mammary epithelial cell differentiation. J Cell Sci 117(Pt 2):271–280. doi:10.1242/jcs.00855

Appendix

Table S1: Secretomics – proteins identified in cell culture supernatants of pbMEC cultured on Matrigel®

Identified Protein	Uniprot ID	Gene	Biological function[1]
Onset of lactation			
Alpha-S1-Casein	P02662	CSN1S1	Response to estradiol and progesterone
Beta-Casein	P02666	CSN2	Lactation
			Calcium ion transport
Alpha-Lactalbumin	P00711	LALBA	Lactose biosynthetic process
Beta-lactoglobulin	P02754	PAEP	Retinol binding
			Small molecule binding
Fatty acid synthase	F1N647	FASN	Mammary gland development
Cell proliferation and differentiation			
14-3-3 protein sigma	Q0VC36	SFN	Regulation of epidermal cell division
			Positive regulation of cell growth
Transgelin-2	Q5E9F5	TAGLN2	Epithelial cell differentiation
Galectin	A6QLZ0	LGALS3	Epithelial cell differentiation
SPARC	P13213	SPARC	Regulation of cell proliferation
Cysteine-rich, angiogenic inducer, 61	Q3ZC35	CYR61	Cell adhesion
			Signal transduction
			Cell-cell signaling
			ECM[2] organization
			Positive regulation of cell differentiation
Pro-opiomelanocortin	P01190	POMC	Cell-cell signaling
Colony stimulating factor 1	F1MGS9	CSF1	Positive regulation of cell-matrix adhesion
			Positive regulator of cell proliferation
			Branching involved in mammary gland duct morphogenesis
			Mammary gland fat development
			Mammary duct terminal end bud growth
Protein FAM3C	A5PKI3	FAM3C	Multicellular organism development
Insulin-like growth factor-binding protein 6	Q05718	IGFBP6	Regulation of cell growth
			Regulation of insulin-like growth factor
Transforming growth factor beta-induced protein ig-h3	F1MBS3	TGFB1	Cell adhesion
			Cell proliferation
			ECM[2] organization
MSLN protein	A6QP39	MSLN	Cell-matrix adhesion
Follistatin-related protein 1	Q58D84	FSTL1	14-3-3 protein binding

Identified Protein	Uniprot ID	Gene	Biological function[1]
Cytoskeletal organization			
Myosin light polypeptide 6	P60661	MYL6	Calcium ion binding
Alpha-actinin-1	Q3B7N2	ACTN1	Actin filament organization
			Actin filament bundle assembly
Destrin	Q5E9D5	DSTN	Actin filament de-polymerization
Tubulin alpha-1D chain	Q2HJ86	TUBA1D	Metabolic process
Calponin-2	Q3SYU6	CNN2	Cytoskeleton organization
Actin-related protein 2/3 complex subunit 2	Q3MHR7	ARPC2	Positive regulation of actin filament
Macrophage-capping protein	Q865V6	CAPG	Actin filament capping
Dystroglycan	F1N7D7	DAG1	Cytoskeletal anchoring at plasma membrane
Collagen alpha-2(I) chain	P02465	COL1A2	Collagen fibril organization,
			Cellular response to amino acids
Fascin actin-bundling protein 1	Q3MHK9	FSCN1	Actin filament organization
			Cell-cell junction assembly
			Cell migration
			Establishment of apical/basal polarity
Cofilin-1	Q5E9F7	CFL1	Cytoskeleton organization
			Regulation of cell morphogenesis
			Actin filament de-polymerization
Immune system			
Beta-2-microglobulin	P01888	B2M	Immune system process
			Antibacterial humoral response
Lactotransferrin	P24627	LTF	Regulation of cytokine production
			Antibacterial and antifungal
			Innate immune response in mucosa
C-X-C motif chemokine 3	F1MD23	CXCL3	Chemotaxis
			Inflammatory response
C-X-C motif chemokine 6	P80221	CXCL6	Chemotaxis
			LPS response
Secretogranin-2	P20616	SCG2	Induction of positive chemotaxis
Protein S100-A8	P28782	S100A8	Immune system process Leukocyte migration involved in
			Inflammatory response
			Chemotaxis
			Innate immune response
Translation			
Ribosome-releasing factor 2, mitochondrial	A6QNM2	GFM2	Translation
			Ribosome disassembly
40S ribosomal protein S12	Q76I81	RPS12	Translation
40S ribosomal protein S21	Q32PB8	RPS21	Translation
60S ribosomal protein L6	Q58DQ3	RPL6	Translation

Identified Protein	Uniprot ID	Gene	Biological function[1]
			Ribosomal large subunit assembly
Ornithine aminotransferase, mitochondrial	F1MYG0	OAT	Arginine catabolic process to proline via ornithine
Protein folding			
Heat shock protein HSP 90-alpha	Q76LV2	HSP90AA1	Protein folding
			Response to stress and heat
Copper transport protein ATOX1	Q3T0E0	ATOX1	Copper ion transport
			Response to oxidative stress
Heat shock 70 kDa protein 1A	Q27975	HSPA1A	Protein refolding
Prefoldin subunit 6	Q17Q89	PFDN6	Protein folding
Peptidyl-prolyl cis-trans isomerase A	P62935	PPIA	Protein folding
40S ribosomal protein S21	Q32PB8	RPS21	Protein folding
Sulfhydryl oxidase	F1MM32	QSOX1	protein folding
Proteolysis			
Matrix metalloproteinase-9	F1MF56	MMP9	Proteolysis
			Collagen catabolic process
Ubiquitin carboxyl-terminal hydrolase isozyme L3	Q2TBG8	UCHL3	Proteolysis
Proteasome subunit alpha type-5	Q5E987	PSMA5	Proteolysis
			Ubiquitin-dependent protein catabolism
Tissue-type plasminogen activator	Q28198	PLAT	Proteolysis
Cathepsin D	P80209	CTSD	Proteolysis
Peptidase inhibition			
Calpastatin	F1MD74	CAST	Endopeptidase inhibitor
Leukocyte elastase inhibitor	Q1JPB0	SERPINB1	Endopeptidase inhibitor
Cystatin-C	P01035	CST3	Endopeptidase inhibitor
PCSK1N protein	A4IFR2	PCSK1N	Endopeptidase inhibitor
Plasminogen activator inhibitor 1	P13909	SERPINE1	Endopeptidase inhibitor
Serin peptidase inhibitor, Kunitz type 1	Q2KJJ7	SPINT1	Endopeptidase inhibitor

[1]The biological function was determined according to the GO terms of EMBL-EBI QuickGo browser (Binns et al. 2009).

[2]ECM = Extracellular matrix

Appendix II

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BMC Veterinary Research

RESEARCH ARTICLE Open Access

Gene expression profiling in pbMEC – in search of molecular biomarkers to predict immunoglobulin production in bovine milk

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Abstract

Background: Optimization of the immunoglobulin (Ig) yield in bovine milk used as therapeutic immune milk or whey for the prevention of *Clostridium difficile*-associated diarrhea in humans is of great importance to improve the economic efficiency of production. Individual dairy cows have diverse immune responses upon vaccination, resulting in a variable Ig yield in blood and milk. Therefore, it is advisable to pre-select cows with the best ability to produce and secrete high yields of specific Igs.

Results: The gene expression profile of pbMEC (primary bovine mammary epithelial cells), challenged with the gram-positive, non-mastitis, pathogen *Clostridium difficile* showed distinct and significant differences in the gene expression of effector molecules of the innate immune system. A number of genes were identified that could possibly serve as molecular biomarkers to differentiate high responder cows from low responder cows. These identified genes play key roles in the promotion of innate immunity.

Conclusion: Using a gene expression profiling approach, we showed that upon others, especially the gene expression of the pro-inflammatory cytokines was altered between the high and low responder cows. Those genes are indicated as potential molecular biomarkers in the pre-selection of cows that are able to secrete high immunoglobulin yields in milk.

Keywords: Molecular biomarkers, Primary bovine mammary epithelial cells, Innate immunity, Microfluidic gene expression profiling, *Clostridium difficile*-associated diarrhea

Background

Clostridium difficile (*C. diff.*) is a widespread hospital germ that causes severe antibiotic associated gastroenteritis in humans especially in industrialized countries [1, 2]. *C. diff.* is a gram-positive enterotoxic, spore building pathogen that due to its acidic resistance is able to overcome the acidic environment of the stomach [1–3]. The primary reservoirs of this pathogen are asymptomatic carriers and contaminated surfaces, which are important issues especially in hospitals and nursing homes [4]. The progression of the disease is quite diverse, ranging from mild diarrhea to severe life-threatening pseudomembranous colitis [1, 3, 5]. Until now, the treatment of *C. difficile*-associated diarrhea (CDAD) results in a vicious circle, as the antibiotics metronidazole, vancomycin, and fidaxomicin are mainly used [6–8]. As those antibiotics do not exclusively target pathogenic *C. diff.* Bacteria but also commensal gut bacteria, the gut microbiota of the patients is further damaged. Therefore, we aimed to develop a new treatment strategy or better yet, a preventive treatment strategy for CDAD. Inspired by a study by Van Dissel et al. (2005) [9], we wanted to developed immune milk enriched with naturally derived polyclonal immunoglobulin A (IgA) against *C. diff.*. As the production and application of immune milk are quite promising, it is advantageous to optimize the yield of specific IgA against *C. diff.* in the milk. Therefore, molecular and biological methods were employed to identify potential molecular biomarkers for the pre-selection of high responder dairy cows, prior to immune milk production. Brown Swiss cows were immunized against *C. diff.* in order to induce milk production and secretion of specific IgA. As each animal has a fairly

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individual immune status and, hence, response due to the inherited genetic composition of the host [10], we investigated whether animals can be pre-selected to optimize production of specific Igs upon vaccination. Therefore, we searched for molecular markers of the innate immune system of the cows using a gene expression profiling method. As we surmised that besides blood lymphocytes, primary bovine mammary epithelial cells (pbMEC) are quite important for the promotion of innate immunity and subsequent activation of adaptive immunity and later on, transcytosis and secretion of immunoglobulins into milk, a newly developed three-dimensional 3D–cell culture system of pbMEC was used in this study [11, 12]. The elucidation of the underlying gene expression network may be important to identify differences in the innate immune system of low and high responder cows to facilitate the pre-selection of animals before use for immune milk production.

Methods

Immunization of the cows

The animal trial was approved by the government of Upper Bavaria (AZ. 55.2–1–54-2532.6-17-2012). The cows were bought at the cattle market for Brown Swiss cows of the Allgäuer Herdbuchgesellschaft (Cattle's breeders association). Nine healthy Brown Swiss cows in their first lactation were immunized against *C. diff.* (IDT Biologika GmbH, Dessau-Rosslau, Germany) according to a strict scheme of 16 immunizations over a 31-week period. Before and 1 day after each vaccination, the health status of each animal was routinely monitored by a veterinarian. The milk of each udder quarter was tested for bacterial infection and contamination (Tiergesundheitsdienst Bayern e.V., Grub, Germany) before vaccination to detect the incidence of subclinical or clinical mastitis. Somatic cell counts and milk ingredients were analyzed weekly by a commercial facility (Milchprüfring Bayern e.V., Wolnzach, Germany). The average somatic cell count in milk during the vaccination period was 63.000 cells/ml ± 7075.63 cells/ml (n = 279). During the 31-week experimental period, two cows developed symptoms of subclinical mastitis and one was diagnosed with acute mastitis. No pbMEC were sampled from the diseased animals, as only those from healthy cows were used in the experiments. Furthermore, stool analysis of the animals prior to immunization showed that all were *C. diff.* Negative (Leiden University, Medical Center).

IgA against Clostridium Difficile - ELISA

For the detection of *C. diff.* Specific IgA in cow milk, a sandwich ELISA was applied. In brief, each well of a 96-well plate (Maxisorp, Nunc®; Sigma-Aldrich Corporation, St. Louis, MO, USA) was coated with 2.0×10^8 *C. diff.* Cells/ml, IDT Biologika GmbH) in coating buffer (50 mM $NaHCO_3$, pH 9.6; Merck Chemicals GmbH, Darmstadt, Germany) and incubated for 2 h at 70 °C and then overnight at 4 °C. The coating was terminated by incubation with 200 μl blocking buffer in phosphate-buffered saline (PBS)-Tween 20 (PBST; 2% gelatin, Sigma-Aldrich Corporation) for 1 h at 37 °C. The ELISA plate was washed four times with PBST (1 g/l Tween 20; Merck Chemicals GmbH). A *C. diff.* Specific IgA standard was prepared in dilution buffer (0.2% gelatin, Sigma-Aldrich, in PBST, 62.5 ng/ml – $4*10^3$ ng/μl). The skim milk samples were diluted to 1:10 with dilution buffer. Standard dilutions, samples, and intra-assay controls were applied in duplicates to the pre-coated plate and incubated for 1.5 h at 37 °C. Afterwards, the ELISA plate was washed four times. Then, horseradish peroxidase (HRP)-conjugated sheep anti-bovine IgA (dilution, 1:70,000; Bethyl Laboratories, Inc., Montgomery, TX, USA) was added to each well and the plate was incubated for 1.5 h at 37 °C in the dark. Afterwards, the ELISA plate was washed four times and the HRP-conjugated substrate [13] was added to the wells to induce reaction with the substrate. After 40 min, the substrate reaction was stopped by the addition of 2 M H_2SO_4. Extinction was measured after 30 min at 450 nm using a microplate reader (Sunrise™, Tecan Group Ltd., Männedorf, Switzerland). The amount of *C. diff.* Specific IgA was determined based on a standard curve using Magellan™ V6.6 software (Tecan Group Ltd.).

3D cell culture of pbMEC

The pbMEC were isolated from fresh milk of nine healthy Brown Swiss cows in mid-lactation, as described by Sorg et al. (2013a) and Danowski et al. (2013) [14, 15]. In brief, fresh milk was defatted (10 min, 1850×*g*), and the resulting cell pellet was washed several times with 1× Hanks Balanced Salt solution (Sigma-Aldrich) supplemented with antibiotics and antimycotics [14, 15]. The remaining cell suspension was filtered twice (EASYstrainer™; 40 μm; 100 μm; Greiner Bio-One GmbH, Frickenhausen, Germany) to remove lipid droplets and cell aggregates. The pbMEC were afterwards resuspended in Dulbecco's modified Eagle's medium (DMEM)/F-12 Ham solution supplemented with penicillin/streptomycin, amphotericin B, insulin-transferrin-selenium (ITS) liquid media supplement (Sigma-Aldrich) and fetal bovine serum (FBS; Gibco® Lifetechnologies GmbH, Darmstadt, Germany), and cultured (37 °C, 5% CO_2) in 3D cell culture in 6-well plates coated with 2.4 mg/ml Matrigel® (Corning Inc., Corning, New York, USA), until confluency. pbMEC were sub-cultivated using 0.25% Trypsin-EDTA solution (Sigma-Aldrich). After the second passage, the cells were detached with 0.25% Trypsin-EDTA solution and prepared for cryopreservation. The cells were counted using

the TC10™ Automated Cell Counter (Bio-Rad Laboratories GmbH, Munich, Germany). Afterwards, $1*10^5$–$5*10^5$ cells were resuspended in cryopreservation medium containing 70% DMEM F12-Ham, 20% FBS, and 10% DMSO (Sigma-Aldrich) and then stored in liquid nitrogen until pbMEC from all animals had been sampled. For the experimental set-up, pbMEC were thawed and reseeded at $2*10^4$ cells per well of a 6-well plate (Greiner Bio-One GmbH), coated with 2.4 mg/ml Matrigel®, for the immune stimulatory experiments or $1*10^4$ cells per chamber of a 8-well LabTec chamber slide (LAB-Tek, Nunc, GmbH, Langenselbold, Germany) for immunocytochemistry (IC).

Immune stimulation of pbMEC with formalin inactivated Clostridium Difficile

To calculate the multiplicity of infection (MOI) per cultured cell, three wells per animal served as counting wells. Confluent cells were detached using 0.25% Trypsin-EDTA solution and counted using the TC10™ Automated Cell Counter (Bio-Rad Laboratories GmbH), using life-dead staining with 0.4% trypan blue (Bio-Rad Laboratories GmbH). The mean value of the counted living cells served as the estimated cell count for all other cells used in the experiment. Cell culture replicates of pbMEC were then induced with formalin inactivated *C. diff.* (IDT Biologika GmbH) with a MOI of 70 colony forming units per cultured cell. The MOI was chosen based on the findings of preliminary experiments. A greater MOI was chosen, as compared to reports in the literature, as gram-positive pathogens induce only a weak immune response in pbMEC [12, 16, 17]. To target the immediate, intermediate, and late immune response, pbMEC were treated with *C. diff.* For 6 h, 24 h and 72 h respectively. To obtain representative data, control wells with untreated pbMEC were also sampled in biological triplicates at each time-point (0 h, 6 h, 24 h, and 72 h). To avoid the side effects of antibiotics, antimycotics, and FBS, the cells were supplemented with DMEM/F-12 Ham medium with ITS for 48 h pre-infection. This so-called "infection medium" was refreshed immediately before treatment. After treatment, pbMEC were washed with PBS and further lysed in Qiazol (Qiagen, Hilden, Germany), which was included with the miRNeasy Micro Kit (Qiagen).

Mycoplasma test

To detect the presence of contaminant mycoplasma species in the cell culture, the PCR Mycoplasma Test Kit (AppliChem GmbH, Darmstadt, Germany) was used according to the manufacturer's protocol. Cell culture supernatants of each animal were sampled and stored at −80 °C until further processing.

