# Dose-dependent immune response in milk cells and mammary tissue after intramammary administration of lipopolysaccharide in dairy cows

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**ABSTRACT**: The immune response in milk cells and the status of mammary tight junctions (TJ) in response to intramammary (IM) infusion of different doses of *Escherichia coli* lipopolysaccharide (LPS) was investigated. *Experiment I*: Seven German Braunvieh cows were IM infused into one quarter with 1 μg (LPS-1) and 3 μg (LPS-3) of LPS, respectively, and the contralateral control quarter with saline (9 g/l; C). Milk samples were taken immediately before and 12, 24, 36, 48, 60, 84 and 108 h after infusion and analysed for somatic cell counts (SCC), lactose, sodium (Na) and chloride (Cl) ions, and electrical conductivity (EC). Milk cell mRNA expression of various inflammatory factors was quantified by real-time RT-PCR. Blood samples were taken immediately after milking for the analysis of leukocytes (WBC), polymorphonuclear neutrophils (PMN), Na and Cl. Milk SCC, lactose, Na, Cl and EC did not differ significantly between LPS-1 and C quarters after the challenge. In LPS-3 quarters SCC levels increased within the first 12 h, reached peak levels between 12 and 36 h ($P \leq 0.001$) and decreased ($P \leq 0.05$) thereafter to reach baseline at 108 hours. Lactose in LPS-3 quarters decreased ($P \leq 0.05$) to a minimum at 24 h and increased slightly thereafter while EC, Na, and Cl increased transiently in response to LPS-3. WBC and PMN levels in both groups decreased numerically within 24 h after LPS administration. In LPS-1, WBC at 24, 48 and 108 h were significantly lower whereas in LPS-3 they were significantly higher than at time 0. TNFα-mRNA expression in both groups did not change in response to IM LPS-challenge. IL-1β-mRNA expression at 12, 24 and 36 h in LPS-1 quarters increased significantly as compared to time 0. In LPS-3 quarters the mRNA expression values of all tested ILs increased significantly as compared to time 0 within 12 h after LPS-challenge. IL-1β-mRNA expression decreased ($P \leq 0.05$) at 48 and 84 h in LPS quarters. IL-8 mRNA was significantly decreased at 84 h after challenge in LPS-3 quarters. COX-2-mRNA expression in LPS-1 quarters decreased significantly as compared to time 0 at 48, 84 and 108 h, with a minimum at 84 h ($P \leq 0.05$). In LPS-3 quarters COX-2-mRNA levels increased ($P \leq 0.05$) within 48 h after the LPS-challenge. *Experiment II*: Six cows (5 German Braunvieh, 1 Brown Swiss) were injected in one quarter with 100 μg LPS and in the contralateral quarter with saline (9 g/l; C). Mammary biopsy samples of both quarters were taken immediately before and at 3, 6, 9 and 12 h after infusion and mRNA expression of TJ proteins occludin (OCLN) and zonula occludens (ZO-) 1, 2 and 3 were quantified by real-time RT-PCR. OCLN-mRNA expression did not change in response to the IM infusion while that of ZO-1, ZO-2 and ZO-3 decreased significantly within six hours. In conclusion, a dose of 1 μg LPS did not initiate a immune response in the mammary gland. Furthermore the dose of 100 μg of LPS enhanced TJ permeability by reducing TJ plaque proteins density.

**Keywords**: mammary gland; mastitis; lipopolysaccharide; cattle

Infections of the mammary gland and decreased resistance to pathogens frequently cause severe clinical mastitis and are among the increasing health problems in dairy cows (Burvenich et al., 1994; Vangroenweghe et al., 2002). One of the most prevalent pathogens that cause intramammary infection (IMI) is *Escherichia coli* (Bradley, 2002), a Gram-negative bacterium. The lipopolysaccharides

(LPS) located in the outer cell membrane of Gram-negative bacteria are mainly responsible for many of the clinical signs (fever, pain, increasing somatic cells, loss of milk character) in coliform mastitis (Jain et al., 1978; Hill, 1981; Guidry et al., 1983; Pfaffl et al., 2003). In experimental intramammary challenge studies LPS has been used in amounts of up to 500 μg to induce mastitis and to study the inflammatory response in the bovine udder (Hoeben et al., 2000; Mehrzad et al., 2001; Van Oostveldt et al., 2002; Schmitz et al., 2004a).

The immune defence of the bovine udder is mediated by milk somatic cells composed of macrophages (M), polymorphonuclear neutrophils (PMN), lymphocytes and epithelial cells. The number of each cell population is based on the immunological status of the udder. Burvenich et al. (1994), Paape et al. (2002) and Sarikaya et al. (2004) described M as the predominant cell type in the milk of healthy udders. After being activated through a pathogen contact, M release PMN-recruiting cytokines like tumor necrosis factor alpha (TNFα), interleukin-1beta (IL-1β), interleukin-6 (IL-6) and interleukin-8 (IL-8) as well as lipid mediators such as cyclooxygenase-2 (COX-2). Thus, PMN become the predominant cell type in mastitic milk (Kehrli and Shuster, 1994; Paape et al., 2002; Sarikaya et al., 2004).

During lactation, the mammary secretory epithelium is separated from the interstitial fluid by the endothelium to prevent interdiffusion. This sectioning is guaranteed by the tight junctions (TJ). TJ are apically situated in the junctional complex which surrounds each epithelial cell (Nguyen and Neville, 1998; Schneeberger and Lynch, 2004). TJ are associated with transmembrane proteins such as occludin (OCLN) and cytoplasmatic plaque proteins like zonula occludens 1 to 3 (ZO-1, ZO-2, ZO-3) (Nguyen and Neville, 1998; Haskins et al., 1998). As dynamic structures in the mammary gland TJ are leaky during pregnancy and close around parturition to the impermeable state during the course of lactation (Nguyen and Neville, 1998). However, also during ongoing lactation TJ can become leaky due to milk stasis, during mastitis, and in response to high doses of oxytocin (Stelwagen et al., 1997; Nguyen and Neville, 1998).

The aim of this study was to investigate a possible local immune response initiated by intramammarily (im) infused LPS in doses of 1 and 3 μg, respectively. In addition, the influence of 100 μg im infused LPS on the status of TJ was investigated.

## MATERIAL AND METHODS

### Animals and husbandry

This study was approved by the responsible animal care and use committee (Regierung von Oberbayern, Munich, Germany).

The seven German Braunvieh cows used in Experiment I (a + b) were kept in a tethered barn. They were fed maize silage, hay and concentrate. Water was available *ad libitum*. Three cows were in their first, three in their second and one in its fifth lactation. Two animals were in an early stage of lactation (25–72 days), three were in mid lactation (155–168 days) and two in a late stage of lactation (315–487 days). All animals were free of clinical signs of mastitis. Only quarters whose milk samples were cultured negative for mastitis pathogens five days before the experiment were accepted for the study.

