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Streptococcus uberis strains originating from bovine uteri provoke upregulation of pro-inflammatory factors mRNA expression of endometrial epithelial cells *in vitro*

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ABSTRACT

Streptococcus uberis is an opportunistic pathogen involved in various infections of cattle. It is a well-known etiological agent of bovine mastitis and has recently also been linked to postpartum endometritis in dairy cows. *S. uberis* is frequently isolated from the uterus of postpartum cows but its actual contribution to host pathophysiology is unknown and information on *S. uberis* virulence factors potentially involved in the disease is lacking. To gain first insights into the role of *S. uberis* in the pathology of bovine endometritis, a cell-culture-based infection model was employed to study inflammatory host responses and investigate cytotoxic effects. A comprehensive strain panel, comprising 53 strains previously isolated from bovine uteri, was compiled and screened for known virulence factor genes. Isolates showing distinct virulence gene patterns were used to study their impact on cellular viability and influence on mRNA expression of pro-inflammatory factors in endometrial epithelial cells. Our study revealed that *S. uberis* negatively impacts the viability of endometrial epithelial cells and provokes an upregulation of specific pro-inflammatory factors, although with certain strains having a greater effect than others. Especially, mRNA expression of *IL1A* and *CXCL8* as well as *CXCL1/2* and *PTGS2* was found to be stimulated by *S. uberis*. These results suggest that *S. uberis* might indeed contribute to the establishment of bovine endometritis.

1. Introduction

Fertility of cows and heifers is the backbone of every dairy farm with high economic impact. The basis for an excellent reproductive performance is a healthy uterus. Postpartum endometritis is one of the most common reproductive disorders in dairy cattle, impairing animal fertility and resulting in high economic losses (Lewis, 1997; Liang et al., 2017). Postpartum endometritis can be defined as the presence of purulent vaginal discharge at day 21 postpartum or later or muco-purulent vaginal discharge after day 26 postpartum (Sheldon et al., 2006). The contamination of the uterus after parturition with a broad diversity of bacteria is regarded as an inevitable and highly complex and dynamic process (Williams et al., 2005; Carneiro et al., 2016) that depends on a variety of factors, including endometrial tolerance to bacteria or resistance of the endometrium to infection (Sheldon et al., 2019, 2020). Recent culturomic and metagenomic based studies demonstrated that the intrauterine postpartum microbial community is complex and shows health status specific microbial fluctuations (Machado et al., 2012; Wagener et al., 2015; Gomez et al., 2019). Thus, further research is required to identify and characterize opportunistic pathogens that may interact synergistically with major pathogens, such as *Trueperella pyogenes*. The occurrence of endometritis depends on the pathogenicity of bacteria and the bacterial load as well as on the immune response and immune capacity of the cow (LeBlanc et al., 2011). *Escherichia coli, T. pyogenes, Fusobacterium necrophorum* and *Prevotella* spp., which are commonly regarded as major intrauterine pathogens (Carneiro et al., 2016), have been studied in some detail (Sheldon et al., 2010; Bicalho et al., 2012, 2016; Ibrahim et al., 2017) but the role of other bacteria in uterine disorders and uterine health is still unexplored.

Bacteria belonging to the genera *Bacillus*, *Staphylococcus* and *Streptococcus*, which are frequently isolated from bovine uteri postpartum, have been classified as potential pathogens (Carneiro et al., 2016) but information on their mechanism of action on the endometrium (contribution to inflammation and/or tissue damage) is largely lacking. Only recently, it has been shown by *in vitro* studies that *Bacillus pumilus* can stimulate the mRNA expression of pro-

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inflammatory factors in bovine endometrial epithelial cells (Gärtner et al., 2016). Furthermore, a positive correlation between the intrauterine occurrence of *Streptococcus uberis* on day 3 postpartum and subsequent *T. pyogenes* infections was observed *in vivo* in naturally infected dairy cattle (Wagener et al., 2014). These results are fostering the hypothesis that these bacteria, which have been classified as potential pathogens, play a substantial role in the pathology of bovine endometritis by triggering the inflammatory response of the host and by increasing the severity of cellular damage.

Since *S. uberis*, which is a Gram-positive bacterium particularly recognized as a mastitis pathogen in dairy cattle (Schukken et al., 2011; Zadoks et al., 2011), is of high importance for livestock, this study aims to explore its potential contribution to bovine endometritis. Although *S. uberis* is often isolated from postpartum bovine uteri (Williams et al., 2005; Wagener et al., 2014) and was even reported to be the far most frequent *Streptococcus* species isolated from bovine uteri in frame of a microbial community study (Wagener et al., 2015), information about mechanisms of action of *S. uberis* on endometrial epithelial cells is lacking and *S. uberis* virulence factors potentially involved in bovine endometritis are still unknown.

To gain first insights into mechanisms of *S. uberis* contributing to uterine pathology, a panel of *S. uberis* strains isolated from bovine uteri was screened for known *S. uberis* virulence genes, such as capsule and adhesion genes as well as genes encoding surface proteins (Ward et al., 2001; Field et al., 2003; Reinoso et al., 2011; Kerro Dego et al., 2018; Fessia et al., 2019). In order to elucidate cellular mechanisms in the endometrium as a first response to bacterial infections a previously established primary endometrial epithelial cell culture model (Gärtner et al., 2016; Ibrahim et al., 2017) was employed and the effect of selected *S. uberis* strains on the viability of epithelial cells and on the mRNA expression of selected pro-inflammatory factors was assessed.

