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Mastitis severity induced by two *Streptococcus uberis* strains is reflected by the mammary immune response *in vitro*

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Summary

Streptococcus uberis is the most common environmental mastitis pathogen causing udder inflammations of different severities in dairy cows. The aim of the study was to investigate if the different clinical outcome of mastitis induced by different strains of *S. uberis* can be reflected in the mammary immune response. Mammary epithelial cells and somatic milk cells were treated with heat inactivated and living *S. uberis* of strain A and strain B *in vitro*. Strain A was repeatedly isolated from a chronically infected quarter during 8 months, and persisted in the quarter despite antibiotic treatment. Strain B caused an acute clinical mastitis and was not further isolated after a single antibiotic treatment. Treatment with Strain B induced a more pronounced increase of mRNA-expression of various immune factors (interleukin-8, interleukin-1β, RANTES, and lactoferrin) in mammary epithelial cells than strain A. In contrast to mammary epithelial cells the response of removed somatic milk cells showed no differences between the stimulation with two *S. uberis* strains. Tumor necrosis factor-alpha mRNA expression was not differently induced by the two strains. In conclusion, the characteristics of different severities of mastitis that are induced by different *S. uberis* strains *in vivo* can also be reflected at the level of the immune response of the mammary gland *in vitro*.

Keywords: mastitis, *Streptococcus uberis*, mammary immunity, cell culture, milk

Der Mastitisverlauf, verursacht durch zwei *Streptococcus uberis* Stämme, spiegelt sich in der Immunantwort des Euters *in vitro* wieder

Streptococcus uberis ist ein häufiger Umweltkeim, der bei Milchkühen Mastitis mit unterschiedlich klinischem Verlauf hervorruft. In der vorliegenden Studie wurde untersucht, ob sich der Verlauf einer Mastitis durch unterschiedliche *S. uberis* Stämme hervorgerufen in der Immunantwort des Euters auf diese Stämme wiederspiegelt. Die Reaktion von Milchdrüsenepithelzellen und somatischen Milchzellen auf zwei *S. uberis* Stämme, wurde *in vitro* untersucht. Stamm A wurde innerhalb 8 Monaten wiederholt aus einem chronisch infizierten Euterviertel trotz Antibiotikabehandlung isoliert. Stamm B verursachte eine akute Mastitis und konnte nach einer einmaligen Antibiotikabehandlung nicht mehr isoliert werden. Die Behandlung mit Stamm B induzierte im Milchdrüsenepithel stärkere mRNS-Expressionen verschiedener Immunfaktoren (Interleukin-8, und -1β, RANTES, und Laktoferrin) als Stamm A. In Milchzellen wurden diese Faktoren nicht unterschiedlich induziert. Tumor-Nekrose Faktor-alpha zeigte keine unterschiedliche mRNS-Expression nach Stimulation mit beiden Stämmen. Die Ergebnisse dieser Studie zeigen, dass sich der verschiedene Krankheitsverlauf einer Mastitis, der durch unterschiedliche *S. uberis* Stämme hervorgerufen wird, in der Immunantwort des Euters *in vitro* wiederspiegeln kann.

Schlüsselwörter: Mastitis, *Steptococcus uberis,* Euterimmunität, Zellkultur, Milch

Introduction

Streptococcus uberis is known as a common environmental mastitis pathogen in cows causing udder inflammations of different severities, i.e. clinical or subclinical (Todhunter et al., 1995). Different strains of *S. uberis*, characterized by specific Multilocus Sequence Types (MLST), were associated with specific clinical manifesta-

tions (Tomita et al., 2008).The use of mammary epithelial cell cultures was shown to be a suitable method to study the immune response of the mammary gland to different stimuli (Wellnitz and Kerr, 2004) as these cells play an important role in the mammary immune response. Several studies showed pathogen-related responses in mRNA expression of pathogen-recognition factors, proinflammatory cytokines, chemokines, and lactoferrin by mammary epithelial cells (Swanson et al., 2009; Griesbeck-Zilch et al., 2008; Wellnitz et al., 2006). In addition to mammary epithelial cells the somatic cells in milk are of high importance in the mammary gland immune system. They are the effector cells who eliminate the pathogens and were also shown to release chemoattractants and cytokines after contact with a pathogen (Wittmann et al., 2002). Some important immune factors expressed by mammary epithelial cells and somatic milk cells are tumor necrosis factor alpha (TNFα), interleukin (IL) -1β, IL-8, RANTES (regulated upon activation, normal T-cell expressed and secreted), or lactoferrin (Lf) (Bannerman, 2009; Griesbeck-Zilch et al., 2008; Arnold, 2011). The aim of this study was to investigate if the different clinical characteristics of mastitis induced by the two *S. uberis* strains (strain A and strain B) is also reflected on the level of the immune response of mammary epithelial cells and somatic milk cells *in vitro*.