Immunocytochemistry

pbMEC were cultured on 8-well LabTec chamber Slides (LAB-Tec, Nunc, GmbH, Langenselbold, Germany) to confirm the epithelial character of the cells cultured in 3D cell culture with immunocytochemistry. The IC was conducted as described by Sorg et al. (2013a) and Danowski et al. (2013) [12, 14, 15]. The monoclonal mouse anti-cytokeratin pan antibody clone C-11 (1:400 in PBST, Sigma-Aldrich) was used for cytokeratin staining.

RNA extraction and reverse transcription

RNA was extracted using the miRNeasy Micro Kit (Qiagen), according to the manufacturer's protocol with slight modifications. The miRNeasy Micro spin column was incubated for 5 min with buffer RPE after the second addition of buffer RPE to reduce contamination of the RNA with guanidine thiocyanate. The RNA concentration was calculated using a Nanodrop ND-1000 spectrophotometer (Peqlab, Erlangen, Germany). The RNA integrity was analyzed with a 2100 Bioanalyzer on the 6000 nano chips and the RNA 6000 nano Kit (Agilent Technologies, Waldbronn, Germany) according to the manufacturer's instructions. RNA was stored at −80 °C until further analysis. For reverse transcription of RNA to cDNA, 400 ng of RNA were mixed together with a master mix containing 5× buffer, 0.5 mM dNTPs, 0.5 M oligo-d(T) primers (Fermentas GmbH, St. Leon-Rot, Germany), 2.5 μM random hexamer primers (Invitrogen Life Technologies, Darmstadt, Germany) and 100 U of Moloney murine leukemia virus H(−) reverse transcriptase (Promega, Mannheim, Germany) in a total volume of 20 μl. After reverse transcription, the cDNA was diluted 1:1 to a final volume of 40 μl. RNA isolated from the bovine mammary gland and spleen tissues was used as a positive control. Furthermore, a non-template control (NTC) was included with each 96-well plate (4titude, Wotton, Great Britain) to screen for contamination of the reaction mixture. The RT-PCR reactions were conducted in 96-well plates using a T-Personal Thermocycler (Biometra, Göttingen, Germany) (Annealing: 21 °C, 10 min, transcription phase: 48 °C, 50 min, degrading phase: 90 °C, 2 min). The remaining cDNA was stored at −20 °C.

RT-qPCR primer design

Bovine specific primer pairs were designed using published bovine nucleic acid sequences retrieved from the National Center for Biotechnology Information gene database (NCBI, National Library of Medicine, Bethesda MD, USA). 68 bovine specific primer pairs were generated (Sigma-Aldrich), among them were 7 primer pairs for the reference genes *GAPDH*, *YWHAZ*, *H3F3A*, *ACTy1*, *18srRNA*, *Cyt8*, *UBB* and respectively 61 primer pairs for target genes coding for proteins involved in inflammatory

pathways (Additional file 1: Table S1). For the selection of the panel of genes used in this study, we focused on publications that extensively studied the innate immune response of pbMEC in two-dimensional cell culture in vitro studies [12, 14, 16, 18–21]. Primers were designed using Primer3web version 4.0.0 [22, 23]. The specificity and performance of all primers were tested. All primers had an optimal annealing temperature of 60 °C. Each designed assay was tested using cDNA generated from udder parenchyma tissue, spleen tissue, and pbMEC to confirm tissue-specific gene expression. Furthermore, each qPCR assay was tested for amplification efficiency according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [24]. Only assays with a PCR efficiency >85% were used for subsequent RT-qPCR experiments.

RT-qPCR measurements

The RT-qPCR analysis was conducted using the BioMark™ HD 96 × 96 system (Fluidigm, San Francisco, CA, USA) as described by Sorg et al. (2013) with slight optimizations [12]. The cDNA was specifically pre-amplified for 16 cycles using 67 primer pairs. The 18srRNA primer pair was excluded from pre-amplification as it was scored as highly expressed gene. In brief, 2 μl of cDNA (10 ng/μl) were pre-amplified in a total volume of 15 μl with a final primer concentration of 25 nM using the iQ Supermix (Bio-Rad) according to the following temperature protocol: activation of polymerase at 95 °C for 3 min, followed by 16 cycles of denaturation at 95 °C for 15 s and 4 min of annealing and extension at 59 °C. The cDNA was diluted 20-fold after the pre-amplification reaction and stored at −20 °C until further analysis. For the determination of the Cq values, 4 BioMark™ 96 × 96 Gene expression (GE) Dynamic Array chips (Fluidigm) were used. The efficiency of all primer assays was tested on the first 96 × 96 GE dynamic array (Fluidigm). Furthermore, each 96 × 96 GE dynamic array contained positive controls, one no transcription control (NTC) and one control sample to test for possible genomic contaminations, called ValidPrime® (TATAA Biocenter, Gothenburg, Sweden). ValidPrime® is a good alternative to avoid the use of reverse transcriptase controls for RT-qPCR analysis, as it tests all samples for the presence of genomic DNA. Two stably expressed samples of the first 96 × 96 GE dynamic array were chosen as between-chip calibrators and, hence, were measured on all four chips. For the sample pre-mix, 2.5 μl SsoFast™ EvaGreen supermix (Bio-Rad), 0.1 μl of ROX (4× diluted, Invitrogen), 0.25 μl of 20× binding dye loading reagent (Fluidigm), 1 μl pre-amplified and 1:20 diluted cDNA and 1.15 μl water were combined to a final volume of 5 μl. The 5 μl assay mix consisted of 2.5 μl of 5 μM primer pairs (final concentration of primers in an individual reaction: 250 nM) and 2.5 μl of 2× GE assay loading reagent (Fluidigm). The sample and assay pre-mix were transferred to the primed 96 × 96 GE dynamic array and then automatically mixed inside the chip with the Fluidigm® IFC controller. The RT-qPCR assay was conducted using the BioMark™ system with the following protocol: 98 °C for 40 s followed by 30 cycles at 95 °C for 10 s and 60 °C for 40 s, followed by melting curve analysis to reveal the specificity of the primer pairs. Fluidigm Real-Time PCR Analysis Software version 4.1.2 (Fluidigm) was used for data handling and analysis. The RT-qPCR reactions were performed according to the MIQE guidelines [24].

Data pre-processing and data analysis

The qPCR reactions were validated with the Fluidigm Real-Time PCR Analysis Software version 4.1.2 (Fluidigm). Primer pairs with too much missing data were excluded from further analysis (CYP1A1, IL1-B, IL10, CASP1, HP, TAP, LAP, and CCL2). Furthermore, standard curves generated on the first BioMark™ 96 × 96 GE Dynamic Array chip (Fluidigm), were used to determine the efficiency of the primer pairs and the cut-off value for the gene expression data. The dynamic range of the primer assays was tested and the cut-off value was therefore set to 26. The raw data were pre-processed in GenEx Enterprise Version 6 data analysis software (MultiD Analyses AB, Gothenburg, Sweden). Within GenEx values larger than 26 were treated as missing data, the cut-off was set to a Cq-value of 26, and missing data was treated with an offset of "+1". Furthermore, the genomic background of each sample was evaluated, and an inter-plate calibration was conducted using the mean value of the two inter-plate calibrator samples. The pre-processed Cq values were normalized to the values of a set of seven reference genes, as suggested by the 'Normfinder' tool of the GenEx software package (MultiD Analyses AB). Additionally, the normalized Cq values were further normalized to the corresponding reference samples, which were represented by the Cq values of untreated control wells that were sampled at the start of treatment (time point 0 h). The fold changes ($2^{\wedge(-\Delta\Delta Cq)}$) were calculated as described by Livak and Schmittgen (2001) [25]. All statistical analyses were conducted using SigmaPlot 12.0 software (Systat Software, Inc., San Jose, CA, USA). Before *p*-values were calculated, the normal distribution of the data sets was confirmed with the Shapiro–Wilk normality test. The signed-rank test was conducted for data not normally distributed. To evaluate the treatment effect of *C. diff.* The ΔCq values of the treated and untreated groups were compared using the paired *t*-test. Significant differences in the gene expression between the different treatment time points (6 h vs.

24 h, 6 h vs. 72 h, 24 h vs. 72 h) were also evaluated using the paired *t*-test. Furthermore, a normal *t*-test was conducted to identify differentially expressed genes between the high and low responder group. Gene expression changes with *p*-values between 0.1 and 0.05 were considered as distinct changes in gene expression, whereas *p*-values below 0.05 were considered as statistically significant changes in gene expression ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$). As no correction for multiple testing was imposed on the p-values, this study has to be considered as explorative study. For the identification of similar gene expression profiles, a cluster analysis with the self-organizing tree algorithm (SOTA) was conducted with the Multi Experiment Viewer software (MeV 4.9.0, TM4) [26].

Results

C. Diff. Specific IgA in cow milk

The IgA content in milk was determined using an IgA ELISA as described above. To distinguish between high and low responder cows, the threshold of *C. diff.* Specific IgA in secreted milk of the immunized animals was set to 8 μg/ml milk. Therefore, four cows were considered as low responder animals with an average specific IgA content of 2.6 μg/ml ± 1.9 μg/ml and five were considered as high responder animals with an average specific IgA content of 11.1 μg/ml ± 1.2 μg/ml milk ($p \leq 0.001$) (Fig. 1).

pbMEC cell culture – IC and mycoplasma test

IC analysis was conducted to confirm the epithelial character of the 3D cultured pbMEC. All cultured cells were cytokeratin-positive and showed a typical cobblestone-like morphology, which is characteristic for pbMEC (Fig. 2). Therefore, cross-contamination with other cells was excluded. Furthermore, all cells were mycoplasma-free (PCR Mycoplasma Test Kit, AppliChem GmbH, Darmstadt, Germany).

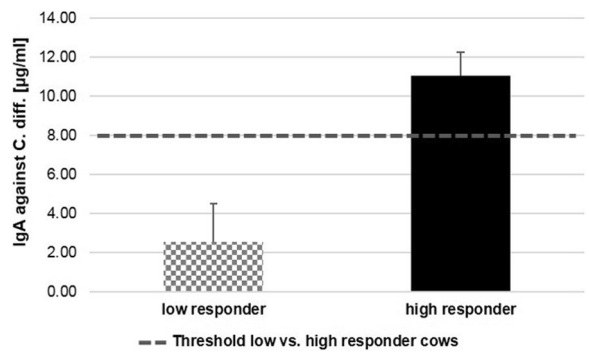

Fig. 1 Determination of the specific immunoglobulin A (IgA) content in milk. The animals ($n = 9$) were classified according to their immune response to the *C. diff.* Vaccine into the low ($n = 4$) and high ($n = 5$) responder group. The specific IgA content in milk was measured using a sandwich ELISA, the threshold to distinguish between low and high responder animals was set to 8 μg/ml specific IgA

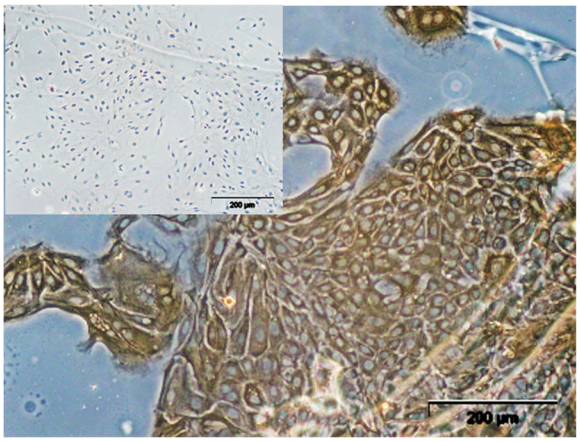

Fig. 2 Light microscopy of pbMEC isolated from fresh milk immunostained against cytokeratin (the inset shows the negative control, magnification ×200). pbMEC were cytokeratin-positive and showed the characteristic cobblestone-like morphology of the cells

Quality control of the extracted RNA and the RT-qPCR assays

The quality of the extracted RNA was assessed as described before. In brief, the total RNA yield was determined using a Nanodrop ND-1000 spectrophotometer. The overall RNA yield was 335.21 ng/μl ± 15.00 ng/μl ($n = 314$). The RNA integrity which was analyzed with the 2100 Bioanalyzer on the 6000 Nano chips was measured for 70 RNA samples that were randomly collected over all 4 time-points. An average RIN value of 9.94 ± 0.13 ($n = 70$) could be determined, indicating very good integrity of all RNA samples, as the highest possible RIN is 10. Furthermore, the designed qPCR assays were tested for their efficiency in qPCR reactions according to the MIQE guidelines [24], using standard curves of serial diluted sample material. The performance of the assays was tested on the BioMark™ 96 × 96 GE dynamic array. Assays with poor PCR amplification efficiency were excluded from further analysis (CYP1A1, IL1-B, IL10, CASP1, HP, TAP, LAP, and CCL2). Analyses of the remaining 60 qPCR assays resulted in an average r^2-value of 0.97 ± 0.01 ($n = 60$) and an average PCR efficiency of 1.14 ± 0.025 ($n = 60$), indicating that the PCR efficiency of the primer pairs was between 90% - 114%.

Effect of the C. Diff. Treatment on the gene expression within the high responder cows

According to the Ig yield obtained in the milk, the vaccinated cows showed a rather diverse immune response. Therefore, a gene expression profiling method was applied

to identify molecular biomarkers of innate immunity of cows with a fast and efficient immune response. A detailed listing of the fold changes in gene expression and the calculated *p*-values that were determined by the paired *t*-test for the treatment effect and the time effect can be found in "Additional file 2: Table S2". For the identification of differences within the expression profiles of low and high responder animals, a cluster analysis with the self-organizing tree algorithm (SOTA) was conducted applying the Multi Experiment Viewer software (MeV 4.9.0, TM4) [26]. The analysis was done based on mean centered fold change values. Genes with higher fold changes than the mean are highlighted in green, whereas fold changes below the mean fold change are highlighted in red. With this method, the time course of gene expression changes within the high and low responder groups could be illustrated whereby the genes were clustered concerning their early, intermediate or late gene expression.

SOTA analysis of RT-qPCR data

Genes coding for FcRn and pIGR were excluded from the SOTA analysis of the high and low responder groups because of no contribution to the scientific question. SOTA analysis of the high responder group revealed three clusters, one composed of early induced genes (Fig. 3a), a second composed of the intermediate induced genes (Fig. 3b), and a third composed of genes that were mostly induced at 72 h after immune stimulation (Fig. 3c). The first cluster contained 16 genes, which were induced early after immune stimulation, which

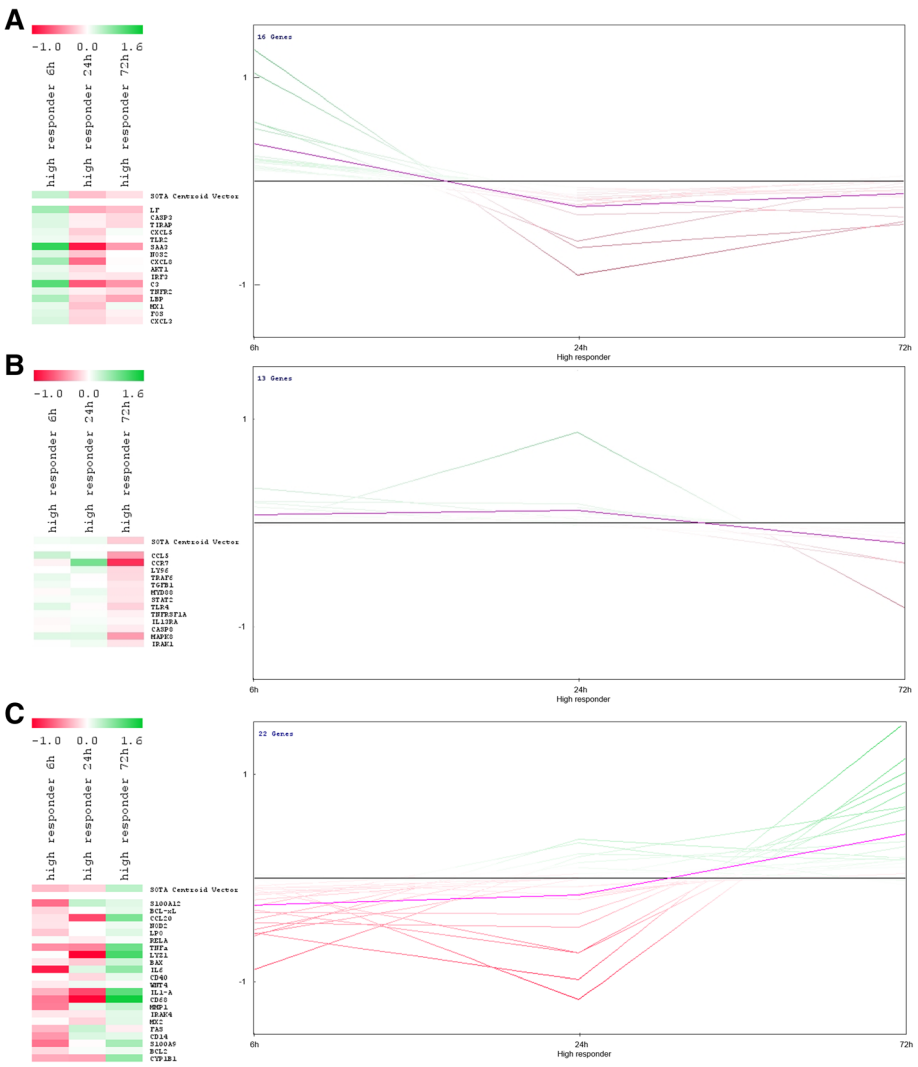

Fig. 3 Self-organizing tree algorithm (SOTA) analysis revealed early (16 genes) (**a**), intermediate (13 genes) (**b**), and late (22 genes) (**c**) expression profiles of genes stimulated with *C. diff.* in the high responder group. Sample fold changes compared to the mean fold change of the whole group are highlighted in the SOTA dendrogram (left) as well as in the SOTA expression profile (right). Fold changes greater than the mean fold change are highlighted in green, whereas fold changes below the mean fold change are highlighted in red

included some really strongly induced genes coding for the acute phase protein SAA3, the antimicrobial peptide lactoferrin (LF), the complement component C3, the components of the Toll-like receptor (TLR) pathway LBP, TLR2, and TIRAP, and the chemokines CXCL5, CXCL3, and CXCL8 (Fig. 3a). The effect of *C. diff. Treatment* was statistically evaluated using the paired *t*-test (Additional file 2: Table S2). The gene expression levels of CXCL8, CXCL3, and TIRAP were up-regulated in response to immune stimulation. The time-dependent effects of immune stimulation on gene expression are shown in the SOTA dendrogram (Fig. 3a) as well Additional file 2: Table S2.