2. Materials and methods

2.1. Bacterial strains collection and cultivation

A panel of 53 *S. uberis* strains, isolated from the uterus of dairy cows in a previous study (Wagener et al., 2014), were included in this study. Samples were collected using the cytobrush technique (Kasimanickam et al., 2004; Westermann et al., 2010) at five predefined time points from day 0 postpartum until day 21 postpartum. To describe the uterine health status, cows were examined 21 days postpartum by use of a speculum and the vaginal discharge was scored according to Williams et al. (2005). *S. uberis* strains were isolated on day 3 (n = 23), 9 (n = 26) and day 15 (n = 3) postpartum. A total of 19 strains originated from healthy animals (clear vaginal discharge; vaginal discharge score 0 or 1) and 34 strains originated from animals that developed endometritis (vaginal discharge containing > 50% pus; vaginal discharge score 3) at 21 days postpartum. Strains were stored at −80 °C, and were recovered from glycerol stocks and routinely grown on Columbia III agar supplemented with 5% sheep blood (Becton Dickinson, Heidelberg, Germany) at 37 °C, 5% CO_2. For co-culture experiments with endometrial epithelial cells, bacteria were prepared as described before (Gärtner et al., 2016) with slight modifications. Bacteria were cultivated in brain heart infusion broth (BHIB, Sigma-Aldrich, Taufkirchen, Germany) at 37 °C, aerobically for 24 h. The cultures were collected by centrifugation at 2000*g* for 10 min, washed once with Dulbecco's phosphate-buffered saline (PBS, Biochrom, Berlin, Germany), re-suspended in BHIB with 20% glycerol and stored at −80 °C. In order to determine the number of colony forming units (CFU) per ml plate counting on brain heart infusion agar (Sigma-Aldrich) was performed. For co-cultivation assays, bacteria were thawed and diluted in epithelial cell culture medium (DMEM/Ham's F-12, supplemented with 10% FBS; Biochrom) without antibiotics for obtaining the required bacterial concentrations for each assay.

For bacterial growth experiments, DMEM/F12 medium supplemented with 10% FBS (both from Biochrom) was inoculated with a standard inoculum (10^3 cfu/mL) of selected *S. uberis* strains and incubated at 37 °C, 5% CO_2, 120 rpm, for 24 h. Optical density (OD_{600}) and pH were recorded for 24 h in 4 h intervals. OD measurements were performed using an Eppendorf BioPhotometer D30 (Eppendorf, Hamburg, Germany).

2.2. DNA isolation and PCR amplification of virulence associated genes from S. uberis

Bacterial DNA was isolated using the MasterPure™ Gram Positive DNA Purification Kit (Epicentre Biotechnologies, Madison, USA), according to the manufacturer's instructions. The presence of genes previously reported to represent the main virulence factors of *S. uberis* (Ward et al., 2001; Field et al., 2003; Reinoso et al., 2011; Kerro Dego et al., 2018; Fessia et al., 2019; Kaczorek et al., 2017) was tested by PCR. The following genes were included: the hyaluronic acid capsule genes (*hasA*, *hasB*, *hasC*), the *S. uberis* adhesion molecule gene (*sua*), surface lipoproteins (*slp*), the plasminogen activator gene (*pauA)*, glyceraldehyde-3-phosphate dehydrogenase surface protein (*gapC*), zinc binding protein Adcs (*acdA*) and a putative sortase-processed surface-anchored protein gene (*fpb*). Information on the oligonucleotide primers used is provided in the supplementary material (Table S1). For the PCR reaction, 1 µL template DNA was added to a mixture consisting of 1 µM of the respective oligonucleotide primers, 0.4 µM dNTPs, 2.5 mM $MgCl_2$, 1 X GoTaq Reaction Buffer, and 0.75 U goTaq polymerase (Promega, Mannheim, Germany). The PCR protocol started with an initial denaturation step for 2 min at 95 °C, followed by 30 cycles of: Denaturation at 95 °C for 35 s, annealing for 30 s at appropriate temperature (as specified in Table S1) and extension at 72 °C for time depending on the length of the amplicon. A final extension step of 4 min at 72 °C was done before the reactions were cooled at 4 °C. The presence of the specific bands was checked by gel electrophoresis.

2.3. Fourier transform infra-red (FTIR) spectroscopy and spectral data analyses

Bacteria were grown on Columbia III agar, supplemented with 5% sheep blood, then transferred to tryptic soy agar (Oxoid, Wesel, Germany) and incubated for 24 h at 30 °C aerobically for FTIR measurements. FTIR sample preparation and measurement was performed as described previously (Oberreuter et al., 2002). In brief, bacterial cells grown as confluent lawn were suspended in sterile deionized water and aliquots containing 30 µL of the suspension were transferred onto a 96-well zinc selenite (ZnSe) optical plate (Bruker Optics GmbH, Ettlingen, Germany) and dried at 40 °C for 40 min to create a transparent film for FTIR measurements. The spectra were recorded by using an HTS-XT microplate adapter paired to a Tensor 27 FTIR spectrometer (Bruker Optics, Ettlingen, Germany), in the spectral range between 4000 to 500/cm. The OPUS software version 7.2 (Bruker Optics) was used for spectral data preprocessing and analyses. For hierarchical clustering analysis (HCA) the following spectral windows were used: 3000 − 2800, 1750 − 1500, 1170 − 900/cm, with a weight factor of 1 and repro-level set at 30. With the use of Savitzky–Golay algorithm, first derivatives with nine smoothing points were calculated and Ward's algorithm was applied to calculate the HCA tree.