Material and Methods

Isolation and characterization of S. uberis strains

Two different *S. uberis* strains were isolated from different bovine mastitis cases: Strain A was isolated from a chronical, persistent mastitis and was repeatedly isolated in milk samples of the same quarter during 8 months despite intramammary antibiotic treatments. Strain B caused an acute clinical mastitis and could no more be isolated after antibiotic treatment. Both strains were isolated from bovine milk samples and analyzed using standard procedures according to the guidelines of the National Mastitis Council (NMC, 1999). Furthermore, the isolates were verified as *S. uberis* with API 20 Strept test kits (BioMérieux) and they were typed with the Multilocus sequence Typing method (MLST) (Coffey et al., 2006). Strain A belonged to a predominant MLST type which was found in several cows on the same farm. Strain B MLST type was isolated only from one cow. In contrast to strain A, strain B showed a mucoid appearance on the blood agar plate which is a sign for hyaluronic acid production. Until further processing the isolates were transferred to skimmed milk and kept at –20 °C.

Preparation of bacteria

Bacteria for the challenge were prepared as follows: aliquots of 0.01 ml were streaked on Columbia agar plate containing 5 % sheep blood (COS, BioMérieux) and incubated at 37 °C for 24 h. One colony was transferred to 50ml Brain-Heart-Broth and incubated at 37 °C for 14 h. Bacteria grew up to a concentration of approximately 10^8 colony forming units (cfu)/mL, as was confirmed by plate dilution method. The samples were centrifuged at 1750x g for 10 min. The supernatants were discarded and the pellets re-suspended in 20 ml phosphate buffered saline solution (PBS) and centrifuged again at 1750x g for 10 min. The pellets were re-suspended in 0.5 ml of PBS and stored at 4 °C or heat inactivated in a water bath at 68 °C for 45 min before storage.

Experiment 1: Treatment of mammary epithelial cells with heat inactivated *S. uberis*

Primary cell cultures of mammary gland epithelia of one Swiss Red Holstein dairy cow (5th lactation) with a clinically healthy udder (somatic cell count [SCC] of 114×10^3 cells/ml) was developed as previously described (Wellnitz and Kerr, 2004). In 6-wells tissue culture plates (Falcon, Becton Dickinson) 300 x 10^3 cells per well were grown for 24 h in 2 ml DMEM/F2 medium (SIGMA-Aldrich) containing 5 % FBS, 1 % Penicillin-Streptomycin, and 1 % ITS (SIGMA-Aldrich). Medium was replaced and 200 µl PBS/well with or without living *S. uberis* (strain A or strain B) representing a multiplicity of infection (MOI) of approximately 50 and 500, resp., was added. After 1 or 6 h cells were harvested with TriFast (peqGOLD TriFast™, PEQLAB Biotechnologie GmbH, Erlangen Germany) and total RNA extracted according to manufacturer's instruction. mRNA expression of housekeeping [GAPDH, ubiquitin (UBQ)] and target genes were quantified by real time RT-PCR using Rotor Gene 6000 (Corbett Research, Sydney, Australia) and the Sensimix DNA Kit (Quantace, Biolabo, Châtel St Denis, Switzerland) as described (Wellnitz et al., 2011) using published primer sequences (Griesbeck-Zilch et al., 2008). Target gene expressions were normalized to housekeeping genes according to following equation: ΔCP = CP (arithmetic mean of housekeeping genes) – CP (target gene). Data are presented as ΔΔCP = ΔCP (treatment) – ΔCP (control).

