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Investigation of the pathophysiology of bacterial mastitis using precision-cut bovine udder slices

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ABSTRACT

Mastitis in cattle is a major health problem as well as incurring high costs for the dairy industry. To assess the suitability of precision-cut bovine udder slices (PCBUS) for bovine mastitis studies, we infected PCBUS with 2 different *Staphylococcus aureus* strains. Accordingly, we investigated both the tissue response to infection based on immune mediators at the mRNA and protein levels and the invasion of bacteria within the tissue. The studied proteins represent immune mediators of early inflammation [IL-1β, tumor necrosis factor-α (TNF-α), prostaglandin E_2 (PGE_2)] and showed a time-dependent increase in concentration. Infection of PCBUS with *S. aureus* resulted in increased expression of proinflammatory cytokines and chemokines such as TNF-α, C-C motif chemokine ligand 20 (*CCL20*), IL-1β, IL-6, and IL-10, but not C-X-C motif chemokine ligand 8 (*CXCL8*), lingual antimicrobial peptide (*LAP*), or S100 calcium binding protein A9 (*S100A9*) at the mRNA level. To compare the data acquired with this model, we carried out investigations on primary bovine mammary epithelial cells. Our results showed that the immune responses of both models—PCBUS and primary bovine mammary epithelial cells—were similar. In addition, investigations using PCBUS enabled us to demonstrate adherence of bacteria in the physiological cell network. These findings support the use of PCBUS in studies designed to further understand the complex pathophysiological processes of infection and inflammation in bovine mastitis and to investigate alternative therapies for mastitis.

Key words: bovine mastitis, precision-cut bovine udder slices, primary bovine mammary epithelial cells, *Staphylococcus aureus*, immune response

INTRODUCTION

Bovine mastitis is one of the most important diseases in dairy cows worldwide. It not only causes pain for the animal but also results in considerable financial losses for farmers (Seegers et al., 2003; Sinha et al., 2014; Romero et al., 2018). It is unclear whether costs are higher in clinical or subclinical mastitis. In addition to treatment-related costs, losses due to reduced milk quantity, low milk quality, or premature slaughter of the infected cow must be considered (Huijps et al., 2010; van Soest et al., 2016; Krömker and Leimbach, 2017). Bovine mastitis is usually caused by bacteria (McDougall et al., 2009; Viora et al., 2014; Klaas and Zadoks, 2018), with *Staphylococcus aureus* being one of the major causative pathogens (Ren et al., 2020; Rossi et al., 2020). Subclinical mastitis, with no visible signs of inflammation in the udder but long-term milk losses due to high milk SCC, may also be caused by *S. aureus*. Moreover, subclinical mastitis increases the risk of pathogen spread within the herd (Watts, 1988; Li et al., 2017).

Antibiotic therapy approaches for subclinical *S. aureus* mastitis have proven to be ineffective. In view of antibiotic resistance and consumer health, the use of antibiotics for the treatment of mastitis must be critically questioned, because of their ineffectiveness in successfully treating all cases. The use of antibiotics is not indicated in many cases (McDougall et al., 2009; De Vliegher et al., 2012; Trevisi et al., 2014), and, to date, antimicrobial-resistant microorganisms play a minor role among bovine udder pathogens (Oliver and Murinda, 2012; Krömker and Leimbach, 2017;

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McDougall et al., 2021). Nonetheless, consensus in the literature suggests the presence of increased antimicrobial resistance over time (Abdi et al., 2021; Molineri et al., 2021), and optimization of antimicrobial drugs is a desirable goal. Furthermore, identification of alternative and innovative treatments is necessary (Krömker and Leimbach, 2017). Many studies in this area use different cell lines with various properties to address specific problems. The focus is often on mammary epithelial cells because they are known to play an essential role in existing infections. They contribute to the recruitment of immune cells and thus to the recognition and elimination of the bacteria (Gilbert et al., 2013). Wallis et al. (2019) showed an alternative treatment option to the use of antibiotics that was tested in vitro. They demonstrated that several strains of lactobacilli can suppress pathogenic *S. aureus* biofilms and form a more protective biofilm. These promising findings set the basis for further studies with both bovine udder and ex vivo udder tissue. In vivo research has been conducted to investigate the pathophysiology of the udder, in order to understand the interactions between the individual factors of bovine mastitis (Moosavi et al., 2014; Brodhagen et al., 2019; Schmenger et al., 2020). The results of these studies provide the most comprehensive insight into the infection process of bovine mastitis; however, to obtain sample material for investigation, infected animals must be euthanized (Petzl et al., 2008; Whelehan et al., 2011; Brodhagen et al., 2019). To combine the advantages of in vitro and in vivo approaches in studies, precision-cut tissue slices can be used. This technique has been used successfully to investigate pathophysiological processes and pharmacological mechanisms in several organs (Graaf et al., 2007; Vietmeier et al., 2007; Barton et al., 2010). Moreover, recent data from our group described the possibility of using this approach in the bovine udder for a variety of studies on physiologically related cell structures (Filor et al., 2021).

The aim of this work was to establish an artificial infection model of precision-cut bovine udder slices (**PCBUS**) to study the pathological processes of bovine mastitis ex vivo.

MATERIALS AND METHODS

Ethical approval was not required for this study because only tissues from slaughtered animals were used.

Tissue Sourcing

Tissue was obtained as recently described by Filor et al. (2021). In brief, udders of slaughtered cows (German Holstein Friesian) were examined for udder health and immediately infused with heparinized tyrode solution to prevent clot formation in the vessels. The gland tissue was prepared using a dermatome (Zimmer UK Ltd.) to generate 250-µm-thick slices. To create a uniform surface, slices were then processed with a 6-mm biopsy punch. Preparation of approximately 200 PCBUS from one udder quarter was possible with this method. The tissue was washed several times with a highly concentrated antibiotic solution comprising RPMI-1640 medium supplemented with 20% fetal calf serum (Biochrom GmbH), 10% penicillin-streptomycin (10,000 international units mL; 10,000 µg/mL, Biochrom GmbH), 10% amphotericin B (PAA Laboratories GmbH), and 15 µg/mL gentamicin (PAA Laboratories GmbH), until no more milk residues were visible. The PCBUS were then incubated in 1 mL of maintenance medium (RPMI-1640 medium supplemented with 10% fetal calf serum, 1% penicillin-streptomycin, 1% amphotericin B, and 15 µg/mL gentamicin) in a humidified atmosphere containing 5% CO_2 at 37°C, and the medium was replaced every 48 h.