2.4. Primary endometrial epithelial cell culture

The primary endometrial epithelial cell culture was performed as described previously (Betts and Hansen, 1992), with slight modifications. In brief, uteri with no apparent signs of inflammation were collected from a local slaughterhouse briefly after the animals' slaughter and transported on ice to the laboratory. Small tissue pieces (approximately 0.1 cm^2) were collected from the area between the caruncles. The harvested material was minced finely and then digested in a

solution containing 150 U/mL of collagenase, 150 U/mL of hyaluronidase, 200 U/mL of penicillin and 200 µg/mL of streptomycin (Sigma-Aldrich) in Hank's balanced salt solution (Biochrom) for 2 h at 37 °C, in a rolling shaker under light agitation. Thereafter, the cells were centrifuged and the resulting cell pellet was washed using cell culture medium (DMEM/Ham's F-12 medium supplemented with 10% FBS, 55 µg/mL gentamicin and 1.4 µg/mL amphotericin B; all from Biochrom). The cells were then re-suspended in the aforementioned medium, seeded in 25 cm^2 flasks and incubated at 37 °C with 5% CO_2 for 18 h. During this incubation time most fibroblasts attached and the medium, containing non-attached cells, was transferred to a new 25 cm^2 flask to obtain a pure (> 99%) epithelial cell culture. After some days, when confluence of 80–90% was reached, cells were transferred in a 75 cm^2 flask, and then, after confluence of at least 90% was achieved, the cells were passaged into a 24 well-plate at a final density of 2×10^5 cells per well.

2.5. Co-cultivation of endometrial epithelial cells with S. uberis and test of cytotoxic effects of S. uberis secretomes

Primary endometrial epithelial cell cultures, prepared as described above, were incubated with selected *S. uberis* strains (n = 8), showing different virulence gene profiles. Co-cultivation of bacteria and epithelial cells was carried out in 24 well-plates, using DMEM/Ham's F-12 medium supplemented with 10% FBS without antibiotics and different multiplicities of infection (MOI 1, 5, 10). The epithelial cells were inoculated with a standard inoculum representing the required infectious dose for each MOI and incubated for 24 h at 37 °C, 5% CO_2 in a humidified atmosphere.

The effect of the bacterial secretome on the cells was examined in similar fashion. The secretome (15 mL) of bacterial strains (n = 3) grown in DMEM/Ham's F-12 medium, supplemented with 10% FBS, at 37 °C, 5% CO_2, shaking (120 rpm), was collected after 12 h (start of exponential phase) and 24 h (stationary phase) by centrifugation at 5000 x g for 5 min. To obtain a bacterial free filtrate, the supernatant was passed through 0.2 µm filter (Filtropur S 0.2, Sarstedt, Nümbrecht, Germany) and the filtrate was 10-fold concentrated by centrifugation (Amicon Ultra-15 Centrifugal Filters with a 3 kDA cut-off, Merck, Darmstadt, Germany). 0.5 mL of the concentrated secretome was used to inoculate endometrial primary cells in a 24 well-plate. Cellular viability was assessed after 24, 48 and 72 h of incubation at 37 °C, 5% CO_2, under a humidified atmosphere.

2.6. Viability assay

The viability of the cells was assessed using the trypan blue dye exclusion test described by Strober (Strober, 2015) with slight modifications according to Gärnter et al. (Gärtner et al., 2016). Trypan blue cannot penetrate living cells with intact membranes (dye exclusion) but dead cells, thus it can be used to determine the percentage of living cells present in a cell suspension. In brief, the cell medium was removed, cells were washed with PBS (Biochrom) and mixed with a 0.5% Trypan blue solution (SciencCell, California, USA). Subsequently, it was determined how many cells did take up the blue dye and how many did not by light microscopy. Multiple optical fields (at least 5) per sample were analyzed and the number of unstained cells was calculated as the percentage of the total number of cells. Unstained cells were considered as viable while cells stained blue as non-viable.

2.7. Messenger RNA expression in endometrial epithelial cells co-cultured with S. uberis

To explore the effect of *S. uberis* on pro-inflammatory factors mRNA expression in uterine cells, endometrial epithelial cells isolated from five different animals were grown to the second passage and co-cultured with three *S. uberis* strains, namely LMM 1717, LMM 1719 and LMM 1724, in medium without antibiotics, using the MOI model in 24-well plates as described before (Gärtner et al., 2016). Cells infected with *S. uberis* were incubated at 37 °C, 5% CO_2, lysed with lysis buffer TR (Stratec, Birkenfeld, Germany) and collected at 2, 4 and 8 h post-infection. Epithelial cells, used as a control, were also lysed at 0 h. The lysed cells were frozen at −80 °C until further use.

2.8. RNA extraction and reverse transcription-qPCR

RNA was extracted from the thawed, lysed cells using the InviTrap Spin Cell RNA Mini Kit (Stratec, Birkenfeld, Germany) according to the manufacturer's protocol. Total RNA yield was estimated spectrophotometrically at 260 nm. Verification of the RNA integrity and quality was performed by using an RNA 6000 Nano LabChip on an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). 150 ng total RNA were treated with DNase and reverse transcription was carried out using hexamers in a total volume of 60 µL as described before (Odau et al., 2006). The generated cDNA was aliquoted and stored at −20 °C for further use. QPCR was performed as described previously (Gärtner et al., 2016). In brief, 1 µL cDNA was

Table 1
Primer sequences targeting selected pro inflammatory factors genes used for qRT-PCR, including annealing temperature and amplicon length.