The six dairy cows used in Experiment II were in their first to fourth lactation and were free of clinical udder health problems as described previously (Schmitz et al., 2004a). Only quarters with a SCC < 150 000 cells per ml and milk samples cultured negative for mastitis pathogens were accepted.

### Experimental and laboratory procedures

#### Experiment I

On day one of the experiment the milk of the two selected quarters of every cow was collected in a quarter milker (GEMA – Bruno Gelle GmbH, Wangen, Germany) during routine machine milking. The mammary glands were cleaned and the teats were disinfected. Thereafter cows were injected in one quarter with 1 μg (a) or 3 μg (b) *Escherichia coli* lipopolysaccharide (LPS; Serotype O26:B6, Nr. L 8274, Sigma Chem. Co., St. Louis, USA) diluted in 10 ml saline and with 10 ml of saline (9 g/l) in the control quarters (C). Further quarter milk samples were taken at 12, 24, 36, 48, 60, 84 and 108 h after the injection during routine machine milkings. Blood samples for haematological analyses were taken together with the milk samples. One aliquot each was anti-coagulated by ethylenediaminetetraacetic acid (EDTA) for the analysis of leukocytes (WBC) and PMN. Another aliquot was taken without anticoagulans and was centrifuged for serum production to analyse serum

electrolytes. Rectal temperature was measured at 6 h after the im injection.

### Measurement of milk constituents

Milk lactose concentration and somatic cell counts (SCC) in every sample were analysed in the laboratory of the Milchprufring Bayern e.V. (Wolnzach, Germany), by using a MilkoScan 4500 analyser (FOSS Electrik, Hillerod, Denmark). Aliquots of each sample were frozen at –20°C immediately after sampling for the determination electrical conductivity (EC), sodium (Na) and chloride (Cl) ion concentrations. Na and Cl in milk were measured by potentiometer determination with ion selective electrodes model 9811 and model 9617BN (pH/Ise Meter, Modell 720 Aplus, Orion Research, Beverly, MA, USA). EC was measured at 25°C using the LDM electrode from WTW (LDM 130, Wissenschaftlich-Technische Werkstatten GmbH, Weilheim, Germany).

### Determination of blood parameters

Total and differential WBC were measured with ADVIA 120 (Bayer Diagnostics, Fernwald, Germany), and blood Na and Cl were determined with a MODULAR E170 (Roche Diagnostics GmbH, Mannheim, Germany) at Vet Med Labor GmbH (Ludwigsburg, Germany). Blood lactose was measured by an enzymatic method (R-Biopharm AG, Darmstadt, Germany).

### Milk cell isolation

200 g of each quarter milk sample were centrifuged to separate the cell pellet according to Sarikaya et al. (2004). After washing twice with PBS the pellet was resuspended in 500 µl peqGOLD TriFast™ (PEQLAB Biotechnologie GmbH, 91052 Erlangen, Germany) for total RNA isolation.

### RNA isolation

Total RNA was isolated using peqGOLD TriFast™ (PEQLAB Biotechnologie GmbH, Erlangen, Germany) according to the manufacturer's instruction. The optical density (OD) was determined at three different dilutions of the final RNA preparations at 260 nm, corrected by the 320 nm background absorption, in order to quantify the extracted RNA. RNA integrity was verified by the OD260/OD280 absorption ratio 1.6–1.8.

### Oligonucleotide primers

All used primers for each reference or target gene were synthesized commercially (MWG Biotech, Ebersberg, Germany) using already published bovine specific primer sequences (Schmitz et al., 2004a). All primer information is listed in Table 1.

### mRNA quantification by real-time RT-PCR

The quantitative analysis of the different PCR-products was worked out on the Rotor-Gene 3000 (Corbett Life Science, NSW 2137, Sydney, Australia) via one-step qRT-PCR. 3.8 µl of extracted mRNA solutions (15 ng/µl) were used. According to the manufacturers instruction 6.2 µl Master Mix (Super ScriptTM III Platinum SYBR® Green One-Step qRT-PCR Kit, Invitrogen GmbH, Karlsruhe, Germany) was prepared including 5 µl 2 × SYBR® Green Reaction Mix, 0.2 µl SYBR® Green One-Step Enzyme Mix, 0.5 µl (10pM) forward primer and 0.5 µl (10pM) reverse primer.

Crossing points (CP) were achieved by Rotor-Gene software 5.0. A normalisation of the target genes was performed with an endogenous standard. Therefore the expression levels of two reference genes glyceraldehyde-3-phosphatedehydrogenase (GAPDH) and Ubiquitin (UbQ) were measured. The relative mRNA levels were calculated by normalisation of the CP of the target gene to the mean CP of the two reference genes UbQ and GAPDH.

### Experiment II

Cows were injected in one quarter with 100 µg LPS (Serotype O26:B6, Sigma Chem. Co., St. Louis, MO, USA) in 10 ml of saline and in the contralateral quarter with 10 ml saline (9 g/l). Mammary biopsy samples of both quarters were taken immediately before (0) and at 3, 6, 9 and 12 h after injection. Biopsies were taken by using a human Bard® Magnum™ Biopsy Instrument with a Bard®

Magnum® Core Tissue Biopsy Needle (12 g × 10 cm) (BARD Inc., Covington, Georgia, USA), a core of 30 to 60 mg mammary tissue was extracted.

From the mammary biopsy samples total RNA was isolated. The synthesis of first strand complementary DNA (cDNA) was conducted with reverse transcriptase (MMLV-RT, Promega Corporation, Madison, WI, USA) and random hexamer primers (MBI Fermentas, St. Leon-Rot, Germany). RT-PCR in a LightCycler (Roche Diagnostics, Mannheim, Germany) accomplished the amplification of the target nucleic acids. Primer sequences on factors which have not been published before are shown in Table 1.