Within the second cluster, 13 genes were detected, which were rather early (6 h) and intermediately (24 h) induced (Fig. 3b). Genes coding for the chemokines CCL5, CCR7, and IL13RA, the components of the TLR pathway (i.e., TLR4, LY96, MYD88, TRAF6, and IRAK1), and the gene coding for MAPK8 were strongly induced either early or intermediately. According to the paired *t*-test results, the gene expression of genes coding for IRAK1 and TRAF6 were differentially up-regulated in response to immune stimulation (Fig. 3b; Additional file 1: Table S1). Regulation of gene expression at different treatment time points in this cluster was directly observed with the SOTA dendrogram and the expression graph presented in Fig. 3. These results were verified with a paired *t*-test (Additional file 2: Table S2).

Within the third cluster, 22 intermediate to late induced genes were detected, which included strongly induced genes coding for TNFα, CD68, CD14, and CYP1B1. Furthermore, the genes coding for the so-called "danger-associated molecular pattern molecules" S100A9 and S100A12, the antimicrobial peptides lysozyme 1 (LYZ1) and lacto-peroxidase (LPO), the chemokines and inflammatory cytokines CCL20, IL6, and IL1-A, the components of the apoptotic pathway (e.g., FAS), the scavenger receptor CD68, and the gene coding for MMP1 were differentially induced in response to *C. diff.* Stimulation (Additional file 2: Table S2). This trend was also observed in the SOTA dendrogram and expression profile (Fig. 3c), where again the temporal regulation of the gene expression of immune-related genes was clearly determined (Fig. 3c; Additional file 2: Table S2).

Comparison of the gene expression pattern of low and high responder cows during different treatment time-points

A direct comparison of the gene expression pattern of high and low responder cows indicated a distinct greater induction of the gene expression within the high responder group during all three-time points. Genes were clustered together according to the induction time of the immune response. The first cluster consisted of 19 genes that were early expressed. The SOTA dendrogram (Fig. 4a) was used to identify genes in the high and low responder groups that were induced in response to *C. diff.* Stimulation, which showed that expression levels of some genes were lower in the low responder group (Figs. 4a, b, and c). The potential lower induction of *gene expression* in the *low responder* group is clearly indicated by the SOTA expression graphs (Figs. 4b and c). Most of the early induced genes within the *high responder* group showed a more distinct up-regulation after 6 h of immune stimulation and a strong down-regulation after 24 h. In the *low responder* group, however, only a few of the 19 genes showed a greater fold change, as compared to the mean fold change of the whole group, *and* the *gene expression within the low responder* group *declined* after 24 h. The strongly induced genes in the *high responder* group included those coding for the antimicrobial peptide LF, the chemokines CXCL8, CXCL5, and CXCL3, the acute phase protein SAA3, and the complement component C3. Furthermore, genes coding for components of the TLR pathway (i.e., TIRAP, TRAF6, and RELA) were differentially induced in the *high responder* group, as compared to the *low responder* group, according to the results of a normal *t*-test (Table 1; Fig. 4 and Additional file 2: Table S2).

Eleven genes in the second cluster were induced either early or intermediately. The dendrogram and expression profile (Figs. 5a and b) clearly indicated that genes within this cluster tended to be up-regulated in the high responder group after 6 h, with the exception of the gene coding for the chemokine receptor CCR7 (Figs. 5a and b). This accounts for an early as well as prolonged induction of the expression of genes coding for important chemokines, like CCL5, and components of the TLR pathway, like LY96, MYD88, TLR2, and IRAK1. Most of these genes, however, were down-regulated 6 h post-immune stimulation in the low responder group and were only induced after 24 h. Some genes, such as those coding for NOS2, LBP, MX1, and MX2 were down-regulated in the high responder group after 24 h, but were induced after this period of time within the low responder group, indicating differences in gene regulation between the groups. By contrast, the genes coding for CCL5, IRAK1, and MAPK8 were up-regulated in the high responder group, but down-regulated in the low responder group. The results of a normal *t*-test (Table 1) revealed distinct and differential up-regulation of the gene expression for LY96, MYD88, IRAK1, CCL5, and MAPK8 between the high and low responder groups (Table 1; Fig. 5; Additional file 2: Table S2).

The third cluster consisted of 21 genes that were induced after 72 h and partly after 24 h of immune stimulation. The SOTA dendrogram revealed that those genes

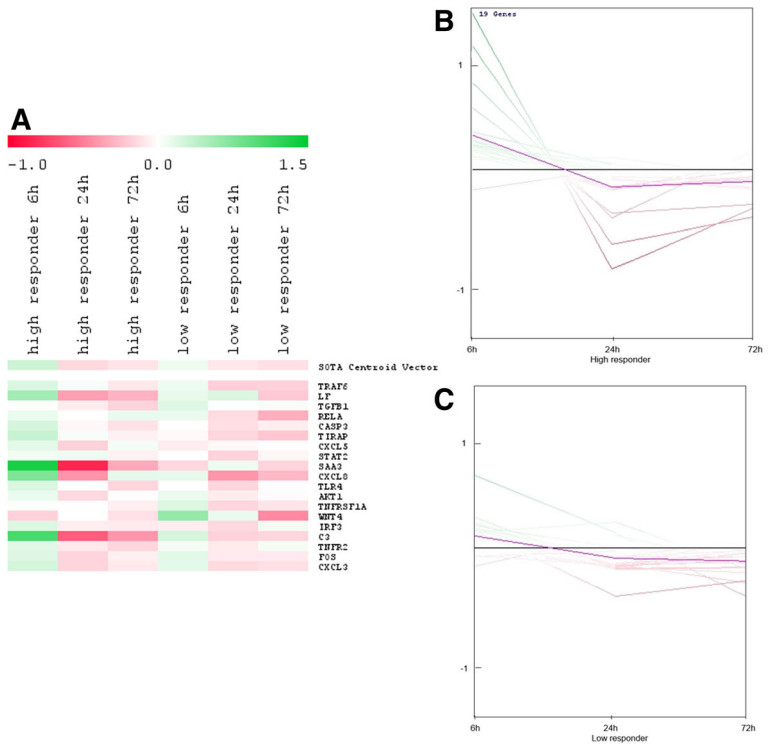

Fig. 4 Differences in the gene expression of immediate early expressed genes (19 genes) between the high (**b**) and low (**c**) responder group. The self-organizing tree algorithm dendrogram (**a**), as well as the expression profile for the high responder (**b**) and low responder (**c**) animals indicated differentially gene expression within both groups

were especially induced in the high responder group after 72 h, as observed by a comparison of color coding with the low responder group at the same time points (Fig. 6a). However, those genes were not induced earlier in the low responder group. The pbMEC in the low responder group hardly showed any induction of immune-related genes after *C. diff.* Stimulation (Table 1). Differentially up-regulated late expressed genes included some that coded for chemokines and inflammatory cytokines, like IL6, IL1-A, and IL13RA, as well as those coding for the danger-associated molecular pattern molecules S100A9 and S100A12, the antimicrobial peptides LYZ1 and LPO, the components of the TLR pathway, like CD14, the pro-apoptotic factors FAS, CASP8, and BAX, and those coding for CD68, CD40, MMP1, and NOD2. Changes in gene expression profiles over-time are depicted in the expression graphs (Table 1; Fig. 6).

Discussion

The gram-positive pathogen *C. diff.* Was chosen in this study as it causes severe CDAD in those with suppressed immunity and the elderly [3]. Treatment of CDAD with immune milk can offer some significant advantages, such as maintenance of the healthy commensal gut microbiota and the prevention of the formation of resistant bacteria due to the use of natural polyclonal animal-derived antibodies. The pathogen-specific polyclonal IgA can specifically neutralize *C. diff.* and minimize the relapse rate and the number of antibiotic treatments.

After stimulation with *C. diff.*, the gene expression profile of pbMECs extracted from milk was compared between high and low responder cows. The milk of high responder cows ($n = 5$) had high amounts of specific IgA and the animals showed a fast immune response, whereas milk of low responder cows ($n = 4$) had lower antibody concentrations after repeated immunization. The term "fast immune response" hereby refers to a rapid increase in the amount of specific IgA in milk after immunization. The aim of this study was to establish a defined gene expression pattern or a special set of genes of chemokines, immune receptors, and acute phase proteins to serve as molecular biomarkers for the pre-selection of cows before immunization to maximize immune milk production. pbMEC were chosen to screen for gene expression responses to antigen exposure, as it is known that bovine mammary epithelial cells play important roles in the bovine mammary gland [11]. The rather low changes in the gene expression levels were expected, as Strandberg et al. (2005), Griesbeck-Zilch et al. (2008), and Sorg et al. (2013) have already reported that gram-positive pathogens provoke only a weak innate immune response [12, 21, 27,].

Table 1 Differences in gene expression of high ($n = 5$) and low ($n = 4$) responder cows, as determined by a normal *t*-test

Time point			
Genes	*C. diff.* 6 h[a]	*C. diff.* 24 h[a]	*C. diff.* 72 h[a]
	Low vs. High[b]	Low vs. High[b]	Low vs. High[b]
TLR pathway			
LY96		***	**
CD14	+	***	*
MYD88		+	
TIRAP	*	**	***
TRAF6		+	
IRAK4		*	
IRAK1		+	
RELA		**	
Chemokines			
CCL5	+	*	
CXCL5	+		
CXCL8	+		+
IL13RA		*	+
Inflammatory cytokines			
IL1-A			+
IL6	**	+	*
Acute phase proteins/danger associated molecular pattern molecules			
S100A9			*
S100A12			*
Antimicrobial peptides			
LYZ1			**
LPO			*
Apoptosis			
FAS	*	**	*
TNFRSF1A		+	
CASP8		**	+
CASP3	*	*	
BAX		**	
BCL-2	+		*
Scavenger Receptor			
CD68	**	*	
CD40	**	**	*
JAK-STAT signaling			
STAT2		**	
MAPK signaling			
MAPK 8	**	*	
Others			
MMP1		*	**
MX2		+	
NOD2			*
AKT1		*	
WNT4			+

[a]Treatment time with *C. diff.* in hours
[b]low responder animals versus high responder animals
*$p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$
+distinct changes ($0.01 \leq p < 0.05$)

TLR pathway

Strandberg et al. (2005) reported that the innate host defense of pbMEC is dependent on germline-encoded receptors that recognize conserved structures expressed by a wide variety of microbes [27]. Since pbMEC express TLRs on the cell surface [16], these cells should be able to recognize the gram-positive pathogen *C. diff.* Upon recognition of the bacterial cell wall component lipoteichoic acid through the pattern recognition receptors CD14, TLR2, and TLR4 [28]. The results of the present study showed that gene expression of TLR2 and TLR4 in response to bacterial stimulation was mainly unaffected in the low and high responder groups. These findings are in accordance with the results reported by Strandberg et al. (2005) [27], who postulated that pbMEC contain a fully functional and constitutively active TLR signaling pathway, which is immediately responsive to a bacterial challenge, so that the gene expression of the receptors was not responsible for the inefficient activation of NFkB and, hence, transcription of cytokines, but rather deficits in the downstream signaling pathways [27]. However, the high responder group showed a distinct and statistically significant greater gene expression of CD14, as compared to the low responder group, which is in accordance with the findings of Lutzow et al. (2008) [29]. To identify differences in the activation and downstream signaling cascades in response to *C. diff.*-stimulation between the high and low responder groups, the gene expression levels of LY96, LBP, CD14, MYD88, TIRAP, TRAF6, IRAK4, IRAK1, and RELA were assessed. The results showed significant and distinct changes in the expression levels of genes coding for LY96, CD14, TIRAP, IRAK1, and RELA, especially in the high responder group. Furthermore, the expression levels of MYD88, TRAF6, LY96, CD14, TIRAP, and RELA were significantly greater in the high responder group, as compared to the low responder group, which was in agreement with the findings of Strandberg et al. (2005) [27] that deficits in the downstream signaling pathways were responsible for the relatively low expression of

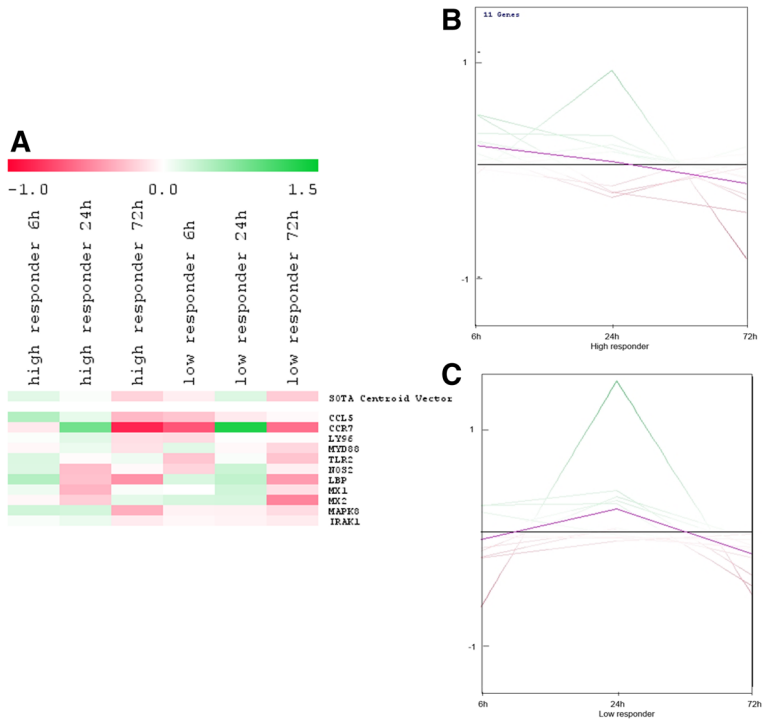

Fig. 5 Differences in the gene expression of immediate early and intermediate early expressed genes (11 genes) in the high (**b**) and low (**c**) responder group. Throughout the SOTA dendrogram (**a**), as well as the expression profiles for the high (**b**) and low (**c**) responder group, indicated differences in gene expression pattern within the high and low responder group

RELA, which is also known as the NF-kappa-B p65 subunit.

Chemokine activation

Upon activation, NF-kB translocates into the nucleus and initiates transcription of a variety of pro-inflammatory factors, such as chemokines and cytokines, as well as genes associated with cell survival and proliferation [30]. Targets also include adhesion molecules, acute phase proteins like SAA-proteins, and inducible enzymes [30, 31]. This effect was observed in the gene expression of prominent chemokines between the high and low responder groups. The gene expression of CXCL8, CCL5, CXCL5, IL6, IL1-A, and IL13RA were distinctly greater in the high responder group. Especially, CXCL8, which is a major initiator of the inflammatory response, has been shown to be essential for the immediate recruitment of leukocytes into the bovine mammary gland and, hence, is responsible for the elimination of invading pathogens [27, 32]. CXCL8 gene expression was substantially greater in the high responder fibroblasts after stimulation of the cell culture with LPS. Additionally, a study by Griesbeck-Zilch et al. (2008) also showed a significant and early induction of CCL5 gene expression after stimulation of pbMEC with *S. aureus* [21]. Furthermore, Lahouassa et al. (2007) showed that bMEC are able to produce and release chemokines, even without up-regulation of the anti-inflammatory cytokine IL10, which we were unable to measure in the present study [33]. As the genes coding for chemokines and inflammatory cytokines were particularly more strongly induced within the high responder group, it could be possible that initiation of the inflammatory reaction and the recruitment of other immune cells to the site of infection was more efficient in the high responder group.