Experiment 2: Comparison of the response of mammary epithelial cells to heat inactivated and living *S. uberis*

Mammary epithelial cells were treated according to experiment 1 with or without 5000 MOI living or heat inactivated bacteria in duplicates. After two hours the medium with the living bacteria was removed, the cells washed with PBS, and medium containing 1,5 % Penicillin-Streptomycin was added to avoid overgrowth. After 1, 6, and 12 h cells were harvested and total RNA was extracted as described above.

Table 1: Somatic cell count (SCC) and multiplicity of infection (MOI) of bacterial treatment of milk samples.

Cow	SCC *10³ cells/mL	Strain A MOI/cell	Strain B MOI/cell
1	150	230	160
2	18	1300	1200
3	400	50	42
4	248	80	65

Experiment 3: Treatment of removed milk cells with living *S. uberis*

Four clinically healthy lactating dairy cows (2nd to 4th lactation) were randomly chosen (3 Red Holstein, 1 Holstein). After teat disinfection and removing of the first milk jets approximately 750 ml milk were aseptically taken as follows: a sterilized dull-edged cannula connected to a tube plugged into a bottle with low pressure was inserted through the teat canal. SCC was measured with an automated milk cell counter (DCC; DeLaval, Tumba, Sweden) and shown in Table 1. The milk was stored on ice until usage within 1 h. Bacteria of strain A or strain B grown in 50 mL broth to a concentration of approximately 5 x 10^8 cfu /ml were centrifuged at 1750x g for 10 min, washed in PBS, and added to the sterile milk representing concentrations shown in Table 1. A third bottle served as control. The bottles were incubated at 37 °C for 3 hours (gently shaken every 20 min). From each bottle 0.1 ml were streaked on COS-Agar plate as purity control. The milk was centrifuged at 1500 x g, and 4 °C for 30 min, the fat layer and supernatant were removed and the pellet washed with 250 ml cold PBS (460 x g, 4 °C, 15 min). From the remaining cell pellet total RNA was extracted and mRNA expression of immune factors measured as described in experiment 1.

Results

Experiment 1

The relative mRNA expression changes of TNFα, IL-8, IL-1β, RANTES, and LF after the stimulation of mammary epithelial cells with 50 and 500 MOI of living bacteria is shown in Table 2. The concentrations of 50 and 500 MOI of living strain A or strain B did not change the mRNA expression of TNFα after 1, and 6h in mammary epithelial cells. The expression of IL-8 mRNA and IL-1β mRNA was higher after the stimulation with 500 MOI of strain B than with strain A for 6 h.

Experiment 2

The relative mRNA expression changes of TNFα, IL-8 and IL-1β after stimulation with 5000 MOI of heat inactivated and living bacteria is shown in Figure 1. The mRNA expression of IL-8 and IL-1β in mammary epithelial cells was higher after stimulation with 5000 MOI of strain B (heat inactivated or living) than after stimulation with 5000 MOI of strain A (heat inactivated or living) for 6 and 12h. The mRNA expression of IL-8 was highest after stimulation with 5000 MOI of living bacteria of strain B for 12 h.

Experiment 3

The relative mRNA expression changes of TNFα, IL-8, IL-1β, RANTES, and LF in milk cells after stimulation with living *S. uberis* are shown in Figure 2. In milk cells from all four cows the expression of TNFα mRNA increased in response to the stimulation with *S. uberis*. The expressions of IL-1β mRNA increased only in cells from two cows where the stimulation was performed with lower concentrations of 42–80 MOI.

Discussion

In this experiment the immune response of the mammary epithelial cells to two different *S. uberis* strains isolated from mastitis cases of different severities was investigated *in vitro* using an established method (Wellnitz and Kerr, 2004). To study the effects of these *S. uberis* strains on somatic cells in milk outside the udder but in their natural environment a novel model was developed using aseptically obtained milk.

The results of this study indicate that the two *S. uberis* strains (living or heat inactivated) are not strong inducers of TNFα mRNA expression in mammary epithelial

Table 2: mRNA expression changes (ΔΔCP) of tumor necrosis factor alpha (TNFα), Interleukin-8 (IL-8), Interleukin-1beta (IL-1β), RANTES, and Lactoferrin (LF) after treatment of mammary epithelial cells with 50 and 500 MOI of two different living *S. uberis* strain A and B for 1 and 6 h.