Gene	Primer sequence (5′ → 3′)	Annealing temperature	Amplicon size (bp)	Reference
18S rRNA	F: GAG AAA CGG CTA CCA CAT CCA A R: GAC ACT CAG CTA AGA GCA TCG A	61 °C	337	(Odau et al., 2006)
ACTB	F: CGG TGC CCA TCT ATG AGG R: GAT GGT GAT GAC CTG CCC	58 °C	266	(Gärtner et al., 2015)
SDHA	F: GGG AGG ACT TCA AGG AGA GG R: CTC CTC AGT AGG AGC GGA TG	60 °C	219	(Gärtner et al., 2015)
CXCL8	F: CGA TGC CAA TGC ATA AAA AC R: CTT TTC CTT GGG GTT TAG GC	56 °C	153	(Fischer et al., 2010)
IL1A	F: TCA TCC ACC AGG AAT GCA TC R: AGC CAT GCT TTT CCC AGA AG	59 °C	300	(Gabler et al., 2009)
TLR2	F: GTA CCC ATG ATG GAA TTG GC R: TGG CCA CTG ACA AGT TTC AG	60 °C	446	(Gärtner et al., 2015)
TLR4	F: ACT GAG TTT CAG CTA CCA AGC C R: AGG TTT GCA GAC TGA CTA AGC C	60 °C	325	(Gärtner et al., 2015)
PTGS2	F: CTC TTC CTC CTG TGC CTG AT R: CTG AGT ATC TTT GAC TGT GGG AG	60 °C	359	(Odau et al., 2006)
CXCL5	F: TGA GAC TGC TAT CCA GCC G R: AGA TCA CTG ACC GTT TTG GG	61 °C	193	(Fischer et al., 2010)
CXCL1/2	F: GAC CTT GCA GGG GAT TCA CCT C R: CGG GGT TGA GAC ACA CTT CCT G	60 °C	125	(Gärtner et al., 2015)

subjected to the amplification reaction containing 1x SensiMix Low-ROX (Bioline, Luckenwalde, Germany) and 0.4 µM of each primer (Table 1) in a total volume of 10 µL. A previously described protocol (Gärtner et al., 2016) was performed for the qPCR using the Rotor Gene 3000 (Corbett Research, Mortlake, Australia): 10 min at 95 °C, 45 cycles of 15 s at 95 °C, 20 s at the annealing temperature indicated for each primer set (Table 1) and 30 s at 72 °C. The mRNA expression of three reference genes was evaluated: 18S rRNA (*18S rRNA*), β-actin (*ACTB*), and succinate dehydrogenase (*SDHA*). Eight genes linked to endometrial inflammatory response were tested: interleukin 1α (*IL1A*), and 8 (*CXCL8*), Toll-like receptor 2 (*TLR2)* and 4 (*TLR4*), prostaglandin-endoperoxide synthase 2 (*PTGS2*) and chemokine (C-X-C motif) ligand 1/2 and 5 (*CXCL1/2* and *CXCL5*). Specific amplification was checked by melting point analysis. Negative controls without using the reverse transcriptase were used for ensuring the absence of any genomic DNA and contaminations. The amplicon identity was further confirmed by sequencing.

2.9. Statistical analysis

Data analysis for the qPCR data was carried out as described previously (Gärtner et al., 2016). In brief, a normalization factor of the stably expressed reference genes *ACTB*, *18S rRNA* and *SDHA* was obtained using geNorm (Vandesompele et al., 2002). The normalized values obtained were used in order to compare the mRNA expression differences between the cells treated with *S. uberis* in a specific MOI and time point to its respective controls. Bar charts were generated and the expression in controls and treatments at each time point was calculated in relation to the 0 h untreated sample for each animal. For the comparison of epithelial cells incubated with *S. uberis* at different MOI and controls at each time point, a Wilcoxon signed-rank test was performed by the use of SPSS (IBM SPSS Statistics for Windows, Version 25. Armonk, NY, USA) and for visualization of the values and creation of bar charts GraphPad Prism version 7.00 for Windows, GraphPad Software (La Jolla, California, USA) was used. A p-value < 0.05 was considered as significant, while p-values $0.05 - 0.1$ were considered as a trend.

3. Results

3.1. S. uberis virulence gene profiling

All 53 *S. uberis* strains included in this study were screened for the presence of major *S. uberis* virulence genes. All strains were tested positive for all of these virulence genes, with the exception of *hasC* and *sua* (Table 2). The latter two genes were found in 83% (*hasC*) and 93% (*sua*) of the strains, respectively. Based on this virulence gene screening, strains could be assigned to four different virulence profiles, designated A–D (see Table 3). The most common virulence gene profile among the strains was the one harboring all tested virulence genes (profile A). 43 out of 53 strains (81.1%) showed this virulence gene profile. The next most common virulence gene profile (profile B) was characterized by the presence of all of the virulence genes included in the screening, except *hasC*, with six out of 53 strains (11.3%) belonging to this virulence gene profile. Three out of the 53 strains (5.6%) exhibited the simultaneous lack of *hasC* and *sua* genes (profile D), while only one strain (1.9%) tested positive for all virulence genes, except *sua* (strain LMM 1724, profile C).

Table 2
Presence of selected virulence factors genes in *S. uberis* strains originating from the uterus of dairy cows.

Gene	Strains tested positive
Hyaluron capsule genes	
hasA	53/53 (100%)
hasB	53/53 (100%)
hasC	44/53 (83%)
S. uberis adhesion molecule (*sua)*	49/53 (93%)
Surface lipoprotein (*slp*)	53/53 (100%)
Plasminogen activator (*pauA*)	53/53 (100%)
Glyceraldehyde-3-phosphate dehydrogenase surface protein (*gapC*)	53/53 (100%)
Zinc binding protein Adcs (*acdA*)	53/53 (100%)
Putative Sortase-processed surface-anchored protein (*fpb*)	53/53 (100%)

Table 3
Virulence gene profiles of *S. uberis* strains originating from the uterus of dairy cows.

Pathotypes	*hasA, hasB, gapC, acdA, fpb, pauA, slp*	*has C*	*sua*	% isolates
A	+	+	+	81.1%
B	+	–	+	11.3%
C	+	+	–	1.9%
D	+	–	–	5.7%

Abbreviations: acdA: zinc binding protein, fpb: sortase-processed surface-anchored protein, gapC: glyceraldehyde-3-phosphate dehydrogenase surface protein, has: hyaluronic acid capsule A–C, pauA: plasminogen activator, sua: *S. uberis* adhesion molecule, slp: surface lipoprotein.