Gene expression pattern of antimicrobial peptides

Activation of the transcription factor NFκB is also known to induce gene expression and production of the antimicrobial peptides LYZ1, LPO, and LF. Normally, antimicrobial peptides are constitutively expressed, even if no direct bacterial stimuli is present. These peptides are mostly constitutively expressed in cells, such as epithelial cells, which are consistently exposed to bacteria. For example, LF shows a bacteriostatic effect through its capability to bind iron, which is essential for bacterial growth [15]. However, in contrast to the report by Griesbeck-Zilch et al. (2008), no up-regulation in LF gene expression was detected in either of the treatment groups [21]. Lysozyme is also a bactericidal protein that cleaves peptidoglycans of the cell wall of gram-positive and gram-negative bacteria. The third antimicrobial peptide analyzed in this study was LPO, which is able to kill or inhibit

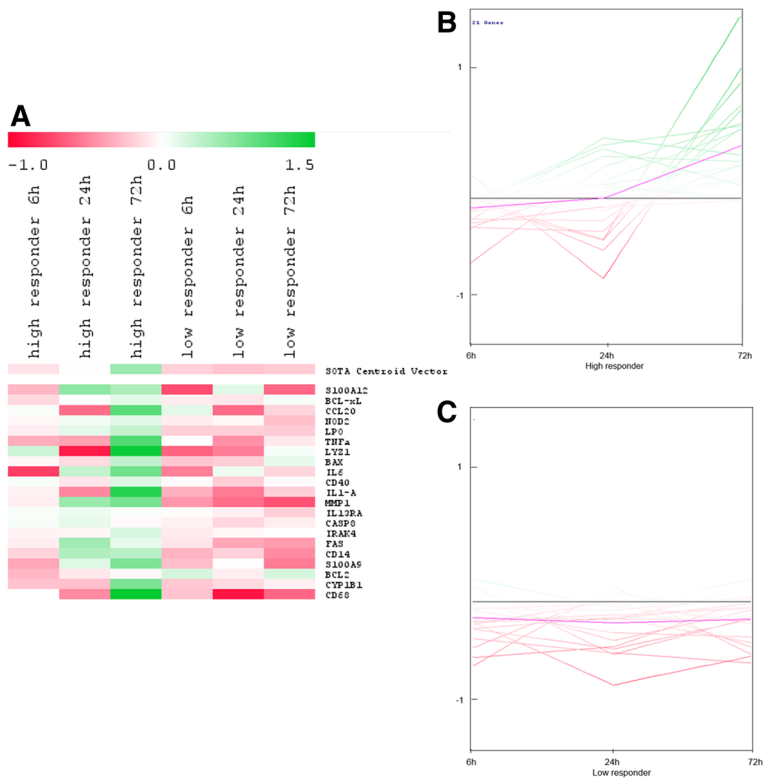

Fig. 6 Differences in the gene expression of late induced genes (21 genes) in the high (**b**) and low (C) responder group. The SOTA dendrogram (**a**) as well as the expression profiles for the high (**b**) and low (**c**) responder group, indicated differences in gene expression pattern within the high and low responder group

bacteria in the presence of thiocyanate and hydrogen peroxide [34]. In the present study, significant induction of the gene expression of LPO and LYZ1 was observed only in the high responder group. Furthermore, the gene expression levels of LPO and LYZ1 were significantly greater in the high responder group, as compared to the low responder group.

Danger associated molecular pattern molecules

The acute phase proteins S100A12 and S100A9 also participate in the regulation of inflammatory processes, as well as the induction of cytokine and chemokine production. The significantly greater gene expresison levels in the high responder group compared to the low responder group could, therefore, together with the chemokines, also contribute to greater activation of immune cells, resulting in a stronger and faster adaptive immune response than in the low responder group [35, 36]. The induction of S100A12 gene expression through gram-positive pathogens has already been reported by Lutzow et al. (2008), Sorg et al. (2013), and Günther et al. (2009), which prompted the question as to whether these molecules are involved in the initial response to bacterial infection [11, 12, 29].

Apoptosis related genes

Apoptosis is an important biochemical process responsible for the proper development and function of the immune system. It has already been shown that apoptosis of bovine mammary epithelial cell lines and primary bovine epithelial cells occurs in response to *S. aureus* infection [37]. Considering the induction of apoptosis, the pbMEC of the high responder group also showed significantly stronger induction of the pro-apoptotic genes Bax, FAS, CASPASE 8, and CASPASE 3 post-infection, as compared to the low responder group. This finding could indicate that the cells in the high responder group were subjected to stronger apoptotic events.

Conclusions

When the expression patterns of genes involved in the TLR signaling pathway and those coding for effector molecules were compared between the low and high responder group, it seems that induction of the innate immune response was quicker in the high responder animals. The greater expression levels of genes involved in the TLR pathway, cytokines, and antimicrobial peptides in pbMEC of the high responder group could be advantageous for the recruitment and activation of immune cells, resulting in a stronger and faster adaptive

immune response than in the low responder group, which in turn leads to a faster induction of antibody producing B-cells and to greater Ig concentrations in milk. It might be possible that the gene expression pattern of the pbMEC during infection together with the gene expression pattern of the bovine lymphocytes is the key to the discovery of new molecular biomarkers to identify cows with an effective immune response and greater amounts of Igs produced in milk. The data obtained from cell culture studies with pbMEC, will be correlated with the gene expression pattern of bovine lymphocytes in our next publication. So, far, genes coding for components of the TLR pathway (LY96, CD14, TIRAP, and RELA), the chemokines CXCL8, CCL5, and CXCL5, the inflammatory cytokines IL6 and IL1-A, the antimicrobial peptides LYZ1 and LPO, and the danger-associated molecular pattern molecules S100A9 and S100A12 appear to be promising as potential candidates for molecular markers, as all were differentially expressed between the low and high immunoglobulin responder group.

Additional files

Additional file 1: Table S1. Primer for RT-qPCR measurements. All primer names, sequences and the NCBI reference sequence number are presented in Additional file 1. (DOCX 45 kb)

Additional file 2: Table S2. Fold changes in gene expression upon C. diff. Treatment - statistical evaluation of the treatment and time-effect with a paired t-test. High responder ($n = 5$), low responder ($n = 4$). (DOCX 40 kb)

Abbreviations

18SrRNA: 18S ribosomal RNA gene; ACTG1: Actin, gamma 1; AKT1 : V-akt murine thymoma viral oncogene homolog 1; BAX: BCL2-associated X protein; BCL-2: B-cell CLL/lymphoma 2; *C. diff.*: *Clostridium difficile*; C3: Complement component 3; CASP1: Caspase 1; CASP3: Caspase 3; CASP8: Caspase 8; CCL2: Chemokine (C-C motif) ligand 2; CCL20: Chemokine (C-C motif) ligand 20; CCL5: Chemokine (C-C motif) ligand 5; CCR7: Chemokine (C-C motif) receptor 7; CD14: CD14 surface receptor; CD40: CD40 surface receptor; CD68: CD68 surface receptor; cDNA: Complementary DNA; Cq: Cycle of quantification; ctr: Control; CXCL3: Chemokine (C-x-C motif) ligand 3; CXCL5: Chemokine (C-x-C motif) ligand 5; CXCL8 : Chemokine (C-X-C motif) ligand 8; CYP1A1: Cytochrome P450, family 1, subfamily A, polypeptide 1; CYP1B1: Cytochrome P450, family 1, subfamily B, polypeptide 1; DMEM/F-12 HAM: Dulbecco's modified eagle's medium nutrient mixture f-12 ham; DMSO: Dimethyl sulfoxide; DNA: Deoxyribonucleic acid; dNTP: Deoxynucleoside triphosphate; *E. coli*: *Escherichia coli*; EDTA: Ethylenediaminetetraacetic acid; FAS: Fas cell surface death receptor; FBS: Fetal bovine Serum; FcRN: IgG Fc receptor; GAPDH: Glyceraldehyd-3-phosphate dehydrogenase; H_2SO_4: Sulfuric acid; H3F3A: H3 histone, family 3A; HBSS: Hank's balanced salt solution; HC: Hydrocortisone; HP: Haptoglobin; HRP: Horse radish peroxidase; IgA: Immunoglobulin A; IL10: Interleukin 10; IL13RA: Interleukin 13 receptor, alpha 1; IL1-A: Interleukin 1, alpha; IL1-B: Interleukin 1, beta; IL6: Interleukin 6; IRAK1: Interleukin-1 receptor-associated kinase 1; IRAK4: Interleukin-1 receptor-associated kinase 4; IRF3: Interferon regulatory factor 3; ITS: Insulin/Transferrin/Sodium selenite solution; KRT8: Cytokeratin 8; LAP: Lingual antimicrobial peptide; LBP: Lipopolysaccharide binding protein; LF: Lactoferrin; LPO: Lactoperoxidase; LPS: Lipopolysaccharide; LTA: Lipoteichoic acid; LY96: Lymphocyte antigen 96; LYS: L-Lysine; LYZ1: Lysozyme 1 K; MAPK1: Mitogen-activated protein kinase 1; MAPK8: Mitogen-activated protein kinase 8; MIQE: Minimum information for publication of quantitative real-time PCR experiments; M-MLV: Moloney murine leukemia virus; MMP1: Matrix metallopeptidase 1; MOI: Multiplicity of infection; mRNA: Messenger RNA; MX1: Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse); MX2: Myxovirus (influenza virus) resistance 2 (mouse); MYD88: Myeloid differentiation primary response gene 88; $NaHCO_3$: Natrium hydrogen carbonate; NCBI: National Library of Medicine; NFκB: Nuclear factor κb; NOS2: Nitric oxide synthase 2, inducible; NTC: Non-template control; PAMP: Pathogen associated molecular pattern; pbMEC: primary bovine mammary epithelial cells; PBS: Phosphate Buffered Saline; PBS-T: PBS-Tween20; PenStrep: Penicillin/Streptomycin; pIGR: Polymeric immunoglobulin receptor; qPCR: Quantitative polymerase chain reaction; RELA: V-rel reticuloendotheliosis viral oncogene homolog A (avian) (NF-kappa-B p65 subunit); RIN: RNA integrity number; RNA: Ribonucleic acid; rpm: Revolutions per minute; rRNA: Ribosomal RNA; RT: Reverse transcription/room temperature; RT-qPCR: Reverse transcription quantitative polymerase chain reaction; S100A12: S100 calcium binding protein A12; S100A9: S100 calcium binding protein A9; *S. aureus*: *Staphylococcus aureus*; SAA3: Serum amyloid A3; SCC: Somatic cell count; SEM: Standard error of the mean; SOTA: Self-organizing tree algorithm; STAT2: Signal transducer and activator of transcription 2; TAP: Tracheal antimicrobial peptide; TGFβ1: Transforming growth factor, beta 1; TIRAP: TCDD-inducible poly(ADP-ribose) polymerase; TLR: Toll-like receptor; TLR2: Toll-like receptor 2; TLR4: Toll-like receptor 4; TNF: Tumor necrosis factor; TNFR2: Tumor necrosis factor receptor 2; TNFRSF1A: Tumor necrosis factor receptor superfamily, member 1A; TNFα: Tumor necrosis factor α; TRAF6: TNF receptor-associated factor 6, E3 ubiquitin protein ligase; UBB: Ubiquitine B; WNT4: Wingless-type MMTV integration site family member 4; YWHAZ: Tyrosine 3-monooxygenase / tryptophan 5-monooxygenase activation protein zeta polypeptide; YY1: Yin Yang 1

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Availability of data and materials

The nucleotide sequences used for primer construction were retrieved from the National Center for Biotechnology Information gene database (http://www.ncbi.nlm.nih.gov/genbank/). The accession numbers are presented in the Additional file 1: Table S1.
The dataset supporting the conclusions of this article is included within the article and its additional files.
Additional file 1: Table S1. Primer for RT-qPCR measurements.
Additional file 2: Table S2. Fold changes in gene expression upon *C. diff.* Treatment - statistical evaluation of the treatment and time-effect with a paired t-test (DOCX 65 kb).

Authors' contributions

MH was responsible for the experimental work, data analysis, and writing of the manuscript. CS contributed to the writing of the manuscript and reviewed the drafts. IB and HK immunized the animals. VK provided knowledge and practicability of the RT-qPCR measurement in the Laboratory of Gene Expression, Institute of Biotechnology, Academy of Sciences of the Czech Republic. MWP and HK coordinated the project, contributed to the experimental design, and reviewed the drafts. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate
This sort of animal trial (production of antibodies) was approved by the government of Upper Bavaria (AZ. 55.2–1–54-2532.6-17-2012), according to the German Animal Welfare Act.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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References
1. Poutanen SM. Clostridium Difficile-associated diarrhea in adults. CMAJ. 2004; 171:51–8. doi:10.1503/cmaj.1031189.
2. Ünal CM, Steinert M. Novel therapeutic strategies for Clostridium Difficile infections. Expert Opin Ther Targets. 2016;20:269–85. doi:10.1517/14728222.2016.1090428.
3. Viswanathan VK, Mallozzi MJ, Vedantam G. Clostridium Difficile infection: an overview of the disease and its pathogenesis, epidemiology and interventions. Gut Microbes. 2010;1:234–42. doi:10.4161/gmic.1.4.12706.
4. Larrainzar-Coghen T, Rodriguez-Pardo D, Puig-Asensio M, Rodríguez V, Ferrer C, Bartolomé R, et al. First recurrence of Clostridium Difficile infection: clinical relevance, risk factors, and prognosis. Eur J Clin Microbiol Infect Dis. 2016;35:371–8. doi:10.1007/s10096-015-2549-9.
5. Elliott B, Chang BJ, Golledge CL, Riley TV. Clostridium Difficile-associated diarrhoea. Intern Med J. 2007;37:561–8. doi:10.1111/j.1445-5994.2007.01403.x.
6. Bagdasarian N, Rao K, Malani PN. Diagnosis and treatment of Clostridium Difficile in adults: a systematic review. JAMA. 2015;313:398–408. doi:10.1001/jama.2014.17103.
7. Mullane K. Fidaxomicin in Clostridium Difficile infection: latest evidence and clinical guidance. Ther Adv Chronic Dis. 2014;5:69–84. doi:10.1177/2040622313511285.
8. Louie TJ, Miller MA, Mullane KM, Weiss K, Lentnek A, Golan Y, et al. Fidaxomicin versus vancomycin for Clostridium Difficile infection. N Engl J Med. 2011;364:422–31. doi:10.1056/NEJMoa0910812.
9. Van Dissel JT, De Groot N, Hensgens CM, Numan S, Kuijper EJ, Veldkamp P, et al. Bovine antibody-enriched whey to aid in the prevention of a relapse of Clostridium Difficile-associated diarrhoea: preclinical and preliminary clinical data. J Med Microbiol. 2005;54:197–205. doi:10.1099/jmm.0.45773-0.
10. Hermann C. Review: variability of host-pathogen interaction. J Endotoxin Res. 2007;13:199–218. doi:10.1177/0968051907082605.
11. Günther J, Koczan D, Yang W, Nürnberg G, Repsilber D, Schuberth HJ, Park Z, Maqbool N, Molenaar A, Seyfert HM. Assessment of the immune capacity of mammary epithelial cells: comparison with mammary tissue after challenge with Escherichia Coli. Vet Res. 2009;40:31. doi:10.1051/vetres/2009014.
12. Sorg D, Danowski K, Korenkova V, Rusnakova V, Küffner R, Zimmer R, Meyer HHD, Kliem H. Microfluidic high-throughput RT-qPCR measurements of the immune response of primary bovine mammary epithelial cells cultured from milk to mastitis pathogens. Animal. 2013;7:799–805. doi:10.1017/S1751731112002315.
13. Prakash BS, Meyer HH, Schallenberger E, Van de Wiel DF. Development of a sensitive enzymeimmunoassay (EIA) for progesterone determination in unextracted bovine plasma using the second antibody technique. J Steroid Biochem. 1987;28:623–7.
14. Sorg D, Fandrey E, Frölich K, Meyer H, Kliem H. Mammary immunity of White Park and highland cattle compared with Brown Swiss and red Holstein. Anim Genet Resour. 2013;52:91–104. doi:10.1017/S2078633612000781.
15. Danowski K, Gross JJ, Meyer HHD, Kliem H. Effects of induced energy deficiency on lactoferrin concentration in milk and the lactoferrin reaction of primary bovine mammary epithelial cells in vitro. J Anim Physiol Anim Nutr (Berl). 2013;97:647–55. doi:10.1111/j.1439-0396.2012.01305.x.
16. Wellnitz O, Arnold ET, Bruckmaier RM. Lipopolysaccharide and lipoteichoic acid induce different immune responses in the bovine mammary gland. J Dairy Sci. 2011;94:5405–12. doi:10.3168/jds.2010-3931.
17. Gilbert FB, Cunha P, Jensen K, Glass EJ, Foucras G, Robert-Granié C, et al. Differential response of bovine mammary epithelial cells to Staphylococcus Aureus or Escherichia Coli agonists of the innate immune system. Vet Res. 2013;44:40. doi:10.1186/1297-9716-44-40.
18. Im J, Lee T, Jeon JH, Baik JE, Kim KW, Kang S-S, et al. Gene expression profiling of bovine mammary gland epithelial cells stimulated with lipoteichoic acid plus peptidoglycan from Staphylococcus Aureus. Int Immunopharmacol. 2014;21:231–40. doi:10.1016/j.intimp.2014.05.002.
19. Günther J, Esch K, Poschadel N, Petzl W, Zerbe H, Mitterhuemer S, et al. Comparative kinetics of Escherichia Coli- and Staphylococcus Aureus-specific activation of key immune pathways in mammary epithelial cells demonstrates that S. Aureus elicits a delayed response dominated by interleukin-6 (IL-6) but not by IL-1A or tumor necrosis factor alpha. Infect Immun. 2011;79:695–707. doi:10.1128/IAI.01071-10.
20. Wellnitz O, Reith P, Haas SC, Meyer HH. Immune relevant gene expression of mammary epithelial cells and their influence on leukocyte chemotaxis in response to different mastitis pathogens. Vet Med (Praha). 2006;51:125–32.
21. Griesbeck-Zilch B, Meyer H, Kühn C, Schwerin M, Wellnitz O. Staphylococcus Aureus and Escherichia Coli cause deviating expression profiles of cytokines and lactoferrin messenger ribonucleic acid in mammary epithelial cells. J Dairy Sci. 2008;91:2215–24. doi:10.3168/jds.2007-0752.
22. Koressaar T, Remm M. Enhancements and modifications of primer design program Primer3. Bioinformatics. 2007;23:1289–91. doi:10.1093/bioinformatics/btm091.
23. Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, et al. Primer3–new capabilities and interfaces. Nucleic Acids Res. 2012;40:e115. doi:10.1093/nar/gks596.
24. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009;55:611–22. doi:10.1373/clinchem.2008.112797.
25. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods. 2001;25:402–8. doi:10.1006/meth.2001.1262.
26. Saeed AI, Sharov V, White J, Li J, Liang W, Bhagabati N, et al. TM4: a free, open-source system for microarray data management and analysis. BioTechniques. 2003;34:374–8.
27. Strandberg Y, Gray C, Vuocolo T, Donaldson L, Broadway M, Tellam R. Lipopolysaccharide and lipoteichoic acid induce different innate immune responses in bovine mammary epithelial cells. Cytokine. 2005;31:72–86. doi:10.1016/j.cyto.2005.02.010.
28. Dziarski R, Wang Q, Miyake K, Kirschning CJ, Gupta D. MD-2 enables toll-like receptor 2 (TLR2)-mediated responses to lipopolysaccharide and enhances TLR2-mediated responses to gram-positive and gram-negative bacteria and their cell wall components. J Immunol. 2001;166:1938–44.
29. Lutzow Y, Strandberg C, Donaldson L, Gray CP, Vuocolo T, Pearson RD, Reverter A, et al. Identification of immune genes and proteins involved in the response of bovine mammary tissue to Staphylococcus Aureus infection. BMC Vet Res. 2008;4:18. doi:10.1186/1746-6148-4-18.
30. Aderem A, Ulevitch RJ. Toll-like receptors in the induction of the innate immune response. Nature. 2000;406:782–7. doi:10.1038/35021228.
31. Janeway C, Murphy KP. Janeway Immunologie. 7th ed. Heidelberg: Spektrum, Akad. Verl; 2009.
32. Boudjellab N, Chan-Tang HS, Zhao X. Bovine interleukin-1 expression by cultured mammary epithelial cells (MAC-T) and its involvement in the release of MAC-T derived interleukin-8. Comp Biochem Physiol, part a Mol Integr. Physiol. 2000;127:191–9.
33. Lahouassa H, Moussay E, Rainard P, Riollet C. Differential cytokine and chemokine responses of bovine mammary epithelial cells to Staphylococcus Aureus and Escherichia Coli. Cytokine. 2007;38:12–21. doi:10.1016/j.cyto.2007.04.006.
34. Rainard P, Riollet C. Innate immunity of the bovine mammary gland. Vet Res. 2006;37:369–400. doi:10.1051/vetres:2006007.