Table 1. Sequences of PCR primers ( for = forward; rev = reverse), PCR product length and EMBL accession number of the used nucleic acid sequences, as well as product specific Rotor-Gene 3000 (Corbett Life Science)[1] and LightCycler (Roche Diagnostics)[2] conditions, respectively

| Primer | Sequence (5' → 3') | Length (bp) | EMBL (access. no.) | Annealing (temp. °C) |
|---|---|---|---|---|
| **Experiment I (a + b)**[1] | | | | |
| UbQ for | AGA TCC AGG ATA AGG AAG GCA T | 198 | Z18245 | 60 |
| UbQ rev | GCT CCA CCT CCA GGG TGA T | | | |
| GAPDH for | GTCTTCACTACCATGGAGAAGG | 197 | U85042 | 58 |
| GAPDH rev | TCATGGATGACCTTGGCCAG | | | |
| TNF-α for | TAA CAA GCC GGT AGC CCA CG | 277 | AF011926 | 64 |
| TNF-α rev | GCA AGG GCT CGG GAT GGC AGA | | | |
| COX-2 for | TCT TCC TCC TGT GCC TGA T | 358 | AF031698 | 64 |
| COX-2 rev | CTG AGT ATC TTT GAC TGT GG | | | |
| IL-1β for | TTC TCT CCA GCC AAC CTT CAT T | 198 | M37211 | 60 |
| IL-1β rev | ATC TGC AGC TGG ATG TTT CCA T | | | |
| IL-6 for | GCT GAA TCT TCC AAA AAT GGA GG | 200 | NM173923 | 60 |
| IL-6 rev | GCT TCA GGA TCT GGA TCA GTG | | | |
| IL-8 for | ATG ACT TCC AAG CTG GCT GTT G | 149 | AF232704 | 60 |
| IL-8 rev | TTG ATA AAT TTG GGG TGG AAA G | | | |
| **Experiment II**[2] | | | | |
| UbQ for | AGA TCC AGG ATA AGG AAG GCA T | 198 | Z18245 | 62 |
| UbQ rev | GCT CCA CCT CCA GGG TGA T | | | |
| GAPDH for | GTCTTCACTACCATGGAGAAGG | 197 | U85042 | 62 |
| GAPDH rev | TCATGGATGACCTTGGCCAG | | | |
| OCLN for | CTG GAT CAG GGA ATA TCC ACC | 176 | AJ313191 | 64 |
| OCLN rev | ACT CTT CAC TTT CTT CTC TAT AGT | | AJ313182 | |
| ZO-1 for | AGA AGA TAG CCC TGC AGC CAA | 272 | AJ313188 | 64 |
| ZO-1 rev | CCT CTC CTT TGT TAA AAC TAA GTC | | AJ313183 | |
| ZO-2 for | GAC CAG ATT CTG AAG GTG AACACA | 197 | AJ313187 | 64 |
| ZO-2 rev | TCC TTC TCA CAT TCA AAG TGG CT | | AJ313184 | |
| ZO-3 for | AAC GAC GTG GGC ATC TTC GT | 251 | AJ313186 | 64 |
| ZO-3 rev | GTG CGG ATG TAG AAG GAG TC | | AJ313185 | |

UbQ = ubiquitin; GAPDH = glyceraldehyde-3-phosphatedehydrogenase; TNFα = tumor necrose factor alpha; COX-2 = cyclooxygenase-2; IL = interleukin; OCLN = occludin; ZO = zonula occludens

### Statistical analysis

Data are presented as means ± SEM. Differences between treated and control quarters were tested for significance ($P \leq 0.05$) by analysis of variance, using the MIXED procedure of the SAS program. The MIXED model included the animal and the sample as class variables. Within each sample the treatment was the repeated term. The matrix structure Compound Symmetry was used. Because SCC values could not be assumed to be normally distributed they were logarithmised ($\log_{10}$) for statistical calculations.

## RESULTS

### Experiment I

#### Milk composition

SCC in quarters treated with 1 µg LPS (LPS-1) showed no significant changes as compared to C after the infusion (Figure 1a), but LPS-1 quarter values at 36, 48 and 60 hours changed significantly compared to time 0. Also C quarter values at 24, 36, 48 and 60 h changed ($P < 0.05$) as compared to time 0. In quarters treated with 3 µg LPS (LPS-3) SCC increased within the first 12 h to maximal levels between 12 and 36 h ($P < 0.001$) and decreased ($P < 0.05$) thereafter by 84 hours. In LPS-3 quarters SCC from 12 to 84 h were significantly higher as compared to time 0. SCC reached baseline values at 108 h after LPS administration (Figure 1b).

Lactose concentration in milk of LPS-1 quarters decreased to a minimum at 48 h after injection and increased thereafter to baseline values until the end of the experiment (Figure 2a). Within C quarters lactose at 36 and 60 h decreased significantly as compared to time 0. The concentration of lactose in the LPS-3 quarters remained significantly decreased until 36 h but started their increase already at 24 h to reach baseline levels at 60 h (Figure 2b).

The course of EC values during the investigation in LPS-1 quarters was similar to values in C (Figure 3a). Values in C quarters showed a significant increase 36 h after the treatment which was significantly different from values in LPS-1 quarters as well as compared to time 0. In LPS-3 quarters EC increased up to maximum values at 12 h ($P < 0.05$), remained significantly elevated as compared to C with a slight decrease until 36 h and decreased further to baseline levels at 48 h (Figure 3b). Within LPS-3 quarters values at 12, 24 and 36 h increased significantly as compared to time 0.

As demonstrated in Table 2 the levels of Na and Cl in LPS-1 quarters did not increase significantly in response to LPS treatment. On the contrary in LPS-3 quarters (Table 2) Na levels increased ($P < 0.05$) to maximum values from 12 to 36 hours. Within LPS-3 quarters values at 12, 24, 36 and 60 h were significantly increased as compared to

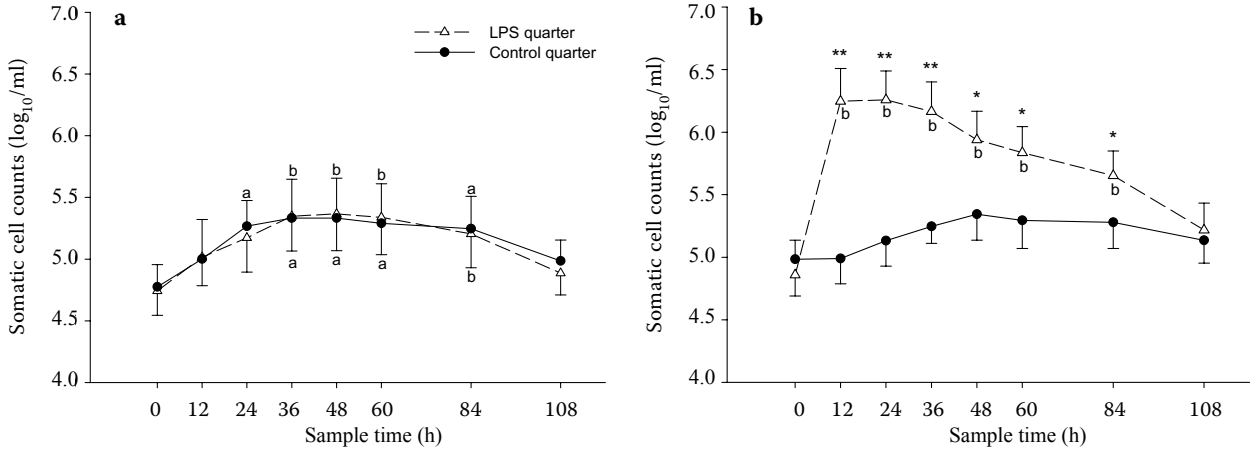


Figure 1. Somatic milk counts (SCC) before and after intramammary infusion of 1 µg (a) and 3 µg (b) *E. coli* endotoxin (LPS quarter) or with saline (control quarter). Data are means ± S.E.M. of seven cows