Viability of PCBUS and Testing for Antimicrobial Contamination

Viability of PCBUS was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (**MTT**) assay, as described by Lambermont et al. (2014), was used with some modifications (Filor et al. 2021). In brief, the MTT assay was performed from the moment the PCBUS were generated until the end of each experiment. The assay was performed on 5 PCBUS of 6 udders each (n = 6). The PCBUS were transferred to a 24-well plate containing 900 µL of RPMI-1640 medium and 100 µL of MTT solution (7 mg/mL; Sigma-Aldrich). After 15 min of incubation, the supernatant was discarded and 200 µL of a mixture of 5% formic acid + 95% propanol (Sigma-Aldrich) was added. After 40 min, 100 µL of supernatant was transferred to a 96-well plate (Greiner Bio-One) for absorbance measurement at 570 nm (MRX-Reader, Dynatech). As a negative control, PCBUS were digested with Triton X-100 (Sigma-Aldrich; 1 mL, 30 min, 37°C, 5% CO_2 atmosphere) 30 min before the start of the MTT assay. All steps were performed at room temperature in the dark.

In preliminary studies, we observed microbial contamination of PCBUS when fewer washing steps were performed; although we performed a California Mastitis Test before tissue preparation, we processed samples in which the milk SCC was <100,000 cells/mL only. Therefore, we washed the sections until no residual milk was seen. To verify the success of the washing steps in the PCBUS generation process, microbiological tests

were performed after 48 and 96 h. For this purpose, 100 μL of maintenance medium from 2 wells per 24-well plate were incubated on Columbia agar plates with 7% sheep blood (Thermo Fisher Scientific) in an incubator at 37°C for 24 h. The examined supernatant was considered free of contamination when no bacterial growth was visible. In the rare event that microbial contamination occurred, we excluded that PCBUS from further experiments. On average, contamination was detected in approximately 5% of the generated PCBUS per run.

Bacterial Strains

Two different *S. aureus* strains were used for infection setup: one from the American Type Culture Collection (*S. aureus* ATCC 29213) and one isolated from the udder of a cow suffering from mastitis (*S. aureus* Rd 5, a multidrug-resistant clinical bovine mastitis isolate; Fessler et al., 2010). In brief, *S. aureus* were subcultured on Columbia agar plates with 7% sheep blood (Thermo Fisher Scientific) under aerobic conditions at 37°C. Then, 1 colony from an overnight culture was resuspended in sterile tryptone-NaCl (0.1% tryptone, 0.85% NaCl) and adjusted to an optical density at 600 nm (OD_{600nm}) of 0.08. To ensure that the immune response during the infection experiment was triggered by the *S. aureus* strain, we examined the supernatants of the infection experiment by MALDI-TOF MS after 1, 4, 6, and 24 h.

Infection of PCBUS

For infection experiments, 8 PCBUS were used for each treatment. We used the supernatants of 3 PCBUS for ELISA and, at each time point (1, 4, 6, and 24 h), one PCBUS was sampled for histological examination after 2, 6, and 24 h and one was saved for quantitative (**q**)PCR after 6 h. One day before infection, PCBUS were washed in sterile PBS (pH 7.4) and incubated in medium without antibiotic additives. For bacterial infection, PCBUS were inoculated with approximately 2.5×10^7 cfu/mL *S. aureus* ATCC 29213 or Rd 5 in 500 μL of RPMI-1640/well for 24 h in a humidified atmosphere containing 5% CO_2 at 37°C. For investigation of cytokine release from the supernatants by ELISA, samples were taken after 1, 4, 6, and 24 h and stored at −20°C. Therefore, we removed 30 μL of the medium and refilled the well with 30 μL of fresh medium without bacteria. This method for sample collection was applied to counteract the variable tissue composition of different PCBUS. Thus, we were able to observe the time course of cytokine release of a single PCBUS. After 2, 6, and 24 h, a single PCBUS infected with each *S. aureus* strain alone (2 PCBUS in total) was collected for histological examination. For histological examination, PCBUS were fixed in 10% buffered formalin for 48 h and embedded in paraffin wax using standard techniques. Additionally, PCBUS infected with the Rd 5 strain were collected for reverse transcription (**RT**)-qPCR after 6 h. All experiments were performed on 6 udders (n = 6). Sample storage for RT-qPCR was done after snap freezing in liquid nitrogen at −80°C until later homogenization.

Isolation of Primary Bovine Mammary Epithelial Cells

Isolation of primary bovine mammary epithelial cells (**pbMEC**) was performed in parallel with PCBUS harvesting, using the same medium. The cells were isolated according to Hu et al. (2016) with some modifications. First, a PCBUS (250 μm thick) was taken and washed until no milk contamination was visible. Then, sterile scalpel blades were used to cut pieces of about 1 × 1 mm, which were treated with PCBUS washing medium and subsequently washed with PBS. The pieces were transferred to a 15-mL tube and incubated overnight at 4°C in 5 mg/mL dispase II solution (Sigma-Aldrich). The next day, the solution was removed, and the pieces were incubated in a 0.05% trypsin, 0.02% EDTA solution (Biochrom GmbH) for 60 min at 37°C and 5% CO_2. After incubation, the cell suspension was passed through a cell strainer (pore size 70 μm, polypropylene; Sarstedt AG and Co. KG), rinsed with maintenance medium, and subsequently centrifuged at 600 × *g* for 5 min at room temperature. Cells were plated at a density of $2 \times 10^4/cm^2$ in a 25-cm^2 flask coated with rat-tail collagenase (Roche Applied Sciences). Medium was changed every 48 h and cells were subcultured at approximately 80% confluence. To separate epithelial cells and fibroblasts, the Percoll density gradient centrifugation method was applied according to Montpetit and Tenniswood (1989). For infection experiments, only cells from the epithelial cell layer were used.

Infection of pbMEC

The infection experiment was performed in the same way as the PCBUS infection experiment. Cells were seeded at a density of 5×10^4 cells/cm^2 in 24-well plates. When cells reached approximately 80% confluence, they were washed with sterile PBS and incubated for an additional 24 h in antibiotic-free medium. Inoculation dose and sampling times were chosen as in the PCBUS infection experiment. Cells from 6 udders (n = 6) were used with 3 technical replicates each.

Cell Viability of pbMEC

After the supernatants from the infection experiment were collected, cell viability was determined by MTS [(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)] assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay), according to the manufacturer's protocol. Briefly, the supernatant containing the infected medium of the samples was removed from the cells. Then, 200 μL of CellTiter 96 Aqueous One Solution Reagent in RPMI-1640 medium (1:6) was added to each well of the 24-well plate and the plate was incubated at 37°C for 1 h in a humidified 5% CO_2 atmosphere. To measure the amount of soluble formazan produced by the cellular reduction of MTS, 100 μL of supernatant was transferred to a 96-well plate and absorbance was determined at 490 nm using a multiwell plate reader (Dynatech Laboratories). Each experiment was performed 6 times. Background absorbance (490 nm) was corrected by using control wells containing the same volumes of culture medium and CellTiter 96 Aqueous One Solution Reagent.