35. Ryckman C, Vandal K, Rouleau P, Talbot M, Tessier PA. Proinflammatory activities of S100: proteins S100A8, S100A9, and S100A8/A9 induce neutrophil chemotaxis and adhesion. J Immunol. 2003;170:3233–42. doi:10.4049/jimmunol.170.6.3233.
36. Yang Z, Tao T, Raftery MJ, Youssef P, Di Girolamo N, Geczy CL. Proinflammatory properties of the human S100 protein S100A12. J Leukoc Biol. 2001;69:986–94.
37. Wesson CA, Deringer J, Liou LE, Bayles KW, Bohach GA, Trumble WR. Apoptosis induced by Staphylococcus Aureus in epithelial cells utilizes a mechanism involving caspases 8 and 3. Infect Immun. 2000;68:2998–3001.

Appendix III

PLOS | ONE

RESEARCH ARTICLE

Effect of the Ketone Body Beta-Hydroxybutyrate on the Innate Defense Capability of Primary Bovine Mammary Epithelial Cells

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Abstract

Negative energy balance and ketosis are thought to cause impaired immune function and to increase the risk of clinical mastitis in dairy cows. The present *in vitro* study aimed to investigate the effect of elevated levels of the predominant ketone body β-hydroxybutyrate on the innate defense capability of primary bovine mammary epithelial cells (pbMEC) challenged with the mastitis pathogen *Escherichia coli* (*E*. *coli*). Therefore, pbMEC of healthy dairy cows in mid- lactation were isolated from milk and challenged in culture with 3 mM BHBA and *E*. *coli*. pbMEC stimulated with *E*. *coli* for 6 h or 30 h showed an up-regulation of several innate immune genes, whereas co-stimulation of pbMEC with 3 mM BHBA and *E*. *coli* resulted in the down-regulation of CCL2, SAA3, LF and C3 gene expression compared to the challenge with solely the bacterial stimulus. These results indicated that increased BHBA concentrations may be partially responsible for the higher mastitis susceptibility of dairy cows in early lactation. Elevated levels of BHBA in blood and milk during negative energy balance and ketosis are likely to impair innate immune function in the bovine mammary gland by attenuating the expression of a broad range of innate immune genes.

Introduction

High-producing dairy cows often experience a state of negative energy balance (NEB) during the first weeks of lactation. Milk production rapidly increases after calving, so that the cows require more energy for maintenance, growth and milk production than they are able to obtain through feed intake and digestion [1–3]. To compensate for the deficiency in energy supply in early lactation, body fat reserves are mobilized resulting in an increase of the plasma concentration of non-esterified fatty acids (NEFA) [4,5]. NEFA are taken up by the liver and further processed via the β-oxidation pathway in mitochondria, where acetyl-CoA is formed, which enters the tricarboxylic acid cycle (TCA cycle) to form citrate by condensation with oxaloacetate. In order to generate essential glucose and carbohydrates in NEB, gluconeogenesis occurs in the liver using oxaloacetate as main substrate. This leads to an insufficient availability of

Competing Interests: The authors have declared that no competing interests exist.

oxaloacetate for the removal of acetyl-CoA and hence an accumulation of acetyl-CoA. Alternatively, acetyl-CoA is metabolized via ketogenesis to the ketone bodies acetoacetate, β-hydroxybutyrate (BHBA) and acetone [1]. Therefore, cows in NEB show increased concentrations of ketone bodies in their blood and milk. The predominant circulating ketone body in dairy cattle that contributes to the development of subclinical or even clinical ketosis is BHBA [2]. Subclinical ketosis is characterized by elevated plasma concentrations of ketone bodies in the absence of the clinical signs of ketosis [6] and is defined at a plasma concentration of 1.4 mM BHBA [5,7], whereby the threshold for clinical ketosis is defined at a plasma concentration of 2 to 3 mM BHBA [5]. Cows suffering from clinical ketosis show signs of indigestion resulting in decreased feed intake, body weight and milk production. In addition, they can be lethargic or abnormally agitated [4]. It has already been discussed that NEB and ketosis might be linked to an increased risk of clinical mastitis in dairy cows, especially in the early phase of lactation. The major mastitis pathogens that cause mastitis are either gram-positive germs, like *Staphylococcus aureus* or gram-negative germs like *Escherichia coli* (*E. coli*). In case of an *E. coli* induced mastitis, the milk producing parenchyma, the milk collecting cistern and the teat show acute symptoms of inflammation, elevated body temperature, decreased milk production and elevated somatic cell counts (SCC) [6]. Mastitis affects dairy cow health and welfare, but is also one of the most costly diseases in the dairy industry due to reduced milk yield and quality [8]. Since primary bovine mammary epithelial cells (pbMEC) are known to be part of the innate immune system of the bovine mammary gland [9], we used a 3D cell culture approach to investigate the effect of elevated BHBA levels on the innate defense capability of pbMEC challenged with the mastitis pathogen *E. coli*. pbMEC represent the first cellular line of defense, after a pathogen manages to cross the teat barrier, therefore pbMEC express transepithelial receptors, like the toll-like receptors (TLR) on their cell surface [10]. Through the activation of the TLR-pathway by pathogen associated molecular patterns, pbMEC are able to induce a downstream signaling cascade, resulting in the activation of the transcription factor NFκB and hence in the production of chemotactic molecules like pro-inflammatory chemokines, cytokines and acute phase proteins [11,12]. Thus, the recruitment of other immune cells, like leucocytes and macrophages to the site of infection is one of the main tasks of pbMEC in innate immunity. Therefore, pbMEC of healthy dairy cows in mid-lactation were isolated from fresh milk and challenged with 3 mM BHBA and *E. coli*. The approach aimed to stimulate the metabolic state of ketosis and, in addition, an inflammation of the mammary epithelium *in vitro*. Results should elucidate the influence of ketosis on the innate immune response of the bovine mammary gland to mastitis.

Materials and Methods

Cultivation of pbMEC

The pbMEC were isolated from the milk of six healthy, first lactating Brown Swiss cows (Veitshof Research Station, Technische Universität München, Freising, Germany) in mid-lactation (100–200 days in lactation). Cows with a somatic cell count (SCC) below 200,000 cells per milliliter milk, were considered healthy. The cell isolation was basically conducted as described in previous studies [12,13], with slight changes in the washing procedure. In brief, milk cells were harvested (10 min, 1850 × g), the cell pellet was washed with 1x HBSS buffer (Sigma-Aldrich, Saint Louis, USA) supplemented with antibiotics and antimycotics (penicillin/streptomycin, amphotericin B, Sigma-Aldrich) and the cell suspension was filtered twice through filters with different pore sizes (100 μm, 40 μm, Greiner Bio-One GmbH, Frickenhausen, Germany). The cells were then re-suspended in 4 ml of pre-warmed DMEM-F12 Ham medium (Sigma-Aldrich) supplemented with ITS liquid media supplement, antibiotics, antimycotics (Sigma-Aldrich) and FBS (gibco® Lifetechnologies GmbH, Darmstadt, Germany) (proliferation

medium), and seeded in one well of a coated (2.4 mg/ml Matrigel®, Corning Inc., Corning, NY) 6-well culture plate. The cells were cultivated at 37°C, 5% CO_2 and 90% humidity. The pbMEC were sub-cultivated twice with 0.25% trypsin-EDTA solution (Sigma-Aldrich), before they were cryopreserved in liquid nitrogen.

Immunocytochemistry

The pbMEC of all animals were seeded at a density of 1.5×10^4 cells per well in 8-well LabTec chamber slides (LAB-Tec, Nunc, GmbH, Langenselbold, Germany) and provided with proliferation medium. At a confluency of 70–80% the medium and the culture chambers were removed and the cells fixed in ice cold methanol/acetone (1:1) for 10 min. Immunocytochemistry (IC) was conducted with the monoclonal mouse anti-cytokeratin pan antibody clone C-11 (1:400 in PBST, Sigma-Aldrich), as described earlier [13] [14].

Mycoplasma Test

The PCR Mycoplasma Test Kit (AppliChem GmbH, Darmstadt, Germany) was used. Cell culture supernatants were sampled for each animal and stored at– 80°C until further processing. The analysis was conducted according to the manufacturer's instructions.

Imitation of the ketotic state and immune stimulation with *E. coli*

Treatment with BHBA and *E. coli* was performed in duplicate. The pbMEC were seeded at a density of 2×10^4 cells per well in 6-well plates coated with 2.4 mg/ml Matrigel® (Corning Inc., Corning, NY). For the *E. coli* treatment, three counting wells were seeded per animal. The pbMEC on those wells were trypsinized with a solution of 0.25% trypsin-EDTA (Sigma-Aldrich) upon reaching confluency and counted (TC10™ Automated Cell Counter, Bio-Rad Laboratories GmbH) using the life-dead staining with 0.4% trypan blue (Bio-Rad Laboratories GmbH, Munich, Germany). The mean value of the cell count served as benchmark for the cell number in all other experimental wells and was the basis for calculating the multiplicity of infection (MOI). After reaching 70–80% confluency, the proliferation medium was replaced with DMEM/F12 Ham medium supplemented with only ITS (Sigma-Aldrich) (challenge medium). After 24 h, the medium was removed and the first samples were taken (0 h time-point). The other cells were treated using different approaches. Control cells were left untreated and were further incubated in a challenge medium for 24 h, 30 h and 54 h. Cells which should be treated with 3 mM BHBA (Sigma-Aldrich), were treated with challenge medium supplemented with 3 mM BHBA for additional 24 h, 30 h and 54 h. For cells to be treated either only with *E. coli* or with both *E. coli* and 3 mM BHBA, pbMEC were infected with a multiplicity of infection (MOI) of 30 colony forming units per cultured cell. The infected pbMEC were further cultivated for 6 h and 30 h with *E. coli* alone, or with *E. coli* and BHBA (30 h BHBA & 6 h *E. coli* and 54 h BHBA & 30 h *E. coli*) (Fig 1). The cell culture samples were lysed in Qiazol (Qiagen, Hilden, Germany) and stored at -80°C until further analysis. For each treatment time-point, cell culture supernatants were also sampled and stored at -80°C.

RNA extraction and reverse transcription (RT)

Total RNA, including mRNA and miRNA, was extracted with the miRNeasy Micro Kit (Qiagen) according to the manufacturer's instructions with slight modifications. An additional incubation for 5 min with buffer RPE was added to the standard protocol in order to reduce contaminations of the RNA with guanidine thiocyanate. The NanoDrop 1000 photometer (Peqlab, Erlangen, Germany) was used to verify RNA concentration and purity, whereas the

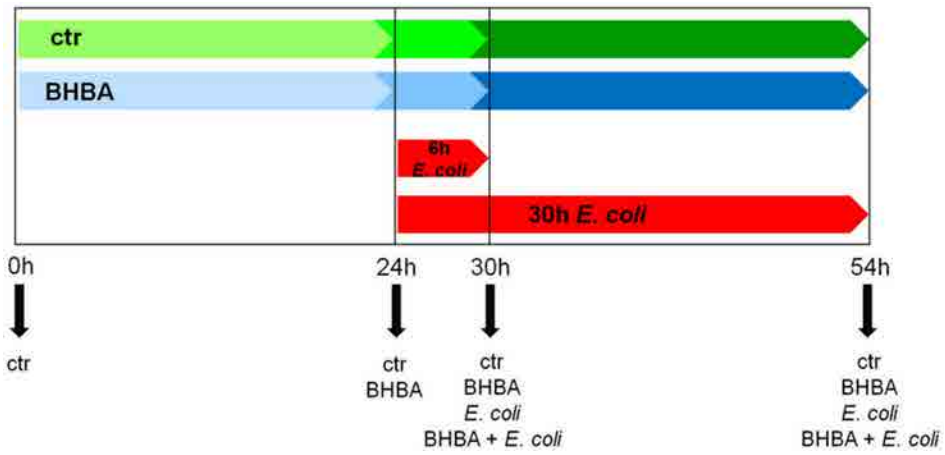

Fig 1. Treatment scheme of the pbMEC *in vitro*. The green arrow indicates the untreated control samples that were taken at every relevant time-point (0 h, 24 h, 30 h and 54 h). The blue arrow indicates the BHBA treatment time-points. The pbMEC that were only treated with 3 mM BHBA were also sampled at every relevant time-point (24 h, 30 h and 54 h). The red arrows indicate the two treatment intervals for the *E. coli* treatment (6 h and 30 h). It can be clearly seen that within the co-stimulated pbMEC, a 24 h adaptation phase to 3 mM BHBA preceded the bacterial treatment, so that pbMEC stimulated with *E. coli* for 6 h, obtained a total BHBA treatment period for 30 h and pbMEC stimulated with E. coli for 30 h obtained a total BHBA treatment period of 54 h.

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2100 Bioanalyzer and the RNA 6000 Nano Kit (Agilent Technologies, Waldbronn, Germany) were used to determine RNA integrity. The analysis was conducted following the manufacturer's instructions. Isolated RNA was stored at -80°C until further processing. The master mix for the reverse transcription of 400 ng RNA to cDNA contained the following components in a total volume of 20 μl: 0.5 mM Oligo-d(T) primers (Fermentas, St Leon-Rot, Germany), 0.5 M dNTPs, 5x buffer, 100U M-MLV H(—) reverse transcriptase (Promega, Mannheim, Germany) and 2.5 μM random hexamer primers (Invitrogen Life Technologies, Darmstadt, Germany). The cDNA was diluted 1:1 to a final concentration of 10 ng/μl. RNA isolated from bovine spleen and bovine udder tissue was used as positive control. Furthermore, a negative control and a non-template control (NTC) were carried along with the RT-PCR. The T-Personal Thermocycler (Biometra, Göttingen, Germany) was used to conduct the RT-PCR according to the following protocol: Annealing: 21°C, 10 min, transcription phase: 48°C, 50 min, degrading phase: 90°C, 2 min. cDNA was stored at– 20°C until further analysis.

RT-qPCR primer design

24 specific primer pairs for *Bos taurus* were designed using Primer3web version 4.0.0 [15,16], based on published bovine nucleic acid sequences of the National Center for Biotechnology Information gene database (NCBI, National Library of Medicine, Bethesda MD), and ordered from Sigma-Aldrich. Amongst primers targeting the genes involved in the innate immune response, 4 primers targeted the reference genes ACTG1, GAPDH, YWHAZ and KRT8 (Table 1).

Expression profiling via RT-qPCR

The RT-qPCR reactions were conducted on the CFX384™ Real-Time System (Bio-Rad Laboratories GmbH). 10 ng/μl cDNA were mixed together with a master mix consisting of SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories GmbH), VisiBlue™ qPCR mix colorant (TATAA Biocenter, Gothenburg, Sweden), RNase/DNase-free DEPC water and 20 μM of each Primer.

Table 1. Primer for RT-qPCR measurements.