		ΔΔTNFα		ΔΔIL-8		ΔΔIL-1β		ΔΔRANTES		ΔΔLF	
MOI	Time h	Strain A	Strain B	Strain A	Strain B	Strain A	Strain B	Strain A	Strain B	Strain A	Strain B
50	1	–0.4	–0.6	–0.6	–0.4	1.2	–0.8	–1.6	–2.7	–1.3	–2.0
50	6	0.8	0.3	–0.8	0.1	–2.4	–0.3	0.1	–0.5	–1.7	–1.5
500	1	0.0	0.1	1.2	0.8	0.2	1.2	–1.0	–0.4	–0.7	–1.1
500	6	0.2	2.0	–1.2	4.4	–2.3	2.0	–1.0	1.3	0.5	–1.0

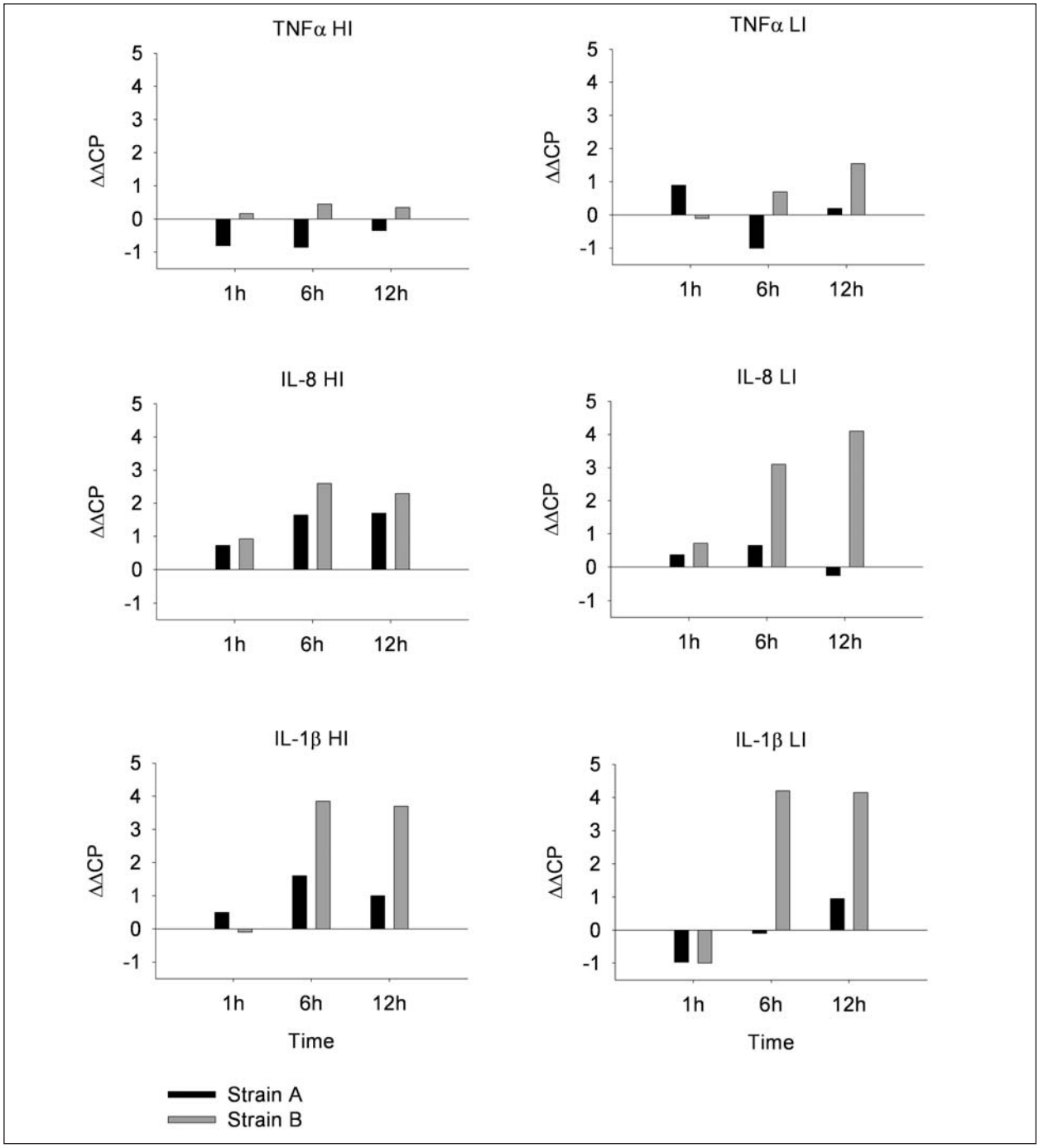

Figure 1: mRNA expression changes (ΔΔCP) of tumor necrosis factor alpha (TNFα), Interleukin-8 (IL-8) and Interleukin-1beta (IL-1β) after stimulation of bovine mammary epithelial cells with 5000 MOI heat inactivated (HI) or living (LI) *S. uberis* strains A and B.

cells. Previous studies showed increased TNFα mRNA expression in mammary epithelial cells after treatment with heat inactivated *Escherichia coli* or *Staphylococcus aureus* (Griesbeck-Zilch et al., 2008; Wellnitz et al., 2006). However, heat inactivated *S. uberis* did not increase the mRNA expression of TNFα (Wellnitz et al., 2006). *In vivo* experiments showed that the increase of TNFα concentration in milk is delayed and this increase was less pronounced after infection with *S. uberis* than with *E. coli* (Bannerman, 2009). The increase of TNFα mRNA expression in somatic milk cells was different between the 4 cows, indicating an individual variability. It seems that *S. uberis* is a stronger inducer of the proinflammatory cytokine TNFα in somatic milk cells than in mammary epithelial cells. However, the different clinical outcome of mastitis induced by strain A or strain B could not be reflected at the level of TNFα mRNA expression in mammary epithelial cells or resident somatic milk cells.