Histological Examination of PCBUS

To examine PCBUS morphology, hematoxylin-eosin (**HE**) staining was performed as previously described by Feldman and Wolfe (2014). Immunofluorescence staining was used to detect and localize the bacteria in PCBUS, and staining was conducted as previously described for histological slices (de Buhr et al., 2019). *Staphylococcus aureus* were stained with a rabbit anti-*S. aureus* antibody (IgG: stock 4 mg/mL, ab20920, 1:100; Abcam). Antibody was diluted in blocking buffer (1% BSA, 5% goat serum, 2% cold water fish gelatin, 0.05% Tween 20, 0.05% Triton X-100 in 1× Tris-buffered saline). Immunoglobulin G from rabbit serum (Sigma I5006, 1.16 mg/mL) was diluted 1:29 as isotype control. As secondary antibody, Alexa Fluor 488 anti-rabbit (1 mg/mL; Thermo Fisher Scientific) was used 1:500 in blocking buffer. Bacteria were visualized using immunofluorescence microscopy and recorded using a Leica TCS SP5 AOBS confocal inverted-base fluorescence microscope with HCX PL APO 40× 0.75 to 1.25 oil immersion objective. The settings were adjusted using isotype control antibodies in separate preparations.

Cytokine Release from PCBUS and pbMEC

The cytokines IL-1β, prostaglandin E_2 (**PGE_2**), and tumor necrosis factor-α (**TNF-α**) were measured in supernatants using ELISA kits according to the manufacturers' specifications, and each sample was tested in technical triplicates (Bovine Interleukin-1β Reagent Kit, Invitrogen/Thermo Fisher Scientific; Prostaglandin E_2 Express ELISA Kit; Cayman Chemical Company; Bovine TNF-α DuoSet ELISA Development Systems, R&D Systems). Dilutions and appropriate time points were determined in preliminary tests (data not shown). Because we were particularly interested in investigating the early phase of infection, we analyzed 3 time points: 1, 4, and 6 h. However, TNF-α was not detected during these time periods, so we analyzed TNF-α at 4, 6, and 24 h. After addition of the bacteria, supernatants were obtained at appropriate times and examined for the release of IL-1β, TNF-α, and PGE_2. This was done with PCBUS from 6 udders (n = 6) with 3 technical replicates. Control PCBUS were treated with medium only.

RT-qPCR of PCBUS and pbMEC

Based on studies by Petzl et al. (2016) and Brand et al. (2021), we chose to examine gene expression of the mediators listed in Table 1. Petzl et al. (2016) investigated the reaction of udder tissue after instillation of *S. aureus* and *Escherichia coli* into the udder in vivo, whereas Brand et al. (2021) performed similar studies on isolated perfused bovine udder. Both studies yielded promising results for the investigation of the early phase of inflammation, making these mediators of interest in our studies as well. Tissue homogenization was performed under liquid nitrogen. RNA extraction, reverse transcription to cDNA, and quantification of expression levels of genes of interest were performed as described in Schenke et al. (2020), with a few modifications concerning reference gene stability assessment and presentation of the data, as described below. Nine random study samples (5 noninfected and 4 infected) were analyzed for expression of 8 candidate reference genes: *EIF3K*, *GAPDH*, *RN18S1*, *ACTB*, *UXT*, *PPIA*, *RPLP0*, and *HPRT1* and were assessed for stable expression and optimal number of reference genes via the geNorm algorithm (Vandesompele et al., 2002) implemented in the qbase+ software, version 3.0 (Biogazelle; www.qbaseplus.com). Mean values of the recommended stably expressed reference genes *RPLP0* and *HPRT1* were used as the reference gene normalization factor for each sample. The target genes were *CCL20*, *CXCL8*, *IL1B*, *IL6*, *IL10*, *LAP*, *S100A9*, and *TNFA* (Table 1). Relative gene expression levels were calculated according to the $2^{-\Delta Cq}$ method (Schmittgen and Livak, 2008). The PCBUS and pbMEC were examined from at least 4 different udders and 3 technical replicates ($n \geq 4$, 3 technical replicates).

Table 1. Primers used for quantification of gene expression (For = forward; Rev = reverse)

Target	Sequence (5′–3′)	Accession number
EIF3K	For: CATGTCGTGGGCATCACGTA Rev: CCTTTAGCTGGCTGTCTGTCA	NM_001034489.2
GAPDH	For: GGCGTGAACCACGAGAAGTA Rev: GGCGTGGACAGTGGTCATAA	NM_001034034.2
RN18S1	For: CGGGGAGGTAGTGACGAAA Rev: CCGCTCCCAAGATCCAACTA	NR_036642.1
ACTB	For: GATCAAGATCATCGCGCCCC Rev: GATCAAGATCATCGCGCCCC	NM_173979.3
UXT	For: GCGACTCCAGGAAGCTAAC Rev: CCAAGGGCCACATAGATCCG	NM_001037471.2
PPIA	For: CGTCTCTTTTGAGCTGTTTGCAG Rev: GGACTTGCCACCAGTACCATT	NM_178320.2
RPLP0	For: TTACCCAACCGTCGCATCTG Rev: GGCCTTGACCTTTTCAGCAAG	NM_001012682.1
HPRT1	For: TATGGACAGGACCGAACGGC Rev: TCCAACAGGTCGGCAAAGAA	NM_001034035.2
CCL20	For: GAATTTGCTCCTGGCTGCTT Rev: AGTTGCTTGCTTCTGACTTGC	NM_174263.2
CXCL8	For: AACGAGGTCTGCCTAAACCC Rev: TTGCTTCTCAGCTCTCTTCACA	NM_173925.2
IL1B	For: AAAAATCCCTGGTGCTGGCT Rev: ATGCAGAACACCACTTCTCGG	NM_174093.1
IL6	For: AAGCGCATGGTCGACAAAAT Rev: AAGCGCATGGTCGACAAAAT	NM_173923.2
IL10	For: TATCCACTTGCCAACCAGCC Rev: GGCAACCCAGGTAACCCTTA	NM_174088.1
LAP	For: TCTGCTGGGTCAGGATTTACTC Rev: GCAGCATTTTACTTGGGCTCC	NM_203435.4
S100A9	For: CTTCTCGGCTTGGTAGGAGGG Rev: GTTTGAGCAACGGAGTTGGG	NM_001046328.2
TNFA	For: GGTTCAAACACTCAGGTCCTCT Rev: CGGAGAGTTGATGTCGGCTA	NM_173966.3

Statistical Analysis

The results were analyzed to determine whether a significant difference in protein and mRNA contents could be detected between uninfected and infected PCBUS or pbMEC at 1, 4, 6, and 24 h. The control group and respective measurement times were compared with those of the infected group using the Kruskal-Wallis test. Differences in mRNA levels between samples to check for both the influence of infection and the model used (PCBUS or pbMEC) were tested for significance using a 2-way ANOVA with a post hoc Tukey's multiple comparison test. $P < 0.05$ was set as the significance level. Statistical evaluation was performed with GraphPad Prism (version 9.0.2, GraphPad Software Inc.).