Gene name	NCBI reference sequence number	Primer sequence (5' -> 3') Forward Reverse	L[1] [bp]
TLR pathway			
Toll-like receptor 4 (*TLR4*)	NM_174198.6	TGCTGGCTGCAAAAAGTATG	213
		TTACGGCTTTTGTGGAAACC	
Myeloid differentiation primary response gene (*MYD88*)	NM_001014382.2	CTGCAAAGCAAGGAATGTGA	122
		AGGATGCTGGGGAACTCTTT	
V-rel reticuloendotheliosis viral oncogene homolog A (avian) (NF-kappa-B p65 subunit) (*RELA*)	NM_001080242.2	ACAGCTTTCAGAACCTGGGG	140
		GACGGCATTCAGGTCGTAG	
Complement system			
Complement component 3 (*C3*)	NM_001040469	AAGTTCATCACCCACATCAAG	191
		CACTGTTTCTGGTTCTCCTC	
Chemokines			
Chemokine (C-C motif) ligand 2 (*CCL2*)	NM_174006.2	TCTCGCTGCAACATGAAGGT	121
		TATAGCAGCAGGCGACTTGG	
Chemokine (C-C motif) ligand 20 (*CCL20*)	NM_174263.2	CTTGTGGGCTTCACACAGC	115
		GTTTCACCCACTTCTTCTTTGG	
Interleukin 8 (*CXCL8*)	NM_173925.2	AAGAATGAGTACAGAACTTCGATGC	160
		GTTTAGGCAGACCTCGTTTCC	
Inflammatory cytokines			
Interleukin 6 (*IL6*)	NM_173923.2	TGGTGATGACTTCTGCTTTCC	109
		AGAGCTTCGGTTTTCTCTGG	
Tumor necrosis factor α (*TNFα*)	NM_173966.2	CCACGTTGTAGCCGACATC	108
		ACCACCAGCTGGTTGTCTTC	
Acute phase proteins / danger associated molecular pattern molecules			
Serum amyloid A3 (*SAA3*)	NM_001242573.1	CACGGGCATCATTTTCTGCTT	179
		GGGCAGCGTCATAGTTTCCA	
Haptoglobin (*HP*)	NM_001040470.1	AATGAACGATGGCTCCTCAC	176
		TTGATGAGCCCAATGTCTACC	
S100 calcium binding protein A9 (*S100A9*)	NM_001046328.1	CTGGTGCAAAAAGAGCTGC	128
		AGCATAATGAACTCCTCGAAGC	
Antimicrobial peptides			
Lactoferrin (*LF*)	NM_180998.2	CGAAGTGTGGATGGCAAGGAA	215
		TTCAAGGTGGTCAAGTAGCGG	
Lysozyme 1 K (*LYZ1*)	NM_001077829.1	AAGAAACTTGGATTGGATGGC	185
		ACTGCTTTTGGGGTTTTGC	
Tracheal antimicrobial peptide (*TAP*)	NM_174776.1	AGGAGTAGGAAATCCTGTAAGCTGTGT	113
		AGCATTTTACTGCCCGCCCGA	
Lingual antimicrobial peptide (*LAP*)	NM_203435.3	AGAAATTCTCAAAGCTGCCG	107
		CAGCATTTTACTTGGGCTCC	
Lactogenesis			
Signal transducer and activator of transcription 5 (*STAT5*)	NM_001012673.1	GTGAAGCCACAGATCAAGCA	176
		TCGAATTCTCCATCCTGGTC	
β-casein (CSN2)[2]	NM_181008.2	GGCTATGGCTCCTAAGCACA	163
		AGTTGGAGGAAGAGGCTGGT	
κ-casein (CSN3)[3]	NM_174294.1	GGAGCCTAAAACCCACAGACA	151

(*Continued*)

Table 1. (*Continued*)

Gene name	NCBI reference sequence number	Primer sequence (5' -> 3') Forward Reverse	L[1] [bp]
		CAGCACAACTTTGGAAGGGC	
Others			
Myxovirus (influenza virus) resistance 2 (mouse) (*MX2*)	NM_173941.2	CTTCAGAGACGCCTCAGTCG	232
		TGAAGCAGCCAGGAATAGTG	
Reference genes			
Actin, gamma 1 (*ACTG1*)	NM_001033618	AACTCCATCATGAAGTGTGAC	234
		GATCCACATCTGCTGGAAGG	
Glyceraldehyd-3-phosphate dehydrogenase (*GAPDH*)	NM_001034034.1	GTCTTCACTACCATGGAGAAGG	197
		TCATGGATGACCTTGGCCAG	
Tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein, zeta polypeptide (*YWHAZ*)	NM_174814.2	CAGGCTGAGCGATATGATGA	141
		GACCCTCCAAGATGACCTAC	
Cytokeratin 8 (*KRT8*)	NM_001033610	TGGTGGAGGACTTCAAGACC	215
		CGTGTCAGAAATCTGAGACTGC	

[1] L: Length in base pair

[2] Tm: 62°C

[3] Tm: 54°C

doi:10.1371/journal.pone.0157774.t001

The RT-qPCR was conducted on the CFX384™ Real-Time System with the following protocol: Activation of the DNA polymerase: 95°C, 30 sec, and 40 cycles of cDNA denaturation: 95°C, 5 sec and primer annealing and elongation: 54°C, 60°C, 62°C (*), 5 sec (* primer specific annealing temperature, Table 1). Primer specificity was verified by checking the melting curves of the RT-qPCR products.

Data pre-processing and data analysis

The cycle of quantification (Cq) was automatically detected by the CFX Manager Software version 2.1 (Bio-Rad Laboratories GmbH). Using the geNorm tool in GenEx 5.4.4 (MultiD; Gothenburg, Sweden), raw Cq values were processed to identify stably expressed reference genes for target gene normalization. Fold changes of gene expression were determined according to the $2^{\wedge(-ddCq)}$ method according to [17] for each sample. Statistics were calculated using SigmaPlot 13.0 (Systat Software GmbH). The dCq values were checked for a normal distribution, before the paired t-test was conducted. In the case of abnormally distributed data, a signed rank test was used. Regulation of gene expression was regarded as differentially expressed for $p \leq 0.05$ in a paired t-test on dCq values between the treatment group and the corresponding control group or between two different treatments. When distinct changes in gene expression are mentioned, a p-value between $0.05 < p < 0.10$ was calculated. The minimum information for the publication of quantitative real-time PCR experiments (MIQE) Guidelines [18] was considered during the entire RT-qPCR quantification workflow.

ELISA measurements

The amount of secreted protein into the cell culture supernatant was evaluated for lactoferrin (LF) and CCL2. The protein content of LF in the cell culture supernatants was determined

using the lactoferrin ELISA established at our institute and described in earlier studies [13]. Slight variations were applied to adapt the protocol to the low concentration of LF obtained in cell culture supernatants. Therefore, the standard curve for LF (colostrum isolate, Sigma-Aldrich) ranged from 12.5 ng/100 µl– 0.049 ng/100 µl and was diluted in phosphate buffered saline-Tween buffer (PBS-T, 1 g/l Tween 20, Merck Chemicals GmbH). For the ELISA measurements, 100 µl of the diluted standard and the undiluted cell culture supernatants, were applied to multiwell plates (96-well, Maxisorp, Nunc®, Sigma-Aldrich, Saint Louis, USA). Additionally 50 µl of the LF antibody (Ak8836, BE Ak8836, BE 08.092009) (1:300000 in PBST) were added to each well of the microplate. The ELISA plate was incubated at 4°C and left slightly shaking overnight. Afterwards, the ELISA plate was set to room temperature for 30 min before 6 ng/100 µl biotinylated LF was applied and incubated on a shaker for 45 min at room temperature. The microplate was then washed four times with PBST and 100 µl streptavidin-HRP working solution (Roche Applied Science, Basel, Switzerland) diluted 1:20000 in PBST was added and incubated for 15 min protected from light. After four additional washing steps, the HRP substrate reaction was started [19] and stopped after 40 min in the dark with 2 M H_2SO_4 before the substrate reaction was evaluated at 450 nm using a Microplate Reader (Sunrise™, Tecan Group Ltd., Männedorf, Switzerland). The mean value of the LF concentration was determined using the OD values of the standard curve within the linear range.

For the determination of the CCL2 amount in cell culture supernatants, the bovine CCL2 VetSet™ ELISA Development Kit (Kingfisher Biotech, St Paul, USA) was used. The ELISA was conducted according to the manufacturer's instructions, with slight changes. As the challenge medium did not contain any carrier protein, the samples and the standard diluent (challenge medium) were supplemented with a solution of 4% BSA (Sigma-Aldrich) in DPBS, so that finally 1% BSA was present in the cell culture samples and the standard diluent. The substrate reaction was induced by the addition of TMB substrate and stopped after 30 min with 0.18 M H_2SO_4 (ELISA Accessory Pack, Kingfisher Biotech). The mean value of the CCL2 concentration was determined using the OD values of the standard curve within the linear range. For the statistical analysis of the treatment effect on CCL2 and LF protein secretion, the mean values of the *E.coli* treated group were compared to the mean values of the co-stimulated treatment group, using a paired t-test.

Results

Confirmation of the epithelial character and purity of the pbMEC cultures

It was proven that none of the pbMEC cultures used in this experiment were contaminated with mycoplasma. Furthermore, an IC confirmed the epithelial character of the cells, as all pbMEC cultures were positively stained with the monoclonal mouse anti-cytokeratin pan antibody clone C-11 and showed the typical cobblestone-like morphology (Fig 2).

Effects of the immune stimulation on pbMEC in ketotic and non-ketotic state

The ketone body BHBA itself had only slight effects on the gene expression of CCL20, CXCL8, IL8, TNFα, LYZ1, TAP, C3, RELA and LF (Table 2). However, due to a normal t-test, a distinct up-regulation in the gene expression of the genes coding for CCL20, LYZ1 and TNFα, and a significant induction of gene expression for the genes coding for CXCL8, IL6, RELA and TAP, could be detected (Table 2). Only the gene coding for LF showed a significant down-regulation 30 h after treatment start (Table 2). All other genes evaluated in this study were not altered by the treatment with 3 mM BHBA itself (Table 2). Treatment with the gram-negative pathogen

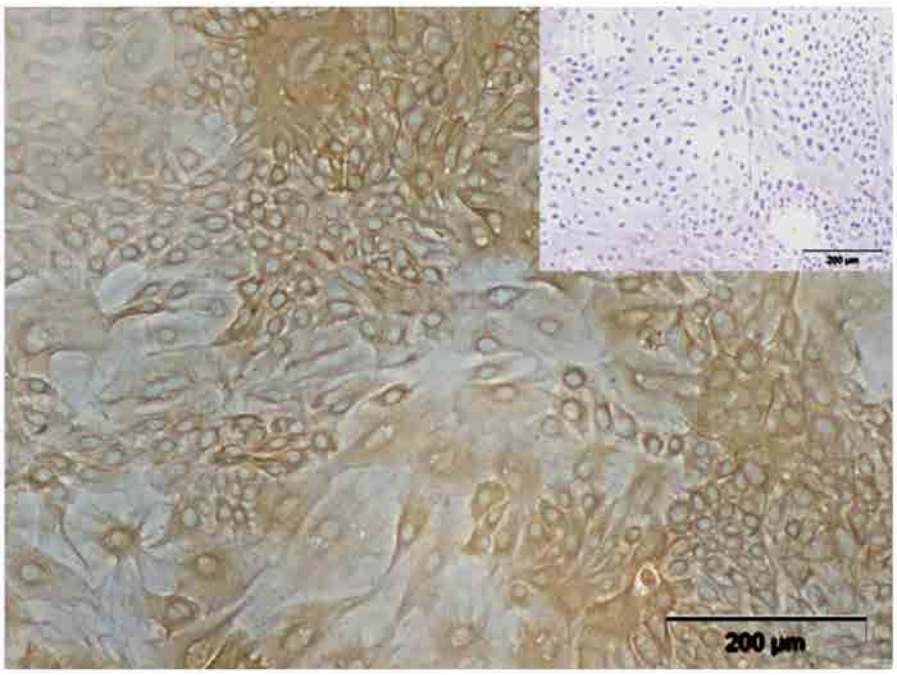

Fig 2. IC of pbMEC. The typical cobblestone-like morphology and the epithelial character were confirmed, by the positive cytokeratin staining, the insert shows the negative control. [Leica light microscope, magnification x200].

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E. coli, however, had a great influence on the gene expression of nearly all immune genes investigated in this study. In both cases, when cells were only treated with *E. coli* or co-stimulated with *E. coli* and 3 mM BHBA, a strong induction of the immune gene expression could be detected. Regarding the gene expression of genes coding for components of the TLR pathway, no differential gene expression could be detected for MyD88 when compared to the untreated control (Table 2). Only the TLR4 gene expression showed a significant up-regulation 30 h after treatment start (Table 2). The gene expression of RELA, the NF-kappa-B p65 subunit, was significantly up-regulated when the pbMEC were only treated with *E. coli* for 6 h. The genes coding for the chemokines, however, were strongly induced by the *E. coli* treatment and co-stimulation with *E. coli* and 3 mM BHBA (Table 2). Therefore, CCL2 and CCL20 gene expression was significantly up-regulated during the 6 h and the 30 h treatment time-point. By contrast, the gene expression of CXCL8 was also strongly induced, however, a significant increase in the gene expression could only be detected 30 h post-infection. The gene expression of the two pro-inflammatory cytokines IL6 and TNFα was also highly significantly induced 30 h post infection in the *E. coli* treated and in the co-stimulated samples (Table 2). Regarding the antimicrobial peptides LF, LYZ1 and TAP, a distinct as well as significant up-regulation of the gene expression could be detected through the *E. coli* treatment and co-stimulation (Table 2). Stimulation with the mastitis pathogen *E. coli* also induced a significant up-regulation of the gene expression of the gene coding for the acute phase protein SAA3 after 6 h and in the co-stimulatory experiment 30 h after treatment start (Table 2). The danger associated molecular pattern molecule S100A9 was distinctly and highly significant altered by the *E. coli* treatment and co-stimulation with *E. coli* and 3 mM BHBA. No effect of the treatment with *E. coli* or the co-stimulation with *E. coli* and 3 mM BHBA could be detected on the gene expression of the

Table 2. Fold changes in gene expression upon immune stimulation.

	Time-point											
	30h						54h					
	Treatment						Treatment					
Genes	BHBA[1]		*E.coli*[2]		BHBA + *E.coli*[3]		BHBA[4]		*E.coli*[5]		BHBA + *E.coli*[6]	
Chemokines												
CCL2												
Fold	0.89		7.56	***	6.29	**	1.16		94.72	***	58.12	***
SEM	0.10		2.00		2.12		0.26		46.64		27.19	
CCL20												
Fold	1.39		49.49	**	46.16	**	1.86	+	205.78	***	185.22	***
SEM	0.34		21.32		23.99		0.30		89.43		77.31	
CXCL8												
Fold	2.12	*	10.13		7.66		1.92	*	29.29	***	30.67	***
SEM	0.44		2.16		1.54		0.49		8.68		9.43	
Inflammatory cytokines												
IL6												
Fold	1.38	*	2.71		2.86		1.52		5.72	***	6.07	***
SEM	0.14		0.49		0.61		0.28		1.99		1.86	
TNFα												
Fold	1.24		17.62		17.04		1.85	+	49.68	***	44.36	***
SEM	0.22		6.06		7.84		0.34		17.76		15.32	
Antimicrobial peptides												
LF												
Fold	1.14		5.49	**	2.39	+	0.71	*	9.59	***	4.99	**
SEM	0.23		1.85		0.68		0.12		4.32		2.04	
LYZ1												
Fold	1.13		2.79	+	2.21	*	2.22	+	25.92	**	16.59	**
SEM	0.18		0.76		0.59		0.55		9.19		6.75	
TAP												
Fold	1.22		3.70	+	3.39	*	2.59	*	66.83	**	38.11	**
SEM	0.44		1.12		1.26		0.59		34.84		15.95	
Acute phase proteins												
SAA3												
Fold	0.94		35.19	***	31.38	**	1.83		2302.14		738.94	***
SEM	0.19		11.15		13.04		0.35		1183.19		361.50	
Complement system												
C3												
Fold	1.02		3.12	**	2.04		0.77	+	10.54	**	3.43	***
SEM	0.16		0.60		0.62		0.16		4.72		0.84	
TLR signaling												
TLR4												
Fold	1.05		1.36	*	1.04		0.90		1.17		1.20	
SEM	0.09		0.14		0.11		0.10		0.11		0.11	
MYD88												
Fold	1.07		1.08		1.02		0.98		1.01		0.99	
SEM	0.08		0.09		0.10		0.09		0.10		0.10	

(*Continued*)

Table 2. (*Continued*)

	Time-point									
	30h					54h				
	Treatment					Treatment				
Genes	**BHBA[1]**		***E.coli*[2]**		**BHBA + *E.coli*[3]**	**BHBA[4]**	***E.coli*[5]**		**BHBA + *E.coli*[6]**	
RELA										
Fold	1.37	**	1.32	*	1.14	1.17	1.42		1.33	
SEM	0.11		0.15		0.12	0.23	0.23		0.32	
Others										
MX2										
Fold	1.81		3.89	**	5.08	*	2.15	13.42	***	8.20	***
SEM	0.39		0.79		1.66		0.88	4.16		2.50	
S100A9										
Fold	1.28		3.88	+	2.94	*	1.43	11.94	**	9.73	**
SEM	0.46		1.52		1.00		0.24	4.33		4.07	
Lactogenesis										
STAT5										
Fold	1.64		1.55		1.84		1.40	1.07		1.25	
SEM	0.48		0.63		0.73		0.26	0.14		0.14	
CSN3										
Fold	1.05		1.40		1.64		1.31	2.36		1.46	
SEM	0.18		0.35		0.57		0.24	0.69		0.37	

The treatment effect was statistically evaluated using a paired t-test.