3.2. Co-culture, viability assay and bacterial growth

To investigate the impact of *S. uberis* on host cell viability, primary endometrial cells were isolated from bovine uteri and subsequently used for co-culture experiments with *S. uberis* strains. The strain selection was based on the different virulence profiles and included strains originating from healthy as well as from diseased cows. The strains LMM 1717 and LMM 1722 were selected as representatives for profile A, strains LMM 1721 and LMM 1723 were chosen as representatives for profile B, LMM 1724 for profile C, while LMM 1718, 1719 and 1720 were used as representatives for profile D.

All tested *S. uberis* strains (n = 8) negatively affected the cellular viability of the host cells, although in a strain dependent manner (Fig. 1). The cellular viability 24 h post-infection ranged from 82% to 43% at MOI 1 and from 47% to 5% to at MOI 10, respectively. For some strains the observed negative impact on host cell viability was found to be MOI dependent (*e.g.* LMM 1717, LMM 1719, LMM 1720) while for other strains there was no clear MOI dependence (*e.g.* LMM 1721, LMM 1723, LMM 1724). To gain insights into the growth characteristics of the strains that may contribute to the observed cytotoxic effects, bacterial strains were grown in DMEM/Ham's F12 medium supplemented with 10% FBS under the same conditions as the co-culture experiments (37 °C, 5% CO_2). Growth of bacteria, measured as OD_{600}, and pH of the cultures were monitored for 24 h in 4-h intervals. No changes in the pH were observed for any of the strains tested, within the first 16 h of growth. After 20 h, the strains started to lower the pH of the cell culture medium but no correlation was found between bacterial growth rate and drop of pH nor between cytotoxicity of a strain and its respective growth rate (indicative for metabolic activity) or between drop in pH and cytotoxicity (see Fig. S1 and Fig. 1). Thus, it is assumed that *S. uberis* strain specific factors, at least partially, contribute to the observed loss of host cell viability, although it could not be excluded that the decrease in pH, as a result of bacterial growth, might also have affected cellular viability after 24 h. Further studies will therefore be necessary to fully decipher and understand the factor contributing to *S. uberis* negative effects on endometrial host cells.

3.3. Typing of S. uberis by Fourier-transform infrared spectroscopy (FTIR)

To further characterize the strains used for the co-culture experiments, metabolic fingerprints of these strains were obtained by means of FTIR spectroscopy and subjected to chemometric analysis. As revealed by hierarchical cluster analysis of pre-processed spectra, the