RESULTS

Viability of PCBUS

The MTT assay was performed to investigate the viability of the generated PCBUS. The assay was performed immediately after sample collection and continuously every 24 h until the end of the infection experiment. As a negative control, PCBUS were treated with Triton X-100. Because cell viability values below 70% are considered to indicate reduced viability according to the ISO standard 10993-5 (ISO, 2009), this cut-off was chosen for analyses. Figure 1 shows the viability of PCBUS over a 4-d period. This time frame corresponds to the total duration of the experiment. When viability values were below the 70% limit, PCBUS from that udder were excluded from trials. Below the self-selected 70% cut-off were 4 of 30 PCBUS tested at d 0, 15 of 30 PCBUS at d 1, 14 of 30 PCBUS at d 2, 15 of 30 at d 3, and 11 of 30 at d 4.

Histological Examination of PCBUS

To confirm that the physiological tissue structure of a bovine udder was preserved after preparation of PCBUS, histological examinations using HE staining were performed. Figure 2 shows the well-preserved morphology and the presence of mammary tissue, consisting of alveolar and ductular structures surrounded by interstitial tissue. This suggests that the procedure used to prepare the PCBUS caused minimal tissue damage.

After infection of PCBUS, the presence of bacteria in PCBUS was examined by immunofluorescence staining after 2, 6, and 24 h. Figure 3 shows that both *S. aureus*

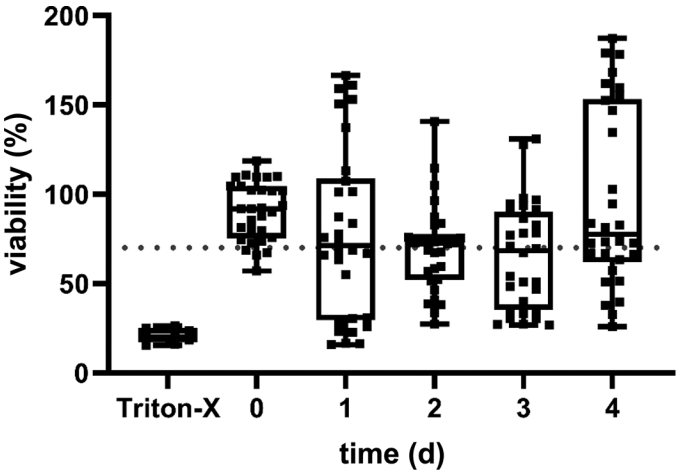

Figure 1. Viability of precision-cut bovine udder slices (PCBUS) from the time of harvesting until the end of the infection trial. Triton X represents the result of the negative control; the red dotted line indicates a viability cut-off of 70% (ISO standard 10993-5:2009; ISO, 2009); data are given as median ± standard deviation of 5 PCBUS from 6 udders for each time point. In the plots presented, the box represents the interquartile range (25th to 75th percentile), and the midline indicates the median. Whiskers extend down to the minimum and up to the maximum value. Square symbols represent each individual value as a point.

strains were mainly localized to the outer edge of the PCBUS, and that the number of bacteria increased during the course of the experiment.

Cytokine Secretion by PCBUS and pbMEC

Figure 4 shows the release profile of the investigated cytokines of pbMEC and PCBUS. In PCBUS infected with *S. aureus* ATCC 29213 and Rd 5, we observed significant increases of IL-1β over a period of 1 to 6 h ($P \leq 0.01$; $P \leq 0.001$). A time-dependent increase in bovine IL-1β was also detected in cell culture experiments in both ATCC 29213- and Rd 5-infected cells. The difference compared with control became significant after 4 h ($P \leq 0.01$; $P \leq 0.001$). Interestingly, the increase in IL-1β concentration of the pbMEC experiments was of similar magnitude to that of the PCBUS experiments.

Significant release of PGE_2 was observed 4 h after infection in PCBUS infected with strain Rd 5 ($P \leq 0.05$). After 6 h, significantly elevated concentrations of PGE_2 were observed in both bacterial treatments ($P \leq 0.05$; $P \leq 0.01$). A significant difference from control was noted after 4 h in pbMEC infected with *S. aureus* ATCC 29213 ($P \leq 0.05$). Figure 4 displays individual data points, which demonstrates the much more pronounced variability in PGE_2 concentration after infection of the PCBUS with *S. aureus* ATCC 29213, as opposed to infection with the Rd 5 strain. Interestingly, this applies equally to infection of pbMEC. In contrast, a reduction in PGE_2 release after infection was noticeable when comparing pbMEC and PCBUS. The PGE_2 concentration in the pbMEC was reduced by a factor of 100 compared with the measured concentration in PCBUS. However, we have to assume different cell numbers in both models, preventing comparison of absolute numbers. Because TNF-α was not detected until 4 h, assessment of TNF-α concentration was performed after 4, 6, and 24 h. After 4 h, the results showed a significant increase in TNF-α concentration in PCBUS treated with the mastitis isolate ($P \leq 0.01$), whereas both bacterial treatments showed a significant difference after 24 h ($P \leq 0.05$; $P \leq 0.001$). The individual values presented in Figure 4 showed high variability, especially in PCBUS infected with the mastitis isolate, which was not detected in PCBUS infected with the ATCC strain. When determining the TNF-α concentration in the pbMEC experiments, we observed a significant increase after 6 and 24 h for pbMEC infected with ATCC 29213 and after 24 h for the pbMEC incubated with Rd 5. Again, cells infected with Rd 5 displayed high variability.

RT-qPCR of PCBUS and pbMEC

Because transcript expression pattern of PCBUS and pbMEC in response to infection was of particular interest in this study, RNA was isolated from PCBUS and pbMEC infected with *S. aureus* Rd 5 and respective control tissues or cells and analyzed by RT-qPCR (Figure 5). Expression of *CCL20* and *IL6* was highly upregulated, (c, $P \leq 0.001$ vs. noninfected control; d, $P \leq 0.0001$ vs. non-infected control), whereas most of the inflammatory genes (*IL1B*, *IL10*, and *TNFA*) were slightly significantly upregulated in infected PCBUS and pbMEC with noninfected control tissue (a, $P \leq 0.05$ vs. non-infected control). In contrast, no significant upregulation was seen in *LAP*, *S100A9*, and *CXCL8* compared with the noninfected control. In some cases (e.g., for *CXCL8*, *IL10*, and *TNFA*), expression of target genes was significantly lower in pbMEC compared with PCBUS, independent of the infected or noninfected status of the samples.