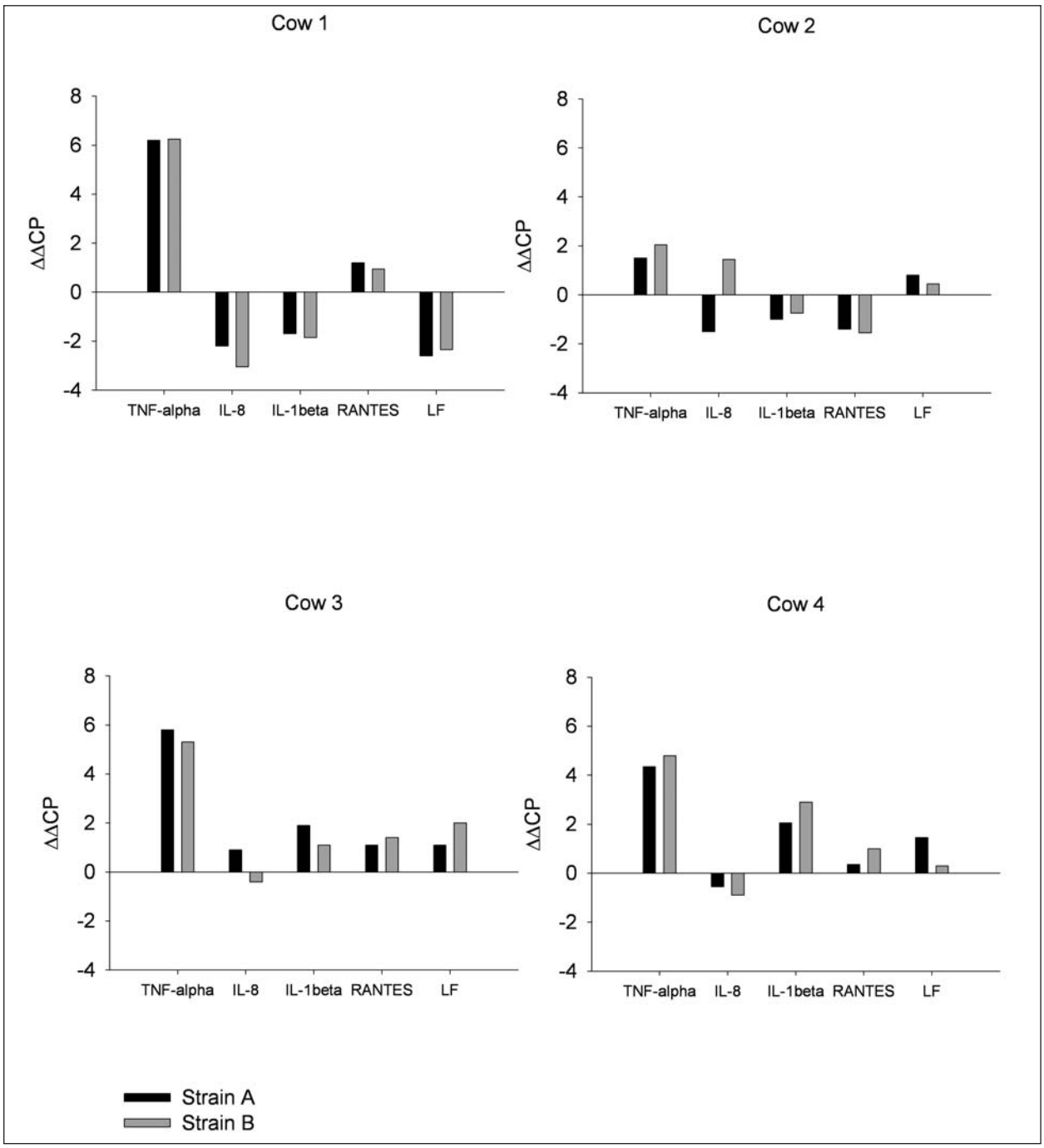

Figure 2: mRNA expression changes (ΔΔCP) of tumor necrosis factor alpha (TNF-alpha), Interleukin-8 (IL-8), Interleukin-1beta (IL-1beta), RANTES, and Lactoferrin (LF) of milk cells after treatment of removed milk cells from 4 cows with two strains of *S. uberis* (strain A and strain B) (3 h at 37 °C).

Interleukin-8 mRNA expression increased in mammary epithelial cells in response to *S. uberis* treatment and this increase was more pronounced after stimulation with strain B than with strain A. Thereby living bacteria seemed to be a stronger inducer than heat inactivated. The inactivation of the bacteria allowed a defined comparable infection pressure and avoided an overgrowth in the cell culture media. Previous studies (Griesbeck-Zilch et al, 2008) have shown the induction of different levels of IL-8 mRNA expression in mammary epithelial cells in response to heat inactivated *E. coli* and *S. aureus*. However, for *S. uberis* the ability to multiply seems to be an important virulence factor to stimulate the response of the epithelial cells. As Il-8 is an important chemoattractant in mammary immunity, it is possible that the induction of different outcomes of mastitis of strain A and strain B are reflected at the level of IL-8 mRNA expression, i. e. strain B induced a stronger increase of mRNA expression of IL-8