*means of LPS quarters are significantly different ($P \leq 0.05$) to control quarters

**means of LPS quarters are significantly different ($P \leq 0.0001$) to control quarters

[a]means within control quarters differ significantly ($P \leq 0.05$) to time 0 h

[b]means within LPS quarters differ significantly ($P \leq 0.05$) to time 0 h

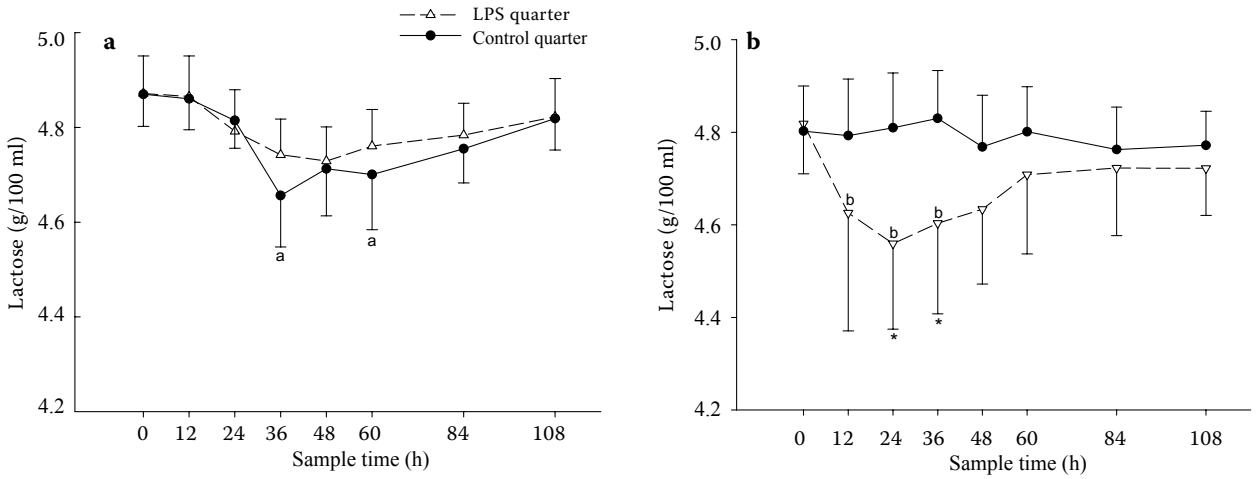


Figure 2. Milk lactose before and after intramammary infusion of 1 µg (a) and 3 µg (b) *E. coli* endotoxin (LPS quarter) or with saline (control quarter). Data are means ± S.E.M. of seven cows

*means of LPS quarters are significantly different ($P \leq 0.05$) to control quarters
[a]means within control quarters differ significantly ($P \leq 0.05$) to time 0 h
[b]means within LPS quarters differ significantly ($P \leq 0.05$) to time 0 h

time 0. Cl values in LPS-3 quarters increased to a maximum at 24 h ($P < 0.05$) after the infusion. Na and Cl values reached basal values at the end of the studied period.

## Blood parameters

**WBC**. In the LPS-1 group WBC at 24, 48 and 108 h after the LPS infusion decreased significantly as compared to time 0 (Table 2). The percentage of PMN in the LPS-1 group showed only a numerical decrease at 24 h but did not differ significantly during the course of the experiment.

WBC and PMN in the LPS-3 group showed a numerical decrease within 24 h after LPS infusion (Table 2). Thereafter the amount of WBC increased as compared to time 0 ($P < 0.05$) up to 60 hours. The percentage of PMN decreased numerically within 24 h and reached its minimum at 48 hours.

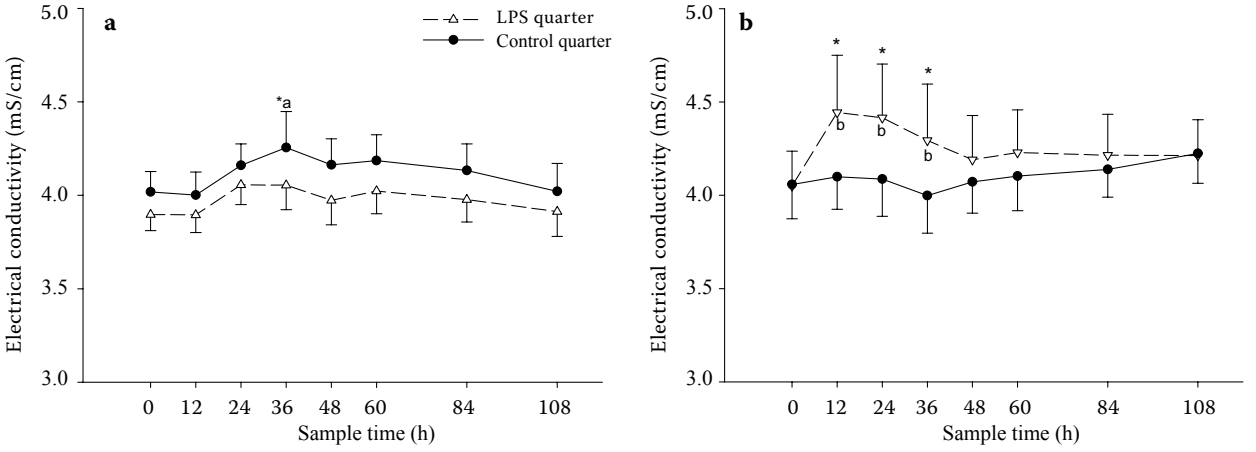


Figure 3. Electrical conductivity before and after intramammary infusion of 1 µg (a) and 3 µg (b) *E. coli* endotoxin (LPS quarter) or with saline (control quarter). Data are means ± S.E.M. of seven cows

*means of LPS quarters are significantly different ($P \leq 0.05$) to control quarters
[a]means within control quarters differ significantly ($P \leq 0.05$) to time 0 h
[b]means within LPS quarters differ significantly ($P \leq 0.05$) to time 0 h