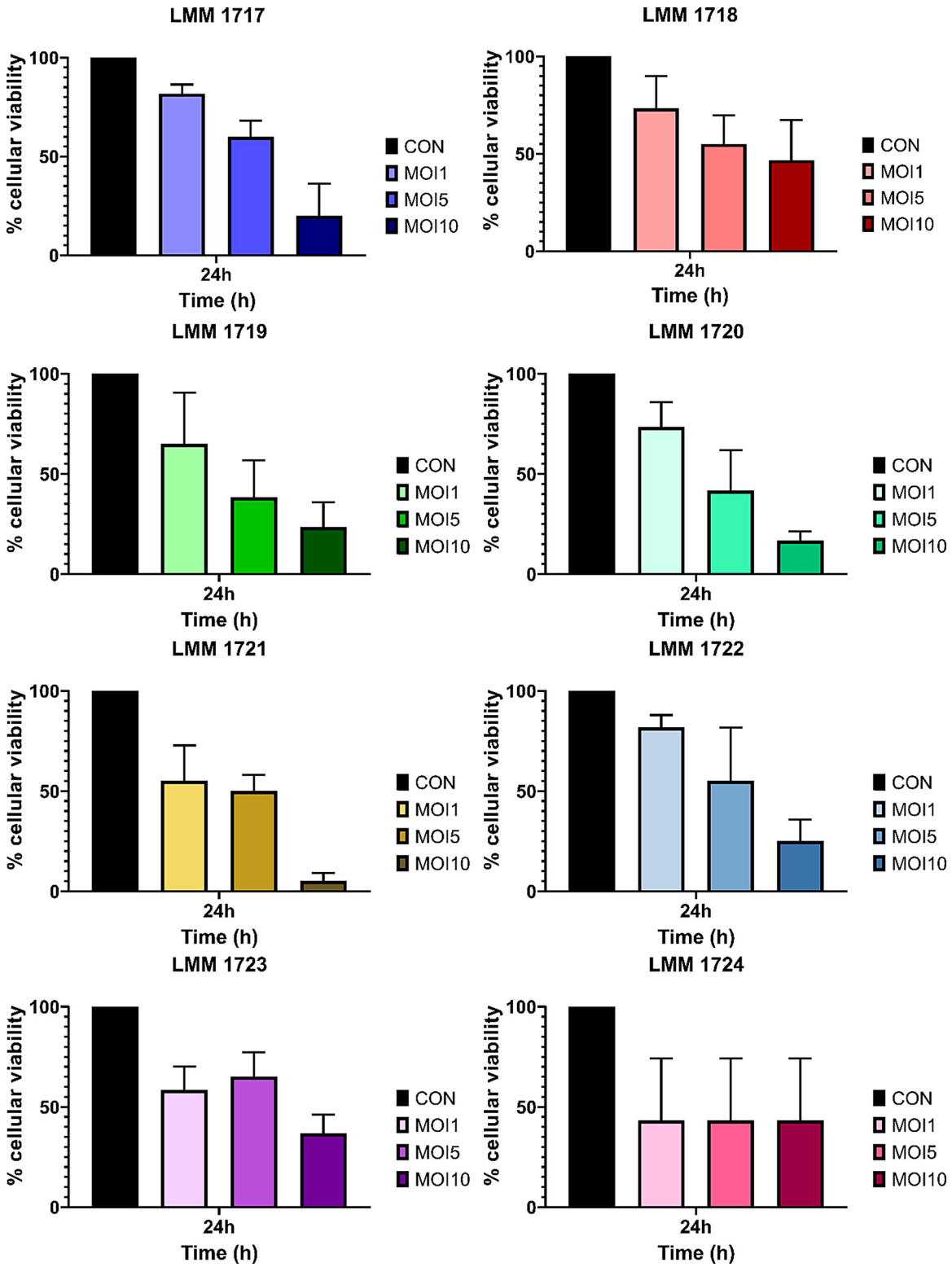

Fig. 1. Endometrial epithelial primary cells viability after co-cultivation with *S. uberis* strains using different MOI (MOI 1, 5 and 10) at 24 h. Viability was assayed by using trypan blue, as an exclusion staining. Bars indicate mean viability of endometrial cells (%) ± SD of cells originating from three individual animals (n = 3) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

strains formed distinct clusters. Notably, these clusters correlated with the potential to reduce cellular viability as well as with the virulence gene pattern (see Fig. 2). Strains with virulence gene profiles A and D clustered in the main cluster I while strains with profiles B and C clustered in the main cluster II. Furthermore, strains in cluster I showed low (< 30% dead cells) to medium (30–50% dead cells) potential to reduce cellular viability in the co-cultivation assay at a MOI 1 after 24 h, while strains belonging to cluster II showed medium to high (> 50% dead cells) capability of reducing cellular viability (see also Fig. 1).

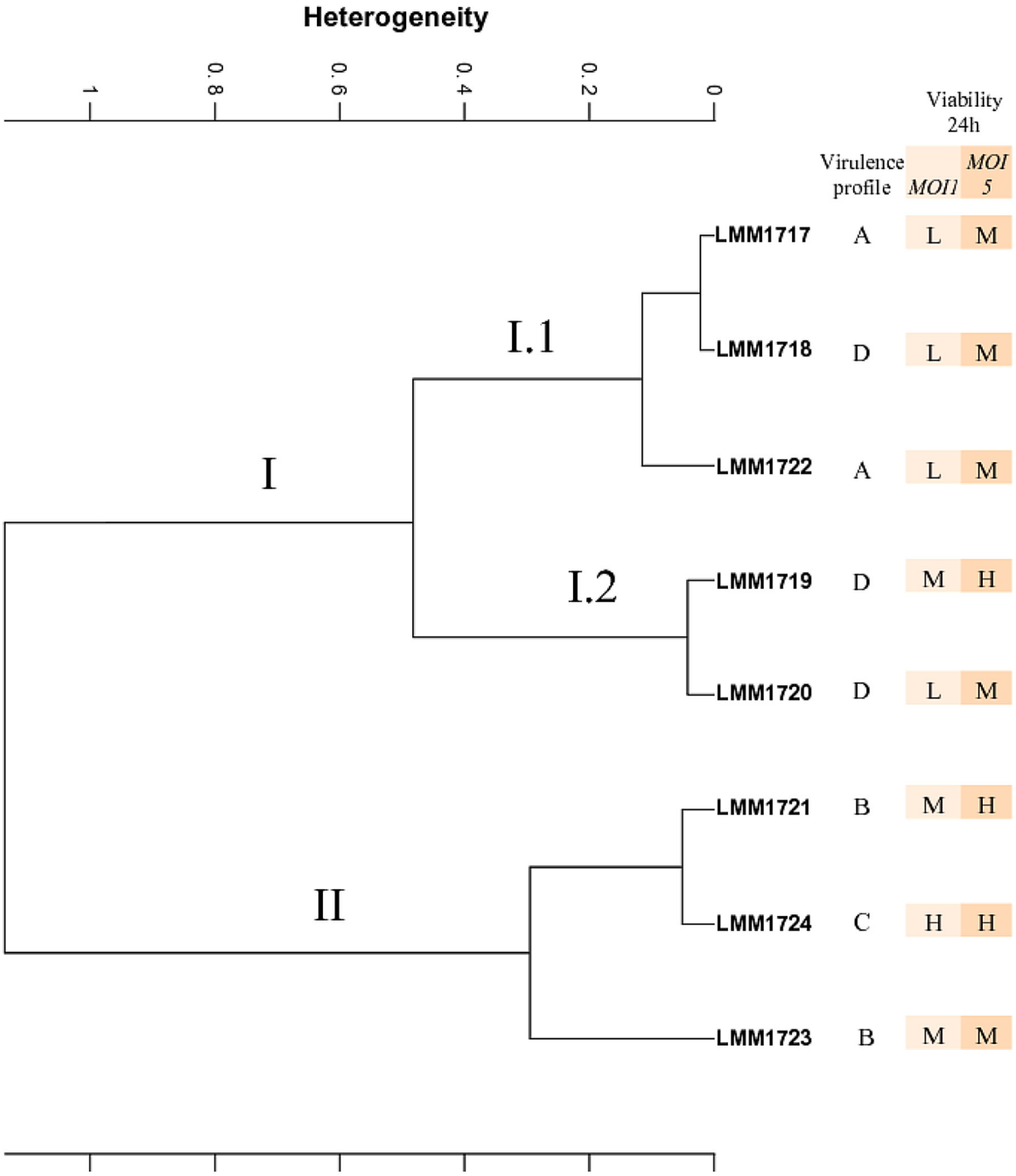

Fig. 2. Dendrogram depicting the results from the hierarchical cluster analysis (HCA) of FTIR spectra derived from selected *S. uberis* strains. First derivatives of spectra, including the spectral windows of 3000–2800/cm, 1750–1500/cm and 1171–900/cm were retrieved and the dendrogram was calculated by using the Ward's algorithm with normalization to repro level 30. Strains group in three clusters, designated as I.1, I.2 and II. Virulence genes profiles of strains are indicated according to Table 3. A classification scheme was used to indicate the effect of the strains on the viability of endometrial cells at MOI 1 and MOI 5: L = Low (< 30%), M = Medium (30–50%), H = High (> 50%) (For details see Fig. 1).