[1] 30h BHBA treatment

[2] 6h *E. coli* treatment

[3] 30h BHBA and 6h *E. coli* treatment

[4] 54h BHBA treatment

[5] 30h *E. coli* treatment

[6] 54h BHBA and 30h *E. coli* treatment

[+] Trend $0.1 \leq p \leq 0.05$

* $p \leq 0.05$

** $p \leq 0.01$

*** $p \leq 0.001$

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gene responsible for the induction of lactogenesis, STAT5, and for the gene coding for the milk protein κ-casein (CSN3) (Table 2).

Differences in the gene expression of pbMEC through the co-stimulation of pbMEC with *E.coli* and 3 mM BHBA or only *E. coli*

The direct comparison of the *E. coli* treatment and the co-stimulation with 3 mM BHBA and *E. coli* indicated a significant down-regulation of the gene expression within the pbMEC that received challenge medium supplemented with *E. coli* and 3 mM BHBA. The p-values were evaluated using a normal t-test that compared the dCq values of the *E. coli* treated pbMEC directly to the dCq values of the co-stimulated pbMEC (Fig 3). Within the genes coding for the chemokines, the gene coding for CCL2 showed a distinct down-regulation 6 h post-infection.

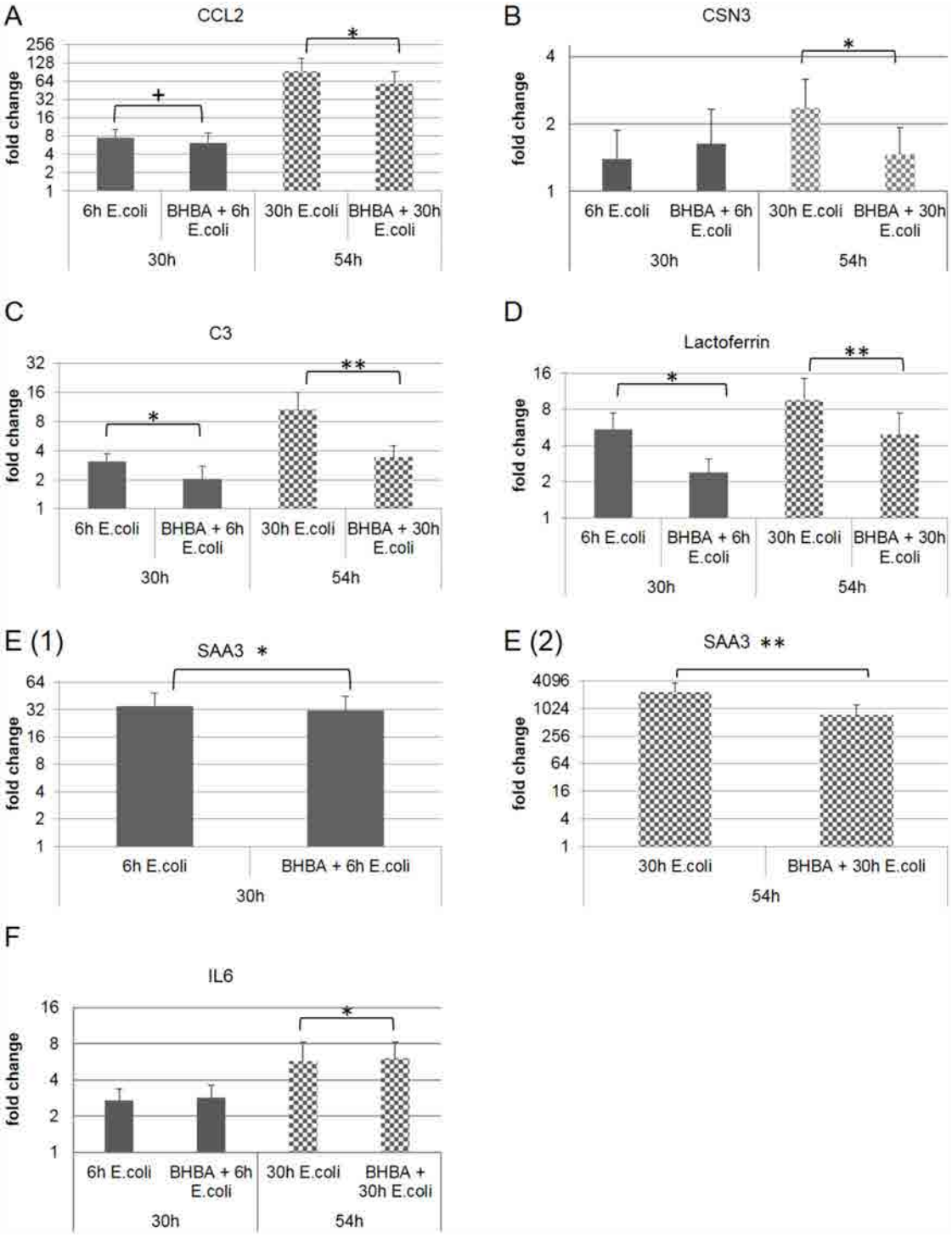

Fig 3. Attenuation of the gene expression through the co-stimulation of pbMEC with *E. coli* and 3 mM BHBA. Fold change of **(A)** the chemokine CCL2, **(B)** the milk protein CSN3, **(C)** the complement component C3, **(D)** the antimicrobial peptide LF, **(E 1–2)** the acute phase protein SAA3, and **(F)** the inflammatory cytokine IL6. Significant changes in the gene expression between the *E. coli* treated pbMEC and the pbMEC co-stimulated with *E. coli* and 3 mM BHBA are indicated by stars: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

doi:10.1371/journal.pone.0157774.g003

There was also a significant attenuation of gene expression 30 h after treatment start in the co-stimulatory group (Fig 3A). The gene expression of IL6 was the only one that was significantly induced due to the co-stimulation 30 h post-infection when directly compared to pbMEC, which were only treated with *E. coli* for 30 h (Fig 3F). The genes coding for the antimicrobial peptide LF, the acute phase protein SAA3, and the complement factor C3 showed the same trend when the p-values were calculated (Fig 3C, 3D, 3E(1) and 3E(2)). All three genes showed a significant and a highly significant down-regulation of the gene expression when pbMEC were treated with both *E. coli* and 3 mM BHBA (Fig 3C, 3D, 3E(1) and 3E(2)). Even if no changes in the gene expression of the gene coding for the milk protein CSN3 could be detected through the *E. coli* stimuli itself, the gene expression of CSN3 in the co-stimulatory experiment was significantly down-regulated 30 h after treatment start compared to the treatment with only *E. coli* (Fig 3B).

Correlation of RT-qPCR data with protein data obtained from ELISAs of the cell culture supernatants

For LF and CCL2, the RT-qPCR results could also be validated with an ELISA measurement. The LF content in cell culture supernatants could be detected using the competitive LF-ELISA, which is routinely used at our institute (S3 Table). The LF content in the cell culture supernatants increased upon treatment of the pbMEC with the mastitis pathogen *E. coli*. However, the amount of secreted LF decreased significantly 6 h and 30 h after treatment start within the cell culture supernatant of the co-stimulated pbMEC (Fig 4B). The same trend could be seen when the RT-qPCR data of CCL2 (Fig 4C, S4 Table) was compared to the protein data obtained by the bovine CCL2 VetSet™ ELISA Development Kit. The amount of secreted CCL2 increased upon stimulation with the gram-negative pathogen *E. coli*, but decreased distinctly and significantly in the cell culture supernatant of the co-stimulated pbMEC (Fig 4D), thus confirming the results obtained by RT-qPCR.

Discussion

Dairy cows are often challenged by mastitis-inducing gram-negative pathogens, like *E. coli*. Despite several physiological defense barriers of the bovine mammary gland, those pathogens are likely to enter the blood circulation via pbMEC, which are part of the blood-mammary gland barrier [20,21]. Therefore, it has already been shown in previous studies that pbMEC are mandatory for the induction of the innate immune response of the bovine mammary gland [22]. It has already been proven that pbMEC bear germline-encoded TLR on their cell surface and are hence able to directly respond to invading pathogens, due to the recognition of so-called pathogen-associated molecular patterns (PAMP) [11,22]. It is known that innate leukocytes are one of the most essential cellular components of innate immunity but bovine innate leukocytes—and especially phagocytic neutrophils and macrophages—are not directly attracted to pathogens or bacterial products [23,24]. Therefore, the induction of the TLR signaling pathway that leads to the activation of the pleiotropic transcription factor NFκB and hence to the induction of the gene expression of genes coding for intracellular signaling molecules and chemotactic molecules, like pro-inflammatory cytokines, chemokines, components of the complement system and acute phase proteins, is crucial for the recruitment of leukocytes to the site of inflammation [25,26]. As several previous studies [9,11,22,26] have already shown that bacterial challenge of pbMEC *in vitro* resulted in differential gene expression of the above-mentioned innate immune gene classes, we also focused on those gene expression profiles. We wanted to elucidate whether the treatment with *E. coli* and/or BHBA could interfere with the gene expression of components of the MYD88 dependent TLR4 signaling cascade that is

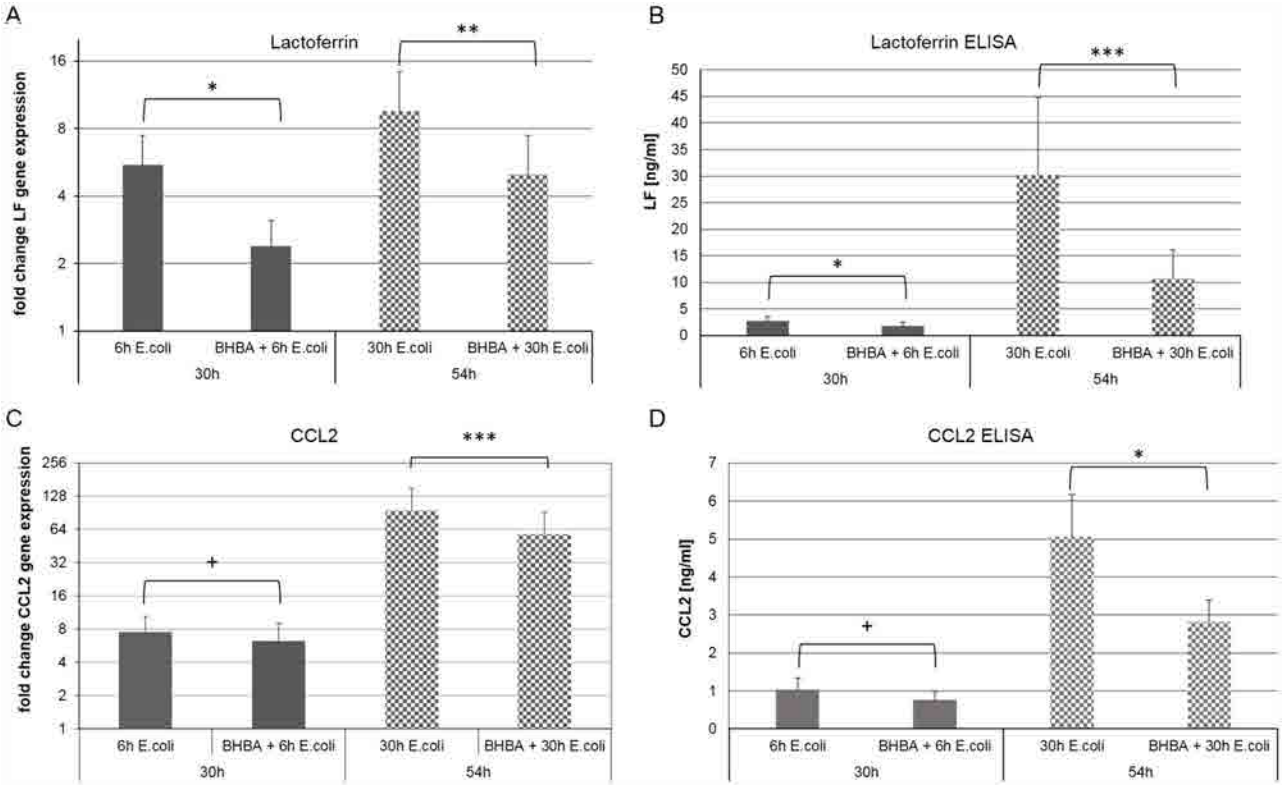

Fig 4. Comparison of the RT-qPCR data of LF and CCL2 with the LF and CCL2 content in pbMEC cell culture supernatants. The fold changes of the LF gene expression (A) indicated a significant down-regulation of LF gene expression when pbMEC where co-stimulated with *E. coli* and 3 mM BHBA. The same effect could be detected within the competitive LF ELISA (B) of pbMEC cell culture supernatants. The amount of secreted LF decreased significantly in case of the co-stimulation. The distinct and significant down-regulation in CCL2 gene expression (C) could also be confirmed by the results of the bovine CCL2 VetSet™ ELISA Kit (D). The CCL2 gene expression and the amount of secreted protein decreased distinctly as well as significantly within the co-stimulatory group. Significant changes: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, distinct changes: + $0.1 \leq p \leq 0.05$.

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responsible for the proper activation of the essential p65 NFκB transcription factor subunit RELA. Furthermore, as it is known that the danger associated molecular pattern molecule S100A9 and chemokines, like CCL2, CCL20 and especially CXCL8, are important mediators of the inflammatory response, we also investigated changes in the gene expression profile of components of those innate immune gene families [9,22,25,27,28]. Especially CXCL8 is known to be essential for the recruitment of leukocytes into the bovine mammary gland [25,29]. Furthermore, CCL2 as well as CXCL8 are discussed to induce locally restricted polarized diapedesis of neutrophils across epithelia and endothelia [9,30]. Furthermore, S100A9 is also discussed to contribute to the recruitment of leucocytes [28]. Despite of the chemokines, we were also interested in changes in the gene expression of genes coding for selected pro-inflammatory cytokines, like IL6 and TNFα. For both, IL6 and TNFα, it has been postulated that they act as acute phase cytokines and therefore are of great importance to the recruitment and activation of neutrophils [31,32]. We further focused on the gene expression of innate immune genes coding for components of the innate humoral defense line. Among them were genes coding for the complement component C3, the acute phase protein SAA3, several β-defensins (HP, TAP, LAP) and antimicrobial peptides (LYZ1, LF). Those genes are normally constitutively expressed, even if no bacterial stimuli is present but can be significantly induced upon pathogen recognition [22,25]. Those humoral defense molecules are more likely to directly act on the pathogen,

while also contributing to the chemotactic gradient like the other classes of innate immune genes presented in this paragraph. It has been shown that LF, LYZ1 TAP, LAP and HP exhibit bacteriostatic and bactericidal properties [11,25]. Furthermore, the proper functionality of the pbMEC used in this study was monitored by the evaluation of the gene expression profile of genes involved in milk protein synthesis (STAT5A, β-casein, κ-casein).

The innate immune response of pbMEC is strongly induced by *E. coli*

Innate pathogen recognition is mainly mediated by the toll-like receptor pathway. In the present study, TLR4 and the signal transducer MYD88 were analyzed. Both were expressed in our pbMEC proving their ability to perceive invading pathogens. However, the expression of TLR4 and MYD88 was largely unaffected by the *E. coli* challenge, although there was a pronounced inflammatory response. Similarly, [10] found no effect of LPS on TLR4 expression in pbMEC and concluded that pbMEC contain a fully functional and constitutively active TLR signaling pathway, which does not need to be up-regulated upon pathogen invasion. It seems that the continuously expressed toll-like receptors are sufficient to induce an adequate inflammatory response by the activation of signaling cascades that lead to the production of cytokines and chemokines [10]. RELA, a subunit of NF-κB, was also not differentially expressed in *E. coli* treated pbMEC. This can be explained by its role as a signal transducer, which needs to be constitutively expressed in order to response immediately upon pathogen invasion.

The genes coding for chemokines like CCL2, CCL20 and CXCL8 were significantly up-regulated in pbMEC stimulated with *E. coli*. The same effect could be found for the pro-inflammatory cytokines TNFα and IL6. This confirmed the contribution of pbMEC to the innate immune system, as they exert immune modulatory functions. Following pathogen invasion, pbMEC secrete signaling molecules like cytokines and chemokines to induce an inflammatory response and to recruit immune effector cells like monocytes and lymphocytes to the site of infection [33]. Our results are consistent with other studies which already mentioned the major role of pbMEC in triggering the innate immune response of the bovine mammary gland [9,13,22]. It has been shown [34] that cell culture supernatants of *E. coli* treated pbMEC enhanced the chemotactic activity of leukocytes, which is most likely a direct effect of the induced chemokine production.