Table 2. Milk electrolytes (Na, Cl), leucocytes (WBC), polymorphonuclear neutrophils (PMN) and blood electrolyte (bNa, bCl) levels after an intramammary infusion ( immediately after sample 0) with 1 µg LPS and 3 µg LPS in 10 ml saline (9 g/l). Means of all treated quarters

| Parameter | Sample time (h) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 12 | 24 | 36 | 48 | 60 | 84 | 108 |
| **LPS-1** | | | | | | | | |
| Na (mmol/l) | 14.2 ± 1.3 | 14.8 ± 1.6 | 16.2 ± 1.6 | 16.2 ± 1.4 | 15.2 ± 1.3 | 15.2 ± 1.1 | 14.8 ± 1.1 | 14.8 ± 1.3 |
| Cl (mmol/l) | 32.0 ± 2.1 | 32.1 ± 2.2 | 33.6 ± 2.4 | 34.2 ± 2.1 | 34.3 ± 2.0 | 34.7 ± 2.3 | 33.7 ± 2.2 | 33.2 ± 2.7 |
| WBC (G/l) | 7.1 ± 0.6 | 7.1 ± 0.7 | 6.6 ± 0.7[a] | 7.0 ± 0.7 | 6.5 ± 0.4[a] | 6.9 ± 0.3 | 6.9 ± 0.6 | 6.2 ± 0.3[a] |
| PMN (%) | 42.0 ± 8.8 | 41.4 ± 4.6 | 37.1 ± 3.1 | 43.0 ± 4.6 | 41.3 ± 6.4 | 44.0 ± 4.2 | 35.0 ± 4.4 | 38.3 ± 4.6 |
| bNa (mmol/l) | 140.0 ± 1.2 | 139.0 ± 2.3 | 131.1 ± 0.6 | 143.0 ± 1.5[a] | 141.0 ± 0.85 | 141.7 ± 1.1 | 140.1 ± 0.7 | 141.7 ± 0.4 |
| bCl (mmol/l) | 104.4 ± 1.3 | 101.4 ± 1.6 | 99.1 ± 1.7[a] | 99.7 ± 2.3[a] | 104.0 ± 2.0 | 103.6 ± 2.2 | 100.4 ± 1.5 | 101.1 ± 1.3 |
| **LPS-3** | | | | | | | | |
| Na (mmol/l) | 14.4 ± 2.0 | 19.7 ± 3.5*[a] | 19.2 ± 2.9*[a] | 18.9 ± 3.2*[a] | 16.6 ± 2.4 | 17.3 ± 2.8[a] | 16.5 ± 2.6 | 15.9 ± 2.6 |
| Cl (mmol/l) | 30.8 ± 3.4 | 36.3 ± 5.5 | 37.4 ± 5.6* | 34.8 ± 5.3 | 33.7 ± 5.1 | 34.3 ± 5.0 | 35.2 ± 4.4 | 32.4 ± 3.7 |
| WBC (G/l) | 6.4 ± 0.5 | 6.3 ± 0.5 | 6.1 ± 0.5 | 6.4 ± 0.6 | 7.0 ± 0.6 | 7.2 ± 0.6[a] | 7.0 ± 0.7 | 6.5 ± 0.7 |
| PMN (%) | 40.4 ± 5.8 | 39.7 ± 4.9 | 36.1 ± 4.5 | 39.7 ± 4.5 | 34.9 ± 5.4 | 36.7 ± 2.5 | 44.7 ± 6.1 | 35.1 ± 5.4 |
| bNa (mmol/l) | 140.9 ± 0.96 | 141.9 ± 0.6 | 141.1 ± 0.5 | 141.3 ± 0.6 | 140.6 ± 1.0 | 141.0 ± 0.6 | 142.6 ± 0.4 | 136.9 ± 4.6[a] |
| bCl (mmol/l) | 99.7 ± 1.2 | 100.1 ± 1.2 | 100.14 ± 0.6 | 99.1 ± 1.0 | 101.0 ± 0.8 | 100.4 ± 0.9 | 104.1 ± 1.1[a] | 97.1 ± 3.3 |

LPS-1 = means of all quarters treated with 1 µg LPS solution

LPS-3 = means of all quarters treated with 3 µg LPS solution

*significant for $P \leq 0.05$ to controls

[a]significant for $P \leq 0.05$ to value at 0 h sample time

Table 3. Changes of mRNA expression ($log^2$) of TNFα, IL-1ß, IL-6, IL-8 and COX-2 in milk cells relative to time = 0 h after an intramammary injection of 1 µg LPS or 10 ml saline (9 g/l) and 3 µg LPS or 10 ml saline (9 g/l)