3.4. Effect of secreted S. uberis factors on viability of bovine endometrial cells

In order to assess whether secreted, non-cell associated factors from *S. uberis* could contribute to the reduced viability observed in the viability assay, three strains with different virulence genes profiles (strains LMM 1717, LMM 1719 and LMM 1724) and representing different FTIR clusters (Fig. 2) were selected for further investigation. *S. uberis* strains were cultivated in cell culture medium at 37 °C, 5% CO_2 and supernatants were collected by centrifugation after 12 h and 24 h. To obtain bacteria-free secretomes, the supernatants were filtrated and concentrated by centrifugation, using 3 kDa cut-off spin filter units. Bovine endometrial primary cells were incubated with the concentrated bacterial secretomes and cell viability was assessed as described above. In contrast to the co-cultivation experiments with living bacteria, the secretomes from the selected *S. uberis* strains showed only minimal cytotoxic effects (see Fig. S2). It is therefore assumed that the negative impact of S. *uberis* on bovine endometrial cells is rather linked to the bacterial cell itself and/or cell bound factors than to secreted factors. Thus, we focused on the interaction of the bacteria and endometrial host cells. To this end, additional co-cultivation experiments were carried out and the effect of *S. uberis* on pro-inflammatory mRNA

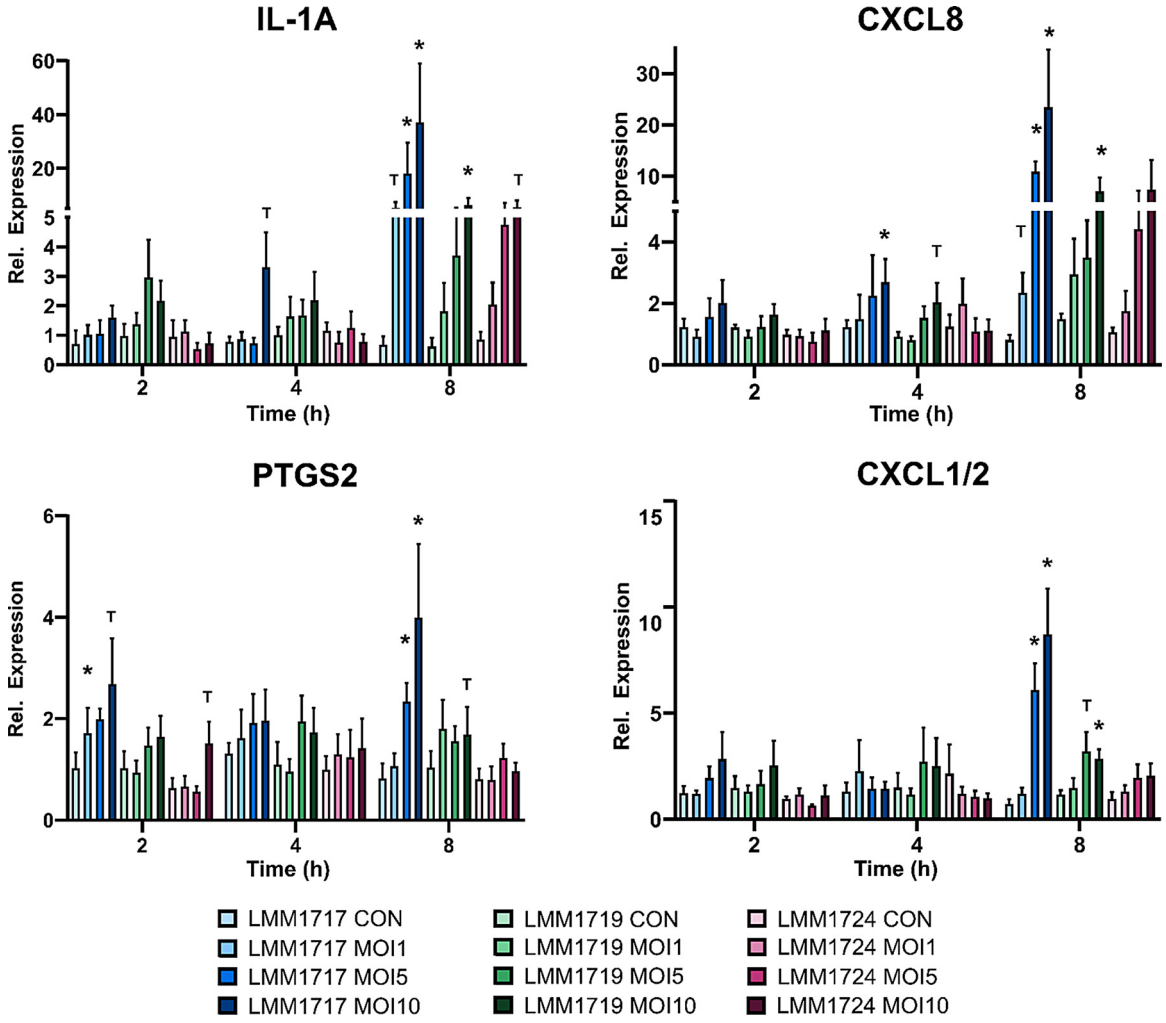

Fig. 3. Relative mRNA expression of selected pro-inflammatory factor genes of bovine endometrial epithelial primary cells infected with *S. uberis* strains LMM 1717, LMM 1719 and LMM 1724 using various MOI (MOI 1, 5 and 10). Bars show the mean ± SEM of cells isolated from five individual animals. mRNA expression in controls and treatments at each time point was calculated in relation to the 0 h untreated sample for each animal Asterisk indicates statistically significant ($p < 0.05$) different expression, while "T" indicates a trend ($p = 0.051–0.1$) of significant difference compared to the control (non-infected cells) at the same time point.

expression in endometrial cells was studied as described below.