DISCUSSION

The aim of the present study was to further characterize the in vitro model of PCBUS. For this purpose, we established an artificial infection to test whether PCBUS is a valuable tool to study infection of bovine mastitis and bridge the gap between simple 2-dimensional cell culture and complex in vivo studies. Our results support the initial hypothesis of obtaining comparable results to previously used study designs when

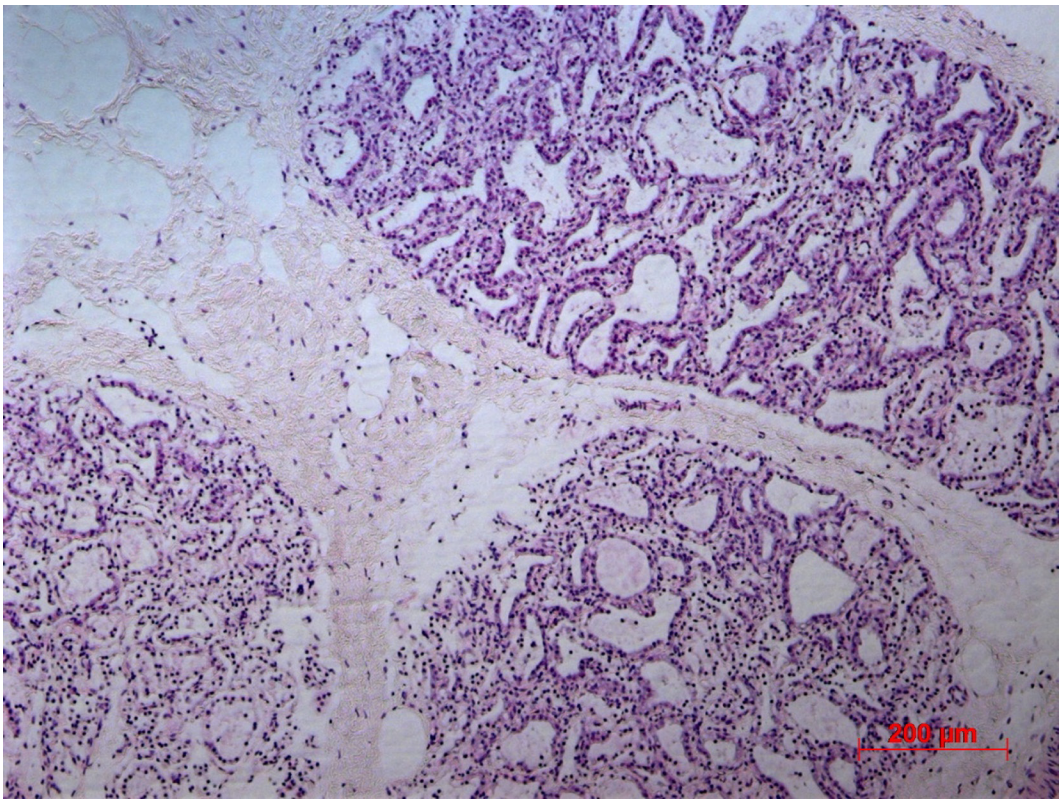

Figure 2. Hematoxylin-eosin staining of precision-cut bovine udder slices showing physiological morphology of bovine udder; magnification 10×; scale bar = 200 µm.

examining the inflammatory process of bovine mastitis in infected PCBUS (Petzl et al., 2016; Magro et al., 2017). Because *S. aureus* plays a major role in the development of subclinical mastitis, we initially chose to investigate this difficult-to-treat form of mastitis. The focus of our study was the artificial infection of PCBUS but because the use of pbMEC is a well-established model (Günther et al., 2012; Zhang et al., 2018; Zaatout et al., 2019), we carried out targeted investigations at the mRNA and protein levels in both models.

We were able to reliably show the viability of PCBUS over a period of 4 d after harvesting, as previously demonstrated by our group (Filor et al., 2021). Moreover, we confirmed that the method used to obtain PCBUS was robust and reproducible. Initially, we looked at the histology of PCBUS and confirmed that the morphology of the bovine udder was preserved after processing. The image in Figure 2 is representative of the obtained PCBUS. Although morphology varied between udders, this fact can be used in further investigations to perform studies at different lactation stages, as described by Magro et al. (2017) with heifer udder explants.

Following ex vivo infection, we visualized *S. aureus* within PCBUS using immunofluorescence microscopy (Figure 3). We observed that the density of bacteria increased over time. Correlated with the results of the supernatant studies, this experiment confirmed an interaction of *S. aureus* and PCBUS after ex vivo infection (Pereyra et al., 2016; Josse et al., 2017).

The main focus of this study was to compare the concentration of proinflammatory cytokines after infection of PCBUS with those of infected pbMEC. Because we were primarily interested in the early phase of infection of bovine mastitis, we decided to investigate IL-1β, PGE_2, and TNF-α by ELISA. The increase in the expression of these inflammatory mediators has previously been detected by administration of the cell wall components LPS and lipoteichoic acid (**LTA**) in PCBUS (Filor et al., 2021). We applied an infection dose of 2.5×10^7 cfu/mL, consistent with published studies on infection of primary mammary epithelial cells, in vivo mastitis experiments, and infection of precision-cut lung slices. For example, Günther et al. (2011) conducted a 24-h event study on inoculated primary mammary epithelial cells with heat-inactivated particles of *E. coli* or *S. aureus* with an infective dose of 10^7 particles/mL. In an in vivo study by Petzl et al. (2016), udder quarters were infected intracisternally