in vitro than strain A. In milk cells the IL-8 mRNA expression showed no difference between stimulation with strain A or strain B. In mammary epithelial cells the increase of IL-8 mRNA expression was highest after 6 h, but stimulation of milk cells was limited to 3 h in our model because of the viability of the cells (Baumert et al., 2009). It is possible that 3 h of incubation was not sufficient to stimulate mRNA expression of IL-8 in milk cells or more IL-8 would have been expressed in fresh recruited neutrophils, which were not investigated in our model.

Interleukin-1β mRNA expression in mammary epithelial cells and somatic milk cells was also more pronounced in response to strain B than to strain A. *In vivo* experiments (Rambeau et al., 2003; Bannerman et al., 2009) showed a delayed increase of IL-1β concentration in milk after stimulation with *S. uberis* compared to *E. coli.* Swanson et al. (2009) showed a higher induction of IL-1β mRNA expression in mammary epithelial cells by heat inactivated hyaluronic acid producing *S. uberis* compared to another strain without hyaluronic acid production. During mastitis caused by strain B the cow had fever but no further clinical signs. As IL-1β is known to be involved in the induction of fever, the stronger induction of IL-1β mRNA expression in mammary epithelial cells *in vitro* by stain B may reflect the clinical development of mastitis that was induced by this strain *in vivo*.