| Factor | Quarter | Time after LPS administration (h) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 12 | 24 | 36 | 48 | 60 | 84 | 108 |
| **LPS-1** | | | | | | | | |
| TNFα | LPS-1 | 0.2 ± 0.8 | 0.2 ± 0.6 | −0.1 ± 0.8 | 1.4 ± 0.7 | 0.6 ± 0.9 | −0.4 ± 1.0 | 0.5 ± 0.7 |
| | C | −0.1 ± 0.4 | −0.2 ± 0.5 | 0.7 ± 0.5 | −0.46 ± 0.3 | 0.1 ± 0.3 | −0.2 ± 0.7 | 0.3 ± 0.3 |
| IL-1ß | LPS-1 | 1.3 ± 0.6[a] | 1.5 ± 0.8 [a] | 1.4 ± 0.8[a] | −1.2 ± 0.8 | 1.0 ± 0.9 | 0.2 ± 0.9 | 0.5 ± 0.7 |
| | C | 0.1 ± 0.3 | 0.3 ± 0.3 | 0.7 ± 0.4 | −0.1 ± 0.5 | 0.1 ± 0.5 | 0.4 ± 0.7 | 0.7 ± 0.4 |
| IL-6 | LPS-1 | 1.1 ± 0.4 | −0.4 ± 0.6 | −0.3 ± 0.7 | −0.9 ± 0.6 | 0.02 ± 0.9 | −1.3 ± 0.8 | −0.5 ± 0.4 |
| | C | 0.8 ± 0.4 | −0.3 ± 0.6 | 0.2 ± 0.7 | −1.3 ± 0.7 | −0.4 ± 0.7 | −1.0 ± 0.9 | −0.2 ± 0.5 |
| IL-8 | LPS-1 | 0.4 ± 0.4 | 1.3 ± 1.0 | 0.9 ± 0.9 | −0.8 ± 0.9 | 0.7 ± 0.7 | 0.2 ± 0.7 | 0.3 ± 0.5 |
| | C | −0.4 ± 0.5 | 0.5 ± 0.5 | 0.1 ± 0.6 | −0.3 ± 0.6 | 0.3 ± 0.7 | 0.4 ± 0.5 | 0.4 ± 0.5 |
| COX-2 | LPS-1 | −0.5 ± 0.7 | 0.8 ± 0.9 | −0.2 ± 0.9 | −1.6 ± 1.0[a] | −0.7 ± 1.0 | −3.1 ± 0.9*[a] | −1.4 ± 1.1[a] |
| | C | 0.2 ± 1.0 | 0.6 ± 0.8 | 1.5 ± 1.1 | 1.0 ± 1.1 | −0.3 ± 2.0 | 1.9 ± 1.2 | 1.3 ± 0.6 |
| **LPS-3** | | | | | | | | |
| TNFα | LPS-3 | −0.2 ± 0.7 | −0.4 ± 0.7 | 0.2 ± 0.7 | −0.2 ± 0.2 | −0.2 ± 0.2 | 0.1 ± 0.5 | 1.0 ± 0.7 |
| | C | 0.6 ± 0.5 | 0.03 ± 0.4 | 0.9 ± 0.4 | −0.5 ± 0.8 | −0.5 ± 0.8 | 0.4 ± 0.7 | 1.1 ± 0.5 |
| IL-1ß | LPS-3 | 1.7 ± 0.7[a] | 0.7 ± 0.8 | 0.6 ± 0.8[a] | −1.0 ± 1.1* | −1.0 ± 1.1* | −1.2 ± 0.7* | 0.2 ± 0.6 |
| | C | 0.5 ± 0.2 | 0.3 ± 0.4 | 1.2 ± 1.0 | 0.3 ± 0.7 | 0.3 ± 0.7 | 0.5 ± 0.5 | 0.4 ± 0.3 |
| IL-6 | LPS-3 | 2.2 ± 1.0[a] | −0.6 ± 1.3 | 0.2 ± 0.6 | −0.8 ± 0.7 | −0.8 ± 0.7 | −0.03 ± 0.9 | 0.9 ± 0.8 |
| | C | 1.7 ± 1.0 | 1.3 ± 1.2 | 2.0 ± 1.2 | 0.7 ± 0.8 | 0.7 ± 0.8 | 0.9 ± 0.6 | 1.3 ± 1.1 |
| IL-8 | LPS-3 | 1.2 ± 0.8[a] | 0.6 ± 0.8 | 0.6 ± 0.7 | 0.2 ± 0.7 | 0.2 ± 0.7 | −0.2 ± 0.8*[a] | −0.5 ± 0.5 |
| | C | 0.4 ± 0.4 | 0.2 ± 0.4 | 0.6 ± 1.0 | 0.4 ± 0.7 | 0.4 ± 0.7 | 0.4 ± 0.5 | 0.2 ± 0.5 |
| COX-2 | LPS-3 | 1.6 ± 0.9 | 1.3 ± 0.9 | 3.3 ± 1.4 | 2.0 ± 1.0* | 2.0 ± 1.0* | 1.8 ± 0.9 | 1.2 ± 1.0 |
| | C | 0.1 ± 1.2 | 0.1 ± 1.7 | 1.5 ± 0.5 | −0.9 ± 1.0 | −0.9 ± 1.0 | −0.5 ± 0.8 | 0.5 ± 1.0 |

TNFα = tumor necrosis factor α, IL-1β = interleukin−1β, IL-6 = interleukin-6, IL-8 = interleukin-8, COX-2 = cyclooxygenase-2

LPS-1 = means of all quarters treated with 1 µg LPS solution, LPS-3 = means of all quarters treated with 3 µg LPS solution, C = means of control quarters treated with 10 ml saline (9 g/l)

*means of LPS and control quarters are significantly different ($P \leq 0.05$)

[a]means within quarters differ significantly ($P \leq 0.05$) from time 0 h

During the further course the percentage of PMN increased to maximum values at 84 h of the investigation and reached baseline levels at the end of the studied period.

**Electrolytes**. Na levels in the LPS-1 group decreased within 24 h, then increased as compared to time 0 ($P < 0.05$) to a maximum at 36 h and decreased thereafter to baseline levels (Table 2). However Cl values in the LPS-1 group decreased significantly (compared to time 0) at 24 h and reached its minimum at 36 hours. Thereafter Cl increased to baseline levels within the next 12 h and remained unchanged until the end of the studied period.

As shown in Table 2 Na and Cl levels in LPS-3 quarters showed no response to LPS infusion, albeit Na values increased ($P < 0.05$ as compared to time 0) at 108 h and Cl levels were elevated ($P < 0.05$ as compared to time 0) at 84 h, respectively.

## Cytokines

The TNFα expression in LPS-1 and LPS-3 quarters did not change in response to im LPS infusion (Table 3).

IL-1β expression levels at 12, 24 and 36 h in LPS-1 quarters increased significantly as compared to time 0 (Table 3), but there were no significant differences between LPS-1 and corresponding C quarters after LPS treatment. In LPS-3 quarters IL-1β values increased to its maximum at 12 h ($P < 0.05$) after infusion. Thereafter IL-1β was decreased ($P \leq 0.05$) at 48 and 84 h as compared to C quarters. IL-1β expression levels in C quarter increased to a maximum ($P < 0.05$) as compared to time 0 at 36 h (Table 3).

As demonstrated in Table 3 the expression of IL-6-mRNA in LPS-1 quarters did not change in response