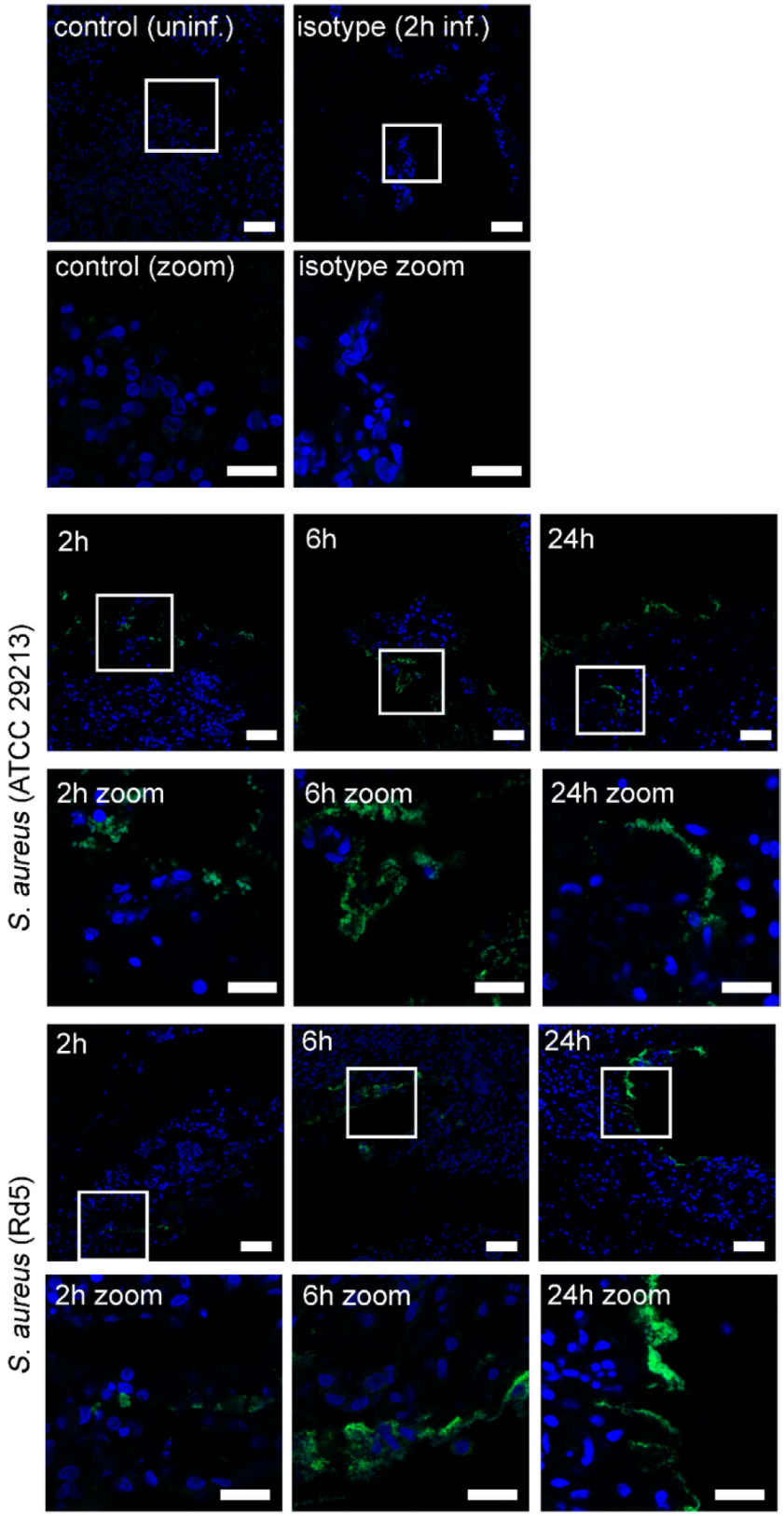

Figure 3. Immunofluorescence staining of *Staphylococcus aureus* in precision-cut bovine udder slices (PCBUS). Uninfected and infected PCBUS slices (4 μm) were analyzed by confocal immunofluorescence microscopy for *S. aureus*. The settings were adjusted to a respective isotype control. Representative images are presented (blue = DNA, green = *S. aureus*). Scale bars: overview = 100 μm, zoom = 20 μm.

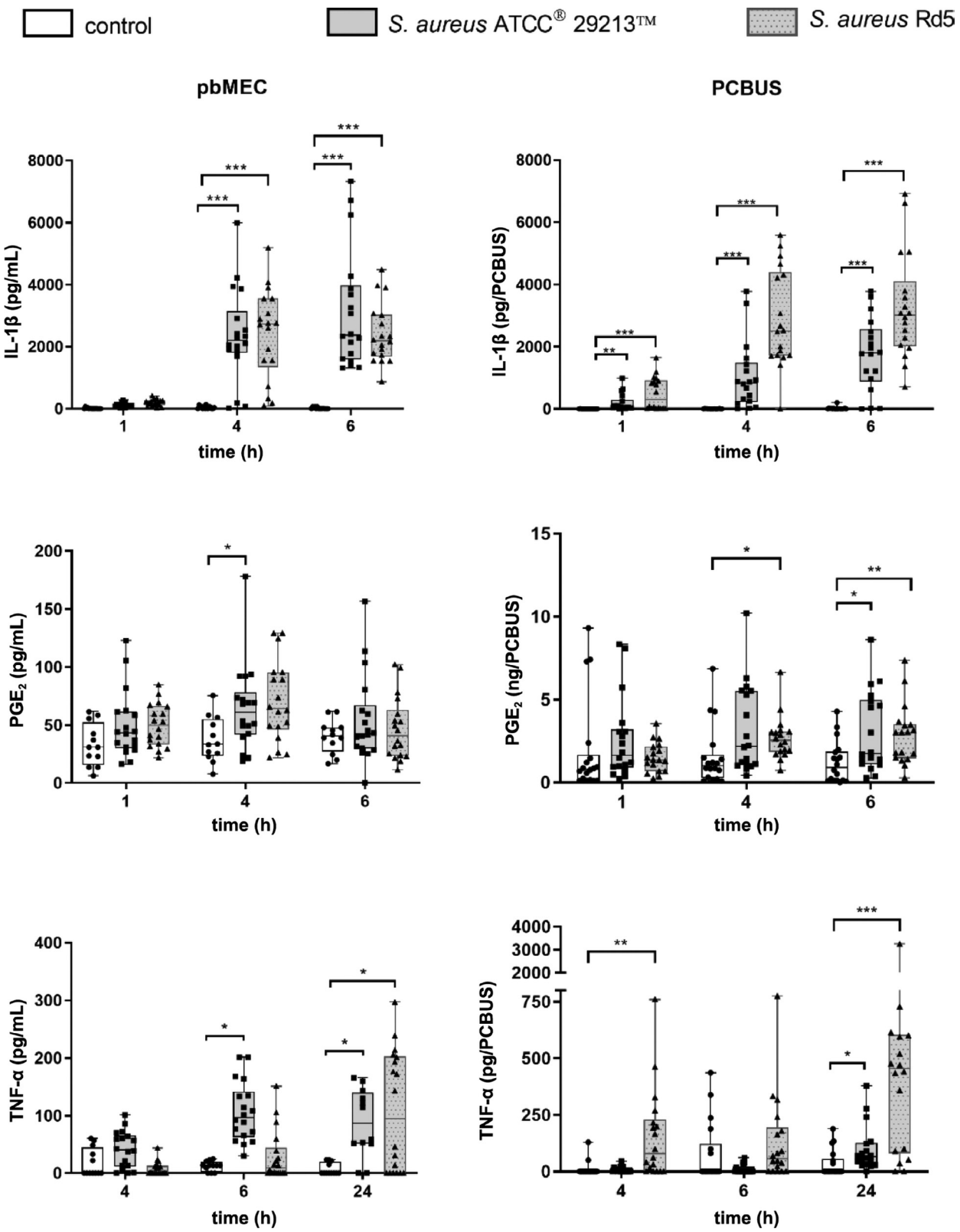

Figure 4. Cytokine profile [IL-1β, prostaglandin E2 (PGE_2), and tumor necrosis factor-α (TNF-α)] after infection of primary bovine mammary epithelial cells (pbMEC) and precision-cut bovine udder slices (PCBUS) with *Staphylococcus aureus* (strain ATCC 29213 or Rd 5). Analysis was performed with 3 PCBUS from 6 udders each; data are given as mean ± SD of cells from 6 different cows and 3 technical replicates or 3 PCBUS from 6 udders each (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$).