The minimal differences in mRNA expression of RANTES after *S. uberis* treatment was in agreement to previous findings with *S. aureus* or the cell wall component of gram-positive bacteria lipoteichoic acid (Griesbeck-Zilch et al., 2008; Arnold et al., 2011). Apparently, RANTES seems to be less important than other chemokines for cell recruitment of leukocytes into the mammary gland during an infection with gram-positive bacteria. In addition, the two tested *S. uberis* strains seemed to be weak stimulants for Lf production in epithelial cells. However, it is possible that the treatment of 12 h was not sufficient to show measurable responses, because it was shown that Lf mRNA expression was highest after stimulation of mammary epithelial cells with *E. coli* or *S. aureus* for 24 h (Griesbeck-Zilch et al., 2008).

Conclusion

S. uberis strains isolated from mastitis cases with different severities induced different immune responses of the mammary gland *in vitro*. Strain B, isolated from an acute clinical mastitis, induced a more pronounced increase of mRNA-expression of some cytokines in mammary epithelial cells than strain A which was isolated from a subclinical, chronic mastitis. In contrast, milk cells showed no differences between the stimulation with the two *S. uberis* strains. Mastitis severity induced by two different *Streptococcus uberis* strains is reflected by a different immune response of the mammary gland.

Il decorso della mastite, causata da due ceppi di *Streptococcus uberis*, è individuabile dalla risposta immunitaria *in vitro* della mammella

Lo *Streptococcus uberis* è un patogeno ambientale comune che provoca mastite nelle vacche da latte, il decorso clinico è variabile. Nel presente studio è stato esaminato se il decorso della mastite provocato da diversi ceppi di *S. uberis*, è individuabile dalla risposta immunitaria nella mammella di questi. La reazione delle cellule epiteliali delle ghiandole mammarie e delle cellule somatiche mammarie è stata studiata in vitro per due ceppi di *S. uberis*. Il ceppo A è stato isolato, nonostante il trattamento antibiotico, negli 8 mesi di ripetute infezioni croniche del quarto. Il ceppo B ha causato una mastite acuta e non è stato più possibile isolarlo dopo un singolo trattamento con antibiotici. Il trattamento con il ceppo B ha indotto nell'epitelio delle ghiandole mammarie delle forti espressioni mRNA dei vari fattori immunitari (interleuchina-8 e1β, RANTES e lattoferrina) in quanto ceppo A. Nelle cellule mammarie questi fattori non differenziati sono stati indotti. Il fattore di necrosi tumorale alfa non ha evidenziato differenze nell'espressione di mRNA dopo la stimolazione con entrambi i ceppi. I risultati

Le cours d'une mammite causée par deux souches de *Streptococcus uberis* se reflète dans la réponse immunitaire de la mamelle *in vitro*

Streptococcus uberis est un germe fréquent dans l'environnement, qui cause chez les vaches laitières des mammites au cours clinique variable. Dans cette étude on examine si le cours d'une mammite causé par différentes souches de *S. uberis* se reflète sur la réponse immunitaire de la mamelle face à ces souches. La réaction des cellules épithéliales de la mamelle et des cellules somatiques du lait face à deux souches de *S. uberis* a été étudiée *in vitro*. La souche A a été isolée à plusieurs reprises durant 8 mois à partir d'un quartier infecté chroniquement malgré un traitement antibiotique. La souche B a causé une mammite aiguë et ne pouvait plus être isolée après un seul traitement antibiotique. Le traitement avec la souche B a induit dans les cellules épithéliales des expressions mRNS de divers facteurs immunitaires (Interleucine-8 et 1β, RANTES et lactoferrine) plus marquée que la souche A. Dans les cellules du lait, ces facteurs ne présentaient pas de différences d'induction. Le facteur de nécrose tumorale alpha ne montrait pas de différence d'expression mRNS après stimulation avec les deux souches. Les résultats

di questo studio mostrano che il diverso decorso di una mastite, indotta da differenti ceppi di *S. uberis,* è individuabile nella risposta immunitaria *in vitro* della mammella.

de cette étude montrent que les cours variables d'une mammite causées par différentes souches de S uberis se reflètent dans la réponse immunitaire de la mamelle *in vitro*.

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