Exposure to *E. coli* activated the gene expression of genes coding for the antimicrobial peptides LF, LYZ1 and TAP. The acute phase protein SAA3 and the complement component C3 were also induced by *E. coli*. This is in accordance with published data [12], where *E. coli* also caused a strong induction of the gene expression of those defense molecules. The antimicrobial peptide LF is known to inhibit the iron-dependent bacterial growth by binding free iron in milk [25] and it is also able to directly kill bacteria by disrupting their cell membrane [35], whereas LYZ1 destroys the outer membrane of gram-negative bacteria, like *E. coli*, by cleaving peptidoglycans [36]. The β-defensin TAP and the acute phase protein SAA3 exert antimicrobial functions as well. SAA3, for instance, facilitates phagocytosis by opsonizing gram-negative bacteria [37]. Activation of the complement cascade is another defense mechanism, as it results in the formation of the membrane attack complex that disrupts the bacterial cell membrane. Cleavage products of complement component C3 exert antimicrobial activity and are able to opsonize microbes, thereby facilitating phagocytosis [38]. However, the most up-regulated gene upon *E. coli* treatment was SAA3. The strong induction of the acute phase protein upon pathogen invasion has also been shown in a study of [9,22,27]. SAA3 has already been proposed as a potential biomarker for mastitis [27]. Furthermore, the gene coding for the danger associated molecular pattern molecule S100A9 tended to be up-regulated by the *E. coli* treatment, but fold changes were not significant. S100A9 belongs to the S100 calgranulins, a group

of anti-infective and anti-inflammatory proteins. Their functions include recruitment of leukocytes, antimicrobial activity and oxidant scavenging [28].

The presence of BHBA attenuates the innate immune response of pbMEC to *E. coli*

The present study is one of the first studies investigating the exclusive effect of the ketone body BHBA on the innate immune response of pbMEC to *E. coli in vitro*. Prior to the immune challenge with the mastitis pathogen *E. coli*, pbMEC were accustomed to the metabolic state of ketosis by exposure to 3 mM BHBA for 24 h. During the immune challenge, the exposure to BHBA was continued. This approach was chosen because we wanted to analyze the influence of an already existing ketosis on the innate immune response of pbMEC. It has been shown in epidemiological studies that there is indeed an association between ketosis and an increased risk of mastitis [39–41]. It is not yet clear by which mechanism ketosis interferes with the immune defense of the bovine mammary gland. However, elevated BHBA levels are considered to have inhibiting effects on immune cells [1]. Our *in vitro* approach aimed to investigate whether this also applies to the innate immune response of bovine mammary epithelial cells. In the present study, 4 of the 15 analyzed innate immune genes showed a significant down-regulation in pbMEC co-stimulated with *E. coli* and 3 mM BHBA. Those were genes coding for the chemokine CCL2, the acute phase protein SAA3, the antimicrobial peptide LF and the complement component C3. ELISA measurements for LF and CCL2 confirmed the finding of the RT-qPCR (Fig 4A, S3 and S4 Tables). Therefore, protein biosynthesis followed the same trend as gene expression when the ketone body BHBA was present in the cell culture supernatant. The only gene that showed a significant induction in gene expression in the co-stimulatory experiment was the pro-inflammatory cytokine IL6. Interestingly, the innate immune genes influenced by BHBA belonged to different gene families, and genes from almost every analyzed gene family were affected by the BHBA treatment in the co-stimulatory approach. This leads to the conclusion that the immunosuppressive effect of BHBA results from an overall suppression of the immune response and is not restricted to a single group of immune response genes. In general, the immunosuppressive effect of BHBA was more pronounced after 54 h, indicating that especially long-term ketosis had a negative effect on the gene expression of innate immune genes. These findings are in agreement with a study on the influence of BHBA on the chemotactic functions of leukocytes [42]. The authors observed an impairment of the chemotactic capacity of leukocytes from naturally-occurring ketotic cows.

We could show that stimulation of pbMEC with the ketone body BHBA not only attenuates the gene expression of the genes coding for CCL2 and LF, but also their protein biosynthesis and hence protein secretion into the cell culture medium. The reduced concentrations of the chemotactic protein CCL2 and the humoral response protein LF, are very likely to lead to a reduced lymphocyte chemotaxis towards the bovine mammary gland, leading to greater mastitis susceptibility in cows.

A previously published study investigated the innate defense capability of pbMEC, which were isolated after an induced negative energy balance *in vivo*, and subsequently challenged with *E. coli in vitro* [13]. In this study, the combination of *E. coli* treatment and the dietary energy deficit induced generally higher expression levels compared to the control-fed group. This is contrary to our findings, as we observed a down-regulation of the gene expression by co-stimulation of pbMEC with *E. coli* and 3 mM BHBA. However, the restriction feeding in the above-mentioned experiment did not induce ketosis as proven by the small change in plasma BHBA concentrations of restricted cows compared to the control cows [13,25,43]. Additionally, pbMEC were cultivated in a common proliferation medium without BHBA, so that they

were likely to regenerate from the previously experienced NEB during cultivation. In contrast, our *in vitro* approach guaranteed a constant concentration of 3 mM BHBA during the whole experiment, as the BHBA containing challenge medium was renewed every day.

Conclusion

The present *in vitro* study aimed to investigate the influence of ketosis on the innate immune response of the bovine mammary gland to mastitis. Therefore, pbMEC isolated from milk were challenged with the ketone body BHBA and the mastitis pathogen *E. coli*. Subsequently, the changes in gene expression of innate immune genes were determined via RT-qPCR. An explicit advantage of the present *in vitro* approach was the fact that it enabled the investigation of the specific effect of BHBA on the innate immune response. Furthermore, experimental conditions like BHBA concentration could be exactly defined. Exposure of pbMEC to the mastitis pathogen *E. coli* strongly induced the gene expression of several genes related to the innate immune response like cytokines, chemokines, antimicrobial peptides, the complement factors and the acute phase proteins. This proves the importance of pbMEC in the induction and promotion of the innate immune response of the bovine mammary gland. Besides their ability to directly combat invading pathogens by secreting various defense molecules, pbMEC are also responsible for the recruitment of immune cells to the site of infection. The results of our study indicated that increased BHBA concentrations may be at least partially responsible for the higher mastitis susceptibility of dairy cows in early lactation. The gene expression of CCL2, SAA3, LF and C3 was significantly down-regulated in pbMEC co-stimulated with 3 mM BHBA and *E. coli* compared to the gene expression of pbMEC stimulated only with *E. coli*. Hence, the immunosuppressive effect of BHBA might be attributed to an overall suppression of immune response genes. Long-term elevated BHBA concentrations, in particular, seem to have a negative effect on the innate immune response.

Supporting Information

S1 Table. Fold change data for Table 2.
(XLSX)

S2 Table. Differences in the gene expression of pbMEC treated with *E. coli* or with *E. coli* and 3 mM BHBA.
(DOCX)

S3 Table. LF ELISA data for generation of Fig 4. (A) Data for 6 h *E. coli* treatment and 30 h of co-stimulation. **(B)** Data for 30 h *E. coli* treatment and 54 h of co-stimulation. **(C)** Standard curve.
(XLSX)

S4 Table. CCL2 ELISA data for generation of Fig 4. (A) Data for 6 h *E. coli* treatment and 30 h of co-stimulation. **(B)** Data for 30 h *E. coli* treatment and 54 h of co-stimulation. **(C)** Standard curve.
(XLSX)

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Author Contributions

Conceived and designed the experiments: MWP HK MH. Performed the experiments: MH CF. Analyzed the data: MH CF. Wrote the paper: MH CF.

References

1. Suriyasathaporn W, Heuer C, Noordhuizen-Stassen EN, Schukken YH. Hyperketonemia and the impairment of udder defense: a review. Vet Res. 2000; 31: 397–412. doi: 10.1051/vetres:2000128 PMID: 10958241
2. Dirksen G, Baumgartner W. Innere Medizin und Chirurgie des Rindes. 5th ed. Stuttgart: Parey; 2006.
3. McArt JAA, Nydam DV, Oetzel GR. Epidemiology of subclinical ketosis in early lactation dairy cattle. J Dairy Sci. 2012; 95: 5056–5066. doi: 10.3168/jds.2012-5443 PMID: 22916909
4. Schultz LH. Ketosis in dairy cattle. J Dairy Sci. 1968; 51: 1133–1140. doi: 10.3168/jds.S0022-0302(68)87141-3 PMID: 5690270
5. Baird GD. Primary ketosis in the high-producing dairy cow: clinical and subclinical disorders, treatment, prevention, and outlook. J Dairy Sci. 1982; 65: 1–10. doi: 10.3168/jds.S0022-0302(82)82146-2 PMID: 7042782
6. Andersson L. Subclinical ketosis in dairy cows. Vet Clin North Am Food Anim Pract. 1988; 4: 233–251. PMID: 3061609
7. Duffield TF, Lissemore KD, McBride BW, Leslie KE. Impact of hyperketonemia in early lactation dairy cows on health and production. J Dairy Sci. 2009; 92: 571–580. doi: 10.3168/jds.2008-1507 PMID: 19164667
8. Seegers H, Fourichon C, Beaudeau F. Production effects related to mastitis and mastitis economics in dairy cattle herds. Vet Res. 2003; 34: 475–491. doi: 10.1051/vetres:2003027 PMID: 14556691
9. Günther J, Koczan D, Yang W, Nürnberg G, Repsilber D, Schuberth HJ, et al. Assessment of the immune capacity of mammary epithelial cells: comparison with mammary tissue after challenge with Escherichia coli. Vet Res. 2009; 40: 31. doi: 10.1051/vetres/2009014 PMID: 19321125
10. Strandberg Y, Gray C, Vuocolo T, Donaldson L, Broadway M, Tellam R. Lipopolysaccharide and lipoteichoic acid induce different innate immune responses in bovine mammary epithelial cells. Cytokine. 2005; 31: 72–86. doi: 10.1016/j.cyto.2005.02.010 PMID: 15882946
11. Griesbeck-Zilch B, Meyer H, Kühn C, Schwerin M, Wellnitz O. Staphylococcus aureus and Escherichia coli cause deviating expression profiles of cytokines and lactoferrin messenger ribonucleic acid in mammary epithelial cells. J Dairy Sci. 2008; 91: 2215–2224. doi: 10.3168/jds.2007-0752 PMID: 18487644
12. Sorg D, Fandrey E, Frölich K, Meyer H, Kliem H. Mammary immunity of White Park and Highland cattle compared with Brown Swiss and Red Holstein. Anim Genet Resour. 2013; 52: 91–104.
13. Danowski K, Gross JJ, Meyer HHD, Kliem H. Effects of induced energy deficiency on lactoferrin concentration in milk and the lactoferrin reaction of primary bovine mammary epithelial cells in vitro. J Anim Physiol Anim Nutr (Berl). 2013; 97: 647–655. doi: 10.1111/j.1439-0396.2012.01305.x
14. Sorg D, Potzel A, Beck M, Meyer HHD, Viturro E, Kliem H. Effects of cell culture techniques on gene expression and cholesterol efflux in primary bovine mammary epithelial cells derived from milk and tissue. In Vitro Cell Dev Biol-Animal. 2012; 48: 550–553. doi: 10.1007/s11626-012-9544-6
15. Koressaar T, Remm M. Enhancements and modifications of primer design program Primer3. Bioinformatics. 2007; 23: 1289–1291. doi: 10.1093/bioinformatics/btm091 PMID: 17379693
16. Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, et al. Primer3—new capabilities and interfaces. Nucleic Acids Res. 2012; 40: e115. doi: 10.1093/nar/gks596 PMID: 22730293
17. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods. 2001; 25: 402–408.
18. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE Guidelines: Minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009; 55: 611–622. doi: 10.1373/clinchem.2008.112797 PMID: 19246619
19. Prakash BS, Meyer HH, Schallenberger E, van de Wiel DF. Development of a sensitive enzymeimmunoassay (EIA) for progesterone determination in unextracted bovine plasma using the second antibody technique. J Steroid Biochem. 1987; 28: 623–627. PMID: 3320573
20. Wellnitz O, Zbinden C, Huang X, Bruckmaier RM. Short communication: Differential loss of bovine mammary epithelial barrier integrity in response to lipopolysaccharide and lipoteichoic acid. J Dairy Sci. 2016. doi: 10.3168/jds.2016-10927

21. Nguyen DA, Neville MC. Tight junction regulation in the mammary gland. J Mammary Gland Biol Neoplasia. 1998; 3: 233–246. PMID: 10819511

22. Sorg D, Danowski K, Korenkova V, Rusnakova V, Küffner R, Zimmer R, et al. Microfluidic high-throughput RT-qPCR measurements of the immune response of primary bovine mammary epithelial cells cultured from milk to mastitis pathogens. Animal. 2013; 7: 799–805. doi: 10.1017/S1751731112002315 PMID: 23228824

23. Gray GD, Knight KA, Nelson RD, Herron MJ. Chemotactic requirements of bovine leukocytes. Am J Vet Res. 1982; 43: 757–759. PMID: 6283962

24. Carroll EJ, Mueller R, Panico L. Chemotactic factors for bovine leukocytes. Am J Vet Res. 1982; 43: 1661–1664. PMID: 6816108

25. Hunziker W, Kraehenbuhl JP. Epithelial transcytosis of immunoglobulins. J Mammary Gland Biol Neoplasia. 1998: 287–302. PMID: 10819515

26. Rainard P, Riollet C. Innate immunity of the bovine mammary gland. Vet Res. 2006; 37: 369–400. doi: 10.1051/vetres:2006007 PMID: 16611554

27. Lutzow YC, Donaldson L, Gray CP, Vuocolo T, Pearson RD, Reverter A, et al. Identification of immune genes and proteins involved in the response of bovine mammary tissue to Staphylococcus aureus infection. BMC Vet Res. 2008; 4: 18. doi: 10.1186/1746-6148-4-18 PMID: 18513449

28. Wellnitz O, Reith P, Haas SC, Meyer HH. Immune relevant gene expression of mammary epithelial cells and their influence on leukocyte chemotaxis in response to different mastitis pathogens. Vet Med. 2006; 51: 125–132.

29. Kutila T, Pyorala S, Saloniemi H, Kaartinen L. Antibacterial effect of bovine lactoferrin against udder pathogens. Acta Vet Scand. 2003; 44: 35–42. doi: 10.1186/1751-0147-44-35 PMID: 14650542

30. Molenaar AJ, Harris DP, Rajan GH, Pearson ML, Callaghan MR, Sommer L, et al. The acute-phase protein serum amyloid A3 is expressed in the bovine mammary gland and plays a role in host defence. Biomarkers. 2009; 14: 26–37. doi: 10.1080/13547500902730714 PMID: 19283521

31. Hsu K, Champaiboon C, Guenther BD, Sorenson BS, Khammanivong A, Ross KF, et al. Anti-infective protective properties of S100 calgranulins. Antiinflamm Antiallergy Agents Med Chem. 2009; 8: 290–305. doi: 10.2174/187152309789838975 PMID: 20523765

32. Sordillo LM, Shafer-Weaver K, DeRosa D. Immunobiology of the mammary gland. J Dairy Sci. 1997; 80: 1851–1865. doi: 10.3168/jds.S0022-0302(97)76121-6 PMID: 9276826

33. Wheeler TT, Smolenski GA, Harris DP, Gupta SK, Haigh BJ, Broadhurst MK, et al. Host-defence-related proteins in cows' milk. Animal. 2012; 6: 415–422. doi: 10.1017/S1751731111002151 PMID: 22436220

34. Zipfel PF, Skerka C. Complement regulators and inhibitory proteins. Nat Rev Immunol. 2009; 9: 729–740. doi: 10.1038/nri2620 PMID: 19730437

35. Boudjellab N, Chan-Tang HS, Zhao X. Bovine interleukin-1 expression by cultured mammary epithelial cells (MAC-T) and its involvement in the release of MAC-T derived interleukin-8. Comp Biochem Physiol. Part A Mol Integr Physiol. 2000; 127: 191–199.

36. Krüger S, Brandt E, Klinger M, Kreft B. Interleukin-8 secretion of cortical tubular epithelial cells is directed to the basolateral environment and is not enhanced by apical exposure to Escherichia coli. Infect. Immun. 2000; 68: 328–334. PMID: 10603405

37. Alluwaimi AM. The cytokines of bovine mammary gland: prospects for diagnosis and therapy. Res Vet Sci. 2004; 77: 211–222. doi: 10.1016/j.rvsc.2004.04.006 PMID: 15276772

38. Pfaffl MW, Wittmann SL, Meyer HHD, Bruckmaier RM. Gene expression of immunologically important factors in blood cells, milk cells, and mammary tissue of cows. J Dairy Sci. 2003; 86: 538–545. doi: 10.3168/jds.S0022-0302(03)73632-7 PMID: 12647960

39. Correa MT, Erb H, Scarlett J. Path analysis for seven postpartum disorders of Holstein cows. J Dairy Sci. 1993; 76: 1305–1312. doi: 10.3168/jds.S0022-0302(93)77461-5 PMID: 8505422

40. Erb HN, Grohn YT. Epidemiology of metabolic disorders in the periparturient dairy cow. J Dairy Sci. 1988; 71: 2557–2571. doi: 10.3168/jds.S0022-0302(88)79845-8 PMID: 3053813

41. Oltenacu PA, Ekesbo I. Epidemiological study of clinical mastitis in dairy cattle. Vet Res. 1994; 25: 208–212. PMID: 8038786

42. Suriyasathaporn W, Daemen AJ, Noordhuizen-Stassen EN, Dieleman SJ, Nielen M, Schukken YH. Beta-hydroxybutyrate levels in peripheral blood and ketone bodies supplemented in culture media affect the in vitro chemotaxis of bovine leukocytes. Vet Immunol Immunopathol. 1999; 68: 177–186.

43. Gross J, van Dorland HA, Bruckmaier RM, Schwarz FJ. Performance and metabolic profile of dairy cows during a lactational and deliberately induced negative energy balance with subsequent realimentation. J Dairy Sci. 2011; 94: 1820–1830. doi: 10.3168/jds.2010-3707 PMID: 21426971