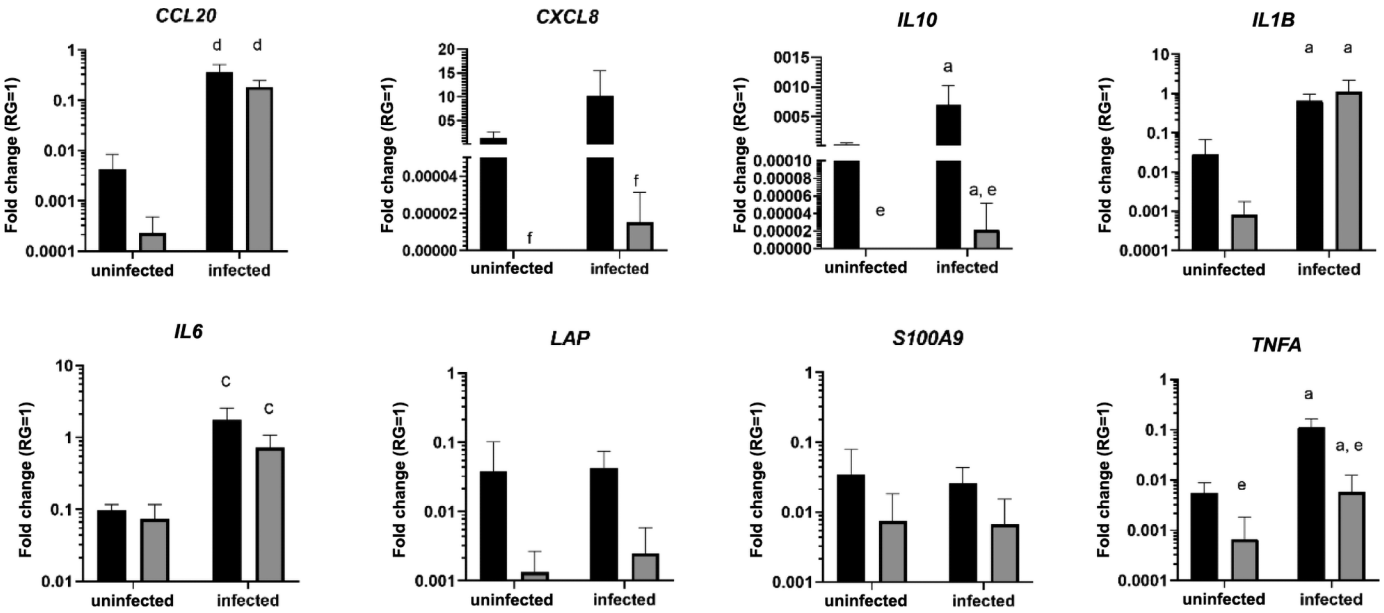

Figure 5. Relative gene expression levels of *CCL20*, *CXCL8*, *IL10*, *IL1B*, *IL6*, *LAP*, *S100A9*, and *TNFA* 6 h after infection of precision-cut bovine udder slices (PCBUS; black bars) and primary bovine mammary epithelial cells (pbMEC; gray bars) with *Staphylococcus aureus* Rd 5. Reference genes (RG) 60S acidic ribosomal protein P0 (*RPLP0*) and hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) were used for normalization of expression. Data are shown as mean ± SD. Gene expression levels in infected and uninfected PCBUS and pbMEC were analyzed with at least 4 biological replicates with 3 technical replicates each. To optimize visualization, the expression of differences on the y-axis is mostly logarithmized to base 10, with 2 exceptions, *CXCL8* and *IL10*, where expression in the uninfected pbMEC was zero and the data could not be logarithmized and are therefore depicted on a linear y-axis. a = $P \leq 0.05$ vs. uninfected control; c = $P \leq 0.001$ vs. uninfected control; d = $P \leq 0.0001$ vs. uninfected control; e = $P \leq 0.05$ PCBUS vs. pbMEC; f = $P \leq 0.01$ PCBUS vs. pbMEC.

with 2.5×10^6 cfu/2 mL of *E. coli* or *S. aureus*, and samples were collected hourly up to 3 h after infection. When precision-cut lung slices were infected with *Streptococcus suis*, Meng et al. (2015) used an infective dose of 10^7 cfu in 500 μL over a 4-h period. Our results showed that the concentrations of the immune mediators generally increased in a time-dependent manner compared with controls. Further studies regarding the amount of administered bacteria are of interest. Magro et al. (2017) performed similar experiments on udder explants from heifers. In addition to the LPS and LTA stimuli, they also used *S. aureus* with infective doses of 10^2 cfu/mL and 10^3 cfu/mL. Although they did not observe any differential expression of *TNFA*, they showed increased mRNA expression of *IL1A* and *IL6* between 1 and 6 h. Thus, adjustment of the infectious dose is possible and, depending on the rationale, it can be adapted to the immune status and general condition of the cow.

We compared the cytokine pattern of PCBUS with that using the well-established pbMEC model. As shown in Figure 4, cytokine concentrations were similar between PCBUS and pbMEC. In particular, the concentration of IL-1β did not differ between the 2 models. The differences in cytokine concentrations between pbMEC and PCBUS could be due to the variability in cell numbers. In future studies, reference to the total protein content would be useful.

Of interest, the concentration of PGE_2 released was 100-fold higher in PCBUS than in pbMEC. Because the cell number of pbMEC was the same in all experiments and the concentration of IL-1β was comparable in pbMEC and PCBUS, the observed difference could be explained by the physiological cell composition of PCBUS rather than by a lower cell number in the pbMEC experiments. In contrast to pbMEC (which contains only epithelial cells), PCBUS also contain endothelial and immune cells. These cells are involved in de novo synthesis of prostaglandins when cells are activated by mechanical trauma or by specific cytokines, growth factors, and other stimuli, which might explain the high PGE_2 concentration in PCBUS compared with pbMEC (Funk, 2001; Park et al., 2006). Furthermore, no increase in PGE_2 concentration over time was observed in pbMEC; the baseline value was constant from 1 to 6 h. In contrast, a significant time-dependent increase in concentration was detected for PGE_2 concentration in PCBUS. However, although this increase was significant, it was less than for the other immune mediators.

In preliminary experiments, we were unable to detect TNF-α in supernatant after 1 h using ELISA.