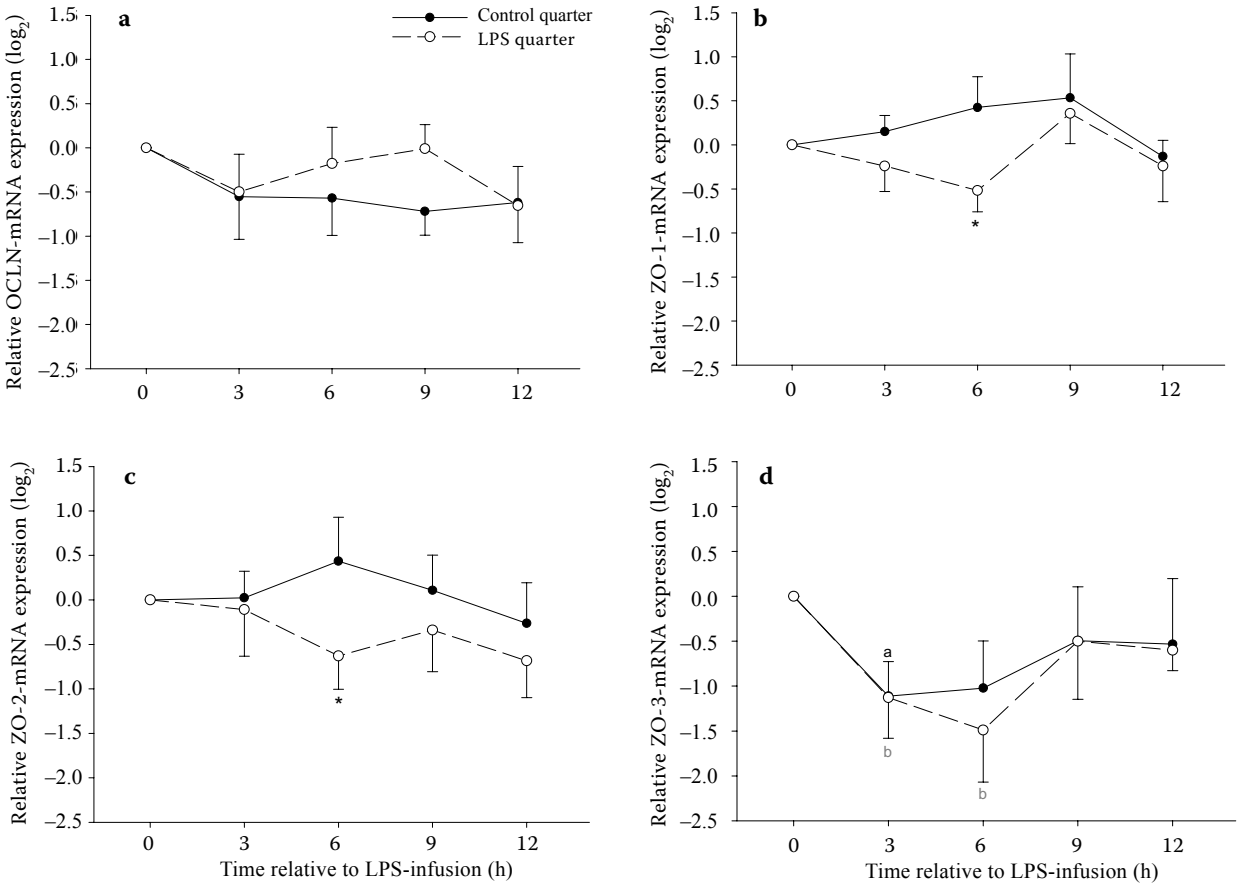


Figure 4. Relative mRNA expression of occludin (OCLN) (a), zonula occludens 1(ZO-1) (b), zonula occludens 2 (ZO-2) (c) and zonula occludens 3 (ZO-3) (d) in mammary biopsy samples of quarters intramammarily infused with 100 μg LPS (LPS quarter) or saline (control quarter). Data are means ± S.E.M. of five cows

*means of LPS-treated quarters are significantly different ($P \leq 0.05$) to control quarters

[a]means within control quarters differ significantly ($P \leq 0.05$) to time 0 h

[b]means within LPS quarters differ significantly ($P \leq 0.05$) to time 0 h

to im LPS infusion. In LPS-3 quarters IL-6 values increased to a maximum ($P < 0.05$) within 12 h after infusion and decreased thereafter (Table 3).

IL-8 did not change within LPS-1 or C quarters and was not different between quarters at any time after the LPS treatment (Table 3). Compared to time 0 IL-8-mRNA expression levels in LPS-3 quarters increased ($P < 0.05$) to highest values at 12 h and decreased thereafter. The measurement at 84 h diminished significantly in comparison to C quarters as well as compared to time 0 (Table 3).

COX-2 expression levels in LPS-1 quarters did not show significant differences within the 36 h of investigation, but thereafter diminished as compared to time 0 from 48 h ($P < 0.05$) to a minimum ($P < 0.0001$) at 84 h and increased ($P < 0.05$) again up to 108 hours. The minimum values at 84 h were also different ($P < 0.05$) from the corresponding COX-2 levels in C quarters (Table 3). COX-2 in LPS-3 quarters was significantly elevated at 48 hours and decreased thereafter (Table 3).

### Experiment II

TJ proteins OCLN, ZO-1, ZO-2 and ZO-3 in mammary biopsy samples are demonstrated in Figure 4. OCLN-mRNA expression did not change significantly in response to the im infusion of 100 µg LPS or saline but there was a numerically elevated value at 9 h after infusion in the LPS quarters. ZO-1- and ZO-2-mRNA expression decreased within the first 6 h after the infusion to their minimum ($P < 0.05$) and increased thereafter to baseline levels.

ZO-3-mRNA expression levels showed no significant differences between LPS and C quarter, whereas expression levels at 3 and 6 h after LPS challenge diminished significantly as compared to time 0.

## DISCUSSION

Former investigators (Vangroenweghe et al., 2004) showed that there are dose dependent changes in milk and blood parameters after im LPS administration. This led to the hypothesis that there might be doses of LPS without any clinical outcome of inflammation but with stimulating effects on the mammary gland immune system. Consequently we analysed factors that were earlier described (Kehrli and Shuster, 1994; Sordillo and Streicher, 2002) to be involved in natural host defence of the bovine mammary gland against invading pathogens.

### Experiment I

In Experiment I we investigated the dose dependent changes in the milk and blood of seven cows after an im infusion with 1 µg and 3 µg of LPS, respectively.

SCC has been established as the generally used parameter for udder health management worldwide. Elevated SCC is an important signal of IMI with pathogens (O'Brien et al., 1999; Pfaffl et al., 2003). Because of the TJ disruption during IMI elevated SCC are associated with increasing amounts of milk electrolytes (Na and Cl) and consequently with augmented EC levels (Stelwagen et al., 1997; Nguyen and Neville, 1998; Bruckmaier et al., 2004). Lactose, the disaccharide produced only by the mammary gland (Wheelock and Rook, 1966; Kuhn and Linzell, 1970), is mainly responsible for osmolarity in milk because of its low molecular weight and is therefore the main determinant of the milk volume (Mielke, 1986; Ontsouka et al., 2003). Therefore changes of lactose concentration might also be associated with leaky TJ and, therefore, the concentration of lactose is mostly in diametrical opposition to milk electrolyte levels (Bruckmaier et al., 2004). All investigated milk parameters (SCC, lactose, EC and the electrolytes Na and Cl) did not change in LPS-1 quarters as compared to C quarters. Our finding of decreasing lactose levels in quarters treated with LPS-1 and C quarters might be due to a great variance amongst animals, but not in response to the treatment. This could mean that we found a LPS dosage that initiates no signs of mastitis but might enhance mammary gland immunity. But it could although mean that there were no signs of mastitis because the LPS was milked out before any reaction developed. These important matters will be discussed later. In contrast to LPS-1 quarters, values of all evaluated parameters in the milk of LPS-3 quarters changed in response to the LPS infusion according to earlier studies (Bruckmaier et al., 1993, 2004; Lee et al., 2003).

During an IMI, WBC and mainly PMN are chemotactically activated by inflammatory mediators, e.g. cytokines, to migrate from peripheral blood into milk (Persson et al., 1993). This is related to decreasing numbers of WBC and PMN in blood after LPS-challenge (Schmitz et al., 2004a) and

increasing numbers of mainly PMN in the SCC of infected quarters (Burvenich et al., 1994; Paape et al., 2002). The same changes are expected in electrolyte values that occur by interdiffusion through a leaky blood milk barrier (Stelwagen et al., 1997; Nguyen and Neville, 1998). During the course of investigation all evaluated blood parameters showed remarkable differences in LPS-1 quarters but not in LPS-3 quarters that were contrary to the expected results but maybe due to a wide range of individual variation amongst all animals.

## Cytokine expression in milk cell mRNA

TNFα released by M is one of the proinflammatory cytokines which is mainly responsible for the endotoxic shock in the acute phase of coliform mastitis (Sordillo and Streicher, 2002). It also enhances the recruitment of PMN from blood into the mammary gland by its chemotactic activity and therefore plays a major role in defence against mastitis (Blum et al., 2000). Pfaffl et al. (2003) demonstrated that milk somatic cells were the major source for TNFα. Although it is previously documented that TNFα concentrations increase after im infusion of LPS (Blum et al., 2000), expression levels in LPS-1 as well as in LPS-3 quarters did not show any remarkable increase in response to the LPS treatment. This finding is obviously due to the very low doses of LPS which did not initiate inflammation. Possibly, some transient changes could not be detected in our experimental design. Previous studies (Blum et al., 2000; Pfaffl et al., 2003; Schmitz et al., 2004b) found maximum expression levels of TNFα already between 2 and 4 h after the LPS challenge. Due to technical reasons no samples were available at this time in the present study.

Besides TNFα which enhances the acute phase inflammatory response, there are other mediators for the first host reaction to invading pathogens: IL-1β and IL-6 (Sordillo and Streicher, 2002; Prgomet et al., 2005), which are also released by M and act as PMN-recruiting chemoattractants (Burvenich et al., 1994; Riollet et al., 2000a; Paape et al., 2002). Our results of more significant changes of the IL-1β expression in the lower LPS dose may be due to a great variation amongst all animals and was not in response to the treatment. The results of a significant increase to the controls in the expression of IL-6 in LPS-3 quarters seems to be in response to the LPS administration but is not in agreement with Prgomet et al. (2005) who found out that IL-6 expression changes analogue to IL-1β levels. In our study IL-6 levels run according to IL-8 expression levels.

In mammary gland immune response IL-8 is responsible for the inflammation and triggers the IL-1 induced PMN recruitment. IL-8 itself is also a very powerful chemoattractant for the accumulation of PMN at sites of inflammation (Riollet et al., 2000b; Sordillo and Streicher, 2002). Persson Waller et al. (2003) found that the induction and the release of IL-8 is independent from the presence of TNFα and IL-1β. Our findings of increasing IL-8 expression levels in LPS-3 quarters 12 h after the LPS-challenge compared to C quarters are according to Riollet et al. (2000b) but different from their results, IL-8 values in our study decreased immediately after the 12 h peak, which may be due to our very low dose of LPS. Cyclooxygenase-1 (COX-1) and COX-2 are the key enzymes in the synthesis of prostanoids i.e. prostaglandins, prostacyclins and thromboxans. COX-1 is expressed in nearly all tissues and it is therefore called the "house-keeping" isoform. COX-2 synthesis is induced by cytokines and therefore it is the predominant enzyme at sites of inflammation (Mitchell et al., 1995; Wittmann et al., 2002; Pfaffl et al., 2003). Our findings in both groups showed no remarkable differences accord to the LPS administration, therefore we have to assume that the wide range of individual variance amongst all animals led to these unspecific results.

## Experiment II

Numerous former investigators (Allen, 1990; Stelwagen et al., 1994, 1997; Nguyen and Neville, 1998; Sloth et al., 2003; Bruckmaier and Meyer, 2005) assumed that changes in TJ protein structure might be existent when SCC and EC levels in milk increased, lactose occurred in blood and a reduced milk yield can be observed. Consequently the influence of 100 μg im infused LPS on TJ proteins OCLN, ZO-1, ZO-2 and ZO-3 in mammary tissue samples was evaluated in order to find confirmations for these suggestions. Earlier publications (Bruckmaier et al., 2004; Schmitz et al., 2004a; Bruckmaier and Meyer, 2005) had shown that the mRNA expression of various factors changed after the LPS-challenge in these mammary tissue samples, which were used in this study.

OCLN was defined to be a TJ associated transmembrane protein (Furuse et al., 1993; Nguyen and Neville, 1998). Fujimoto (1995) found that by using immuno freeze fracture electron microscopy that OCLN is embedded in intramembranous strands of the TJ. Wong and Gumbiner (1997) discovered that a synthetic peptide of OCLN is able to increase TJ permeability but Schneeberger and Lynch (2004) reported that the principal function of OCLN is yet to be determined, but suggested that OCLN is improbably involved in the configuration of ion-selective pores in the TJ. In our study OCLN-mRNA expression showed only a numerical increase 9 h after the LPS-challenge, which might be due to the elevated TNFα levels (Schmitz et al., 2004a) in these tissue samples. According to Mankertz et al. (2000) who found out that the expression of OCLN is regulated by inflammatory cytokines like TNFα or interferon-γ. The cytoplasmatic plaque proteins ZO-1, ZO-2 and ZO-3 are members of the MAGUK (membrane-associated guanylate kinase homologue) family and in contrast to other members of this group each consists of an acidic domain, a basic arginine-rich region and a proline-rich domain (Haskins et al., 1998; Wittchen et al., 1999; Itoh et al., 1999; Schneeberger and Lynch, 2004). All ZO proteins are able to bind directly to OCLN, where as ZO-2 and ZO-3 interacts with ZO-1 but not with each other (Haskins et al., 1998; Wittchen et al., 1999; Schneeberger and Lynch, 2004). Wittchen et al. (1999) allocated the complexity of these protein-protein interactions at the site of TJ to have functional as well as regulatory effects. Our findings of decreasing ($P \leq 0.05$) mRNA expression levels of ZO-1, ZO-2 and ZO-3, respectively, six hours after the LPS administration might be the expression of an increasing TJ permeability. These results might be explained by the protein-protein interaction theory of the Wittchen et al. (1999) group.

## CONCLUSION

In conclusion, on the one hand it can be assumed that a dose of 1 μg im infused LPS does not induce clinical signs of inflammation in the mammary gland but unfortunately has also no clear enhancing effects to mammary gland immunity, whereas the dose of 3 μg im LPS causes signs of IMI. On the other hand the depression in TJ protein expression *in vivo* proves the presumption that during mastitis LPS and increased SCC causes loss of TJ integrity.

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