Therefore, we examined supernatants in a time frame of 4 to 24 h for TNF-α detection. We showed a time-dependent increase in TNF-α concentration in pbMEC compared with PCBUS. The moderate increase in TNF-α concentration over time was significantly different compared with controls. Nevertheless, the results demonstrate that acute inflammation can be mimicked in PCBUS, making this technique attractive for future investigations.

Murphy et al. (2017) advise caution in the use of protein coatings when antibiotics are used to culture mammalian cells on protein gels if the cells are subsequently infected. They observed that residues of antibiotics contained in the medium can affect introduced bacteria, even in the presence of a complete cell layer. We used rat-tail collagenase in our study to ensure good adhesion of pbMEC, which might have affected *S. aureus* infection and led to differences between the *S. aureus* strains used. In any case, this should be investigated in future studies.

Even though the model of primary bovine alveolar epithelial cells is considered well characterized, several factors must be kept in mind when designing and performing experiments. The ability of some *S. aureus* strains to form biofilm represents an important virulence factor. Castilho et al. (2017) investigated host-pathogen interactions between clinical *S. aureus* mastitis isolates, pbMEC, and the well-described HeLa cell line and found that the *S. aureus* strains they used had high invasion potential in bovine mammary epithelial cells, which made it not feasible for studying biofilm formation (Artini et al., 2012; Merghni et al., 2016). In HeLa cells, the formation of a biofilm is possible because the invasion potential is lower; therefore, attention must be paid to the properties of the cells and whether they are suitable for evaluating a specific research question, even when using well-characterized cell culture models.

Differences can also be seen between pbMEC obtained by different methods. Zaatout et al. (2019) investigated the interaction of primary bovine epithelial cells with biofilm-forming staphylococci. By using 2 different techniques to isolate the cells—the explant technique and enzymatic digestion—they were able to internalize bacteria in both models, although the explant technique was more successful.

In all experiments performed in the current study, the variation in results was quite large. As previously described, sample material was obtained from udders of slaughtered cattle. Information on reasons for slaughter, age at slaughter, and the number of lactations and lactation stage of the cows was not available. Thus, the variability in our experimental results may reflect different lactation stages of the slaughtered cows, or their age, health, and immune status. Petzl et al. (2012) conducted an in vivo study in cows administered 1 μg of LPS per udder quarter and then infected with an infectious dose of 250 cfu/mL of *E. coli* via the stalk canal 3 or 10 d later. In contrast to the group of animals that received no LPS pretreatment but the *E. coli* infective dose only, the prestimulated cows showed reduced inflammatory responses. This phenomenon is called endotoxin tolerance and was also described in a study by Petzl et al. (2012). A similar study to investigate endotoxin tolerance was performed by Günther et al. (2012) with primary udder epithelial cells with toll-like receptor (TLR) ligands recognizing a similar effect. Thus, if the cells of the udder tissue were in contact with pathogens a few days before slaughter, these cells may react differently to the bacterial stimulus in the experiment. Consequently, in future studies, it is essential to obtain information about the slaughtered animals from which tissues or cells are derived. However, this applies to both in vitro models investigated in the current study and does not represent a limitation of PCBUS relative to pbMEC.

Another difficulty lies in verifying the viability of the PCBUS. Because the viability assay is a final experiment, the PCBUS used in the MTT assay can no longer be used in other experiments. However, if the PCBUS come into contact with bacteria, checking viability using the MTT assay is no longer possible, because the bacteria reduce the substance of the assay and thus invalidate the assay results. Again, this problem is not exclusive to PCBUS and must be considered for pbMEC as well.

In addition to data collected at the protein level, we decided to conduct initial studies at the mRNA level to examine a broader range of immune mediators. We infected PCBUS and pbMEC with *S. aureus* Rd 5 and set 6 h postinfection as the time point for these studies. We selected cytokines, chemokines, and antimicrobial molecules that have been investigated in other explant, ex vivo, or in vivo studies to compare our pilot data with previous literature (Lind et al., 2015; Petzl et al., 2016; Magro et al., 2017; Brand et al., 2021).

We detected upregulation of all immune mediators (after 6 h) in both pbMEC and PCBUS except for the antimicrobial effector molecules *LAP* and *S100A9*, the levels of which did not differ from those of the control group. In general, PCBUS and pbMEC showed significant changes in gene expression between uninfected and infected samples in both models, with *CXCL8* being the only gene to display differential expression between uninfected and infected pbMEC. However, even if the absolute values of gene expression in PCBUS were much higher than those of the pbMEC, the difference between uninfected and infected PCBUS was not significant.

This leads us to conclude that, in pbMEC, we were able to elicit a differential immune response at the mRNA level by stimulating isolated cells. This is in line with findings reported in the literature (Bauer et al., 2015; Kerro Dego et al., 2018; Ying et al., 2021). Moreover, the PCBUS showed similar or higher gene expression profiles compared with pbMEC; thus, PCBUS can be used for bovine mastitis studies. An important aspect in the study of immune response by PCBUS seems to be the localization of sampling. Petzl et al. (2016) reported higher expression of *S100A9* and *LAP* in the teat cistern than in the alveolar parenchyma and explained this as a natural defense mechanism of the teat cistern against pathogens entering via the teat canal. In agreement, studies by Lind et al. (2015) showed higher constitutive expression of antimicrobial S100 proteins in Fürstenberg's rosette compared with the teat cistern. Thus, depending on when the organism has to intervene against the bacteria, the immune response seems able to adapt, which could plausibly explain the lack of gene upregulation we observed in our study. Importantly, our results are in line those published by Magro et al. (2017). They showed that *LAP* was not detectable in glandular tissue after 6 h. However, in contrast, numerous studies have described the upregulation of *CCL20*, *CXCL8*, *IL1B*, *IL6*, *IL10*, and *TNFA*, making this topic a matter of debate (Petzl et al., 2016; Magro et al., 2017; Islam et al., 2020; Brand et al., 2021).

Based on our results, one advantage of PCBUS over pbMEC is that the udder cells remain in their physiological cell associations during PCBUS experiments. This allows not only the investigation of inflammatory mediators but also histological evaluation, which enables us to examine interactions of bacteria and udder cells and interactions between the different cell types of the udder. When examining udder tissue, it is important to note that it can react differently to pathogens at different locations within the udder. Thus, evaluations of the immune response of tissue at the udder base, the glandular cistern, or the teat cistern can be performed using PCBUS. In contrast to removed explants, tissue of PCBUS is obtained with a standardized, uniform slice thickness, which guarantees a uniform nutrient supply to the tissue. In summary, we were able to establish ex vivo infection of PCBUS, which is similar to the widely used model of pbMEC, and we were able to detect cytokines, chemokines, and antimicrobial peptides in the early phase of infection. The results of our pilot study motivate further investigations, including assessment of pharmacological questions. Our goal is to use PCBUS as an additional tool to shed light on new, urgently needed therapeutic options and to further explore the pathophysiological, pharmacological, and immunological levels of mastitis.

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