3.5. Pro-inflammatory factor expression in bovine endometrial cells co-cultured with S. uberis

Primary endometrial epithelial cells were infected with selected *S. uberis* strains, differing in virulence gene profiles, at different MOIs (1, 5, and 10) and analyzed for mRNA expression of selected pro-inflammatory marker genes after 2, 4 and 8 h of co-cultivation. As depicted in Fig. 3, all three strains chosen (LMM 1717, LMM 1719 and LMM 1724) had a pronounced impact on pro-inflammatory factor mRNA expression in the endometrial epithelial primary cells, although to a different degree (see Fig. 3).

The *IL1A* transcription in the endometrial epithelial cells was significantly upregulated 8 h after infection with LMM 1717 at MOI 5 and MOI 10 ($p = 0.043$) as well as 8 h after infection with LMM 1719 at MOI 10 ($p = 0.043$). Furthermore, a trend of increased *IL1A* transcription was also observed in endometrial epithelial primary cells after infection with LMM 1724 at MOI 10 ($p = 0.080$). Notably, the effect of a low bacterial dose (MOI 1) of LMM 1717 on *IL1A* mRNA expression was similar to the one observed in response to high doses (MOI 10) of LMM 1719 and LMM 1724 8 h post-infection (Fig. 3).

Similar to *IL1A*, *CXCL8* transcription was also strongly upregulated in response to infection with *S. uberis*, showing the most pronounced reactions 8 h post-infection (Fig. 3). In line with the results for *IL1A* mRNA expression, the effect on *CXCL8* mRNA expression was highly infection dose dependent and strain specific. The strongest effects on *CXCL8* mRNA expression were observed in response to infection with LMM 1717. At MOI 10, LMM 1717 led to a significant upregulation of *CXCL8* transcription already after 4 h ($p = 0.043$), which was even more pronounced after 8 h ($p = 0.043$). At MOI 5, a significant upregulation of *CXCL8* transcription was observed 8 h post-infection ($p = 0.043$) and, even at the lowest MOI used (MOI 1), LMM 1717 showed a tendency to provoke an upregulation of the *CXCL8* mRNA expression after 8 h ($p = 0.080$). Furthermore, LMM 1719 caused a significant higher *CXCL8* mRNA expression 8 h post-infection at MOI 10 ($p = 0.043$).

In addition, infection of the epithelial cells with *S. uberis* led to an upregulation of the *CXCL1/2* (Fig. 3). Again, the strongest upregulation of *CXCL1/2* transcription was observed 8 h post-infection in cells infected with LMM 1717 at MOI 5 and MOI 10 ($p = 0.043$, each) as well as for LMM 1719, using the same MOIs (MOI 5, $p = 0.080$; MOI 10, $p = 0.043$). In contrast to *CXCL1/2*, *CXCL5* did not show a clear response (Fig. S3).

Similar to the aforementioned stimulating effect on interleukin mRNA expression, the strain LMM 1717 showed the strongest effect on transcription levels of the *PTGS2* gene (Fig. 3). In case that primary endometrial epithelial cells were infected with LMM 1717, a significant

upregulation of *PTGS2* transcription was already found 2 h after the infection (MOI 1, p = 0.043; MOI 10, p = 0.080) although the strongest effect was again observed 8 h post-infection at higher MOIs (MOI 5 and MOI 10, p = 0.043). A trend towards a higher mRNA expression of *PTGS2* was observed for LMM 1724 at MOI 10 2 h after the infection and for LMM 1719 at MOI 10 8 h after the infection (p = 0.080, each). By contrast, infection with *S. uberis* did not show a clear effect on mRNA expression levels of the Toll-like receptor genes *TLR2* and *TLR 4* (Fig. S3), which are known to be involved in sensing of gram-positive bacteria.

4. Discussion

It is well established that after parturition the bovine uterus is contaminated with a broad variety of bacterial species, resulting in a rather complex postpartum uterine microbiome (Machado et al., 2012; Wagener et al., 2015). This postpartum uterine microbial community is very dynamic with certain species, such as *E. coli* and *T. pyogenes*, playing an important role in the subsequent development of endometritis (Santos et al., 2011; Wagener et al., 2014; Carneiro et al., 2016), although not all infected cows show signs of endometritis, because the development of the disease depends on various mechanisms that are contributing to host resilience (Sheldon et al., 2020). While defined uterine pathogens, such as the above-mentioned species *T. pyogenes* and *E. coli*, are comparatively well studied, information on other, opportunistic pathogens that may contribute to the establishment and progression of uterine diseases is largely lacking. For instance, *S. uberis*, a pathogen commonly found in the farm environment and frequently associated with bovine mastitis (Schukken et al., 2011), is also found in the postpartum uterus (Wagener et al., 2015) but information on its potential contribution and role in the establishment of endometritis is lacking. This lack of information prompted us to employ a previously established primary cell culture model (Gärtner et al., 2016) to investigate the effects of *S. uberis* on endometrial epithelial cells isolated from bovine uteri. From mammary gland infection studies, *in vivo* as well as *in vitro*, it has been reported that host cell response to *S. uberis* infection is highly strain dependent (Tassi et al., 2013). Thus, we compiled a panel of strains originating from postpartum bovine uteri and tested their effect on the viability of endometrial epithelial cells. As revealed by our *in vitro* study, co-cultivation of endometrial epithelial primary cells with *S. uberis* strains resulted in reduced cellular viability 24 h post-infection, indicating a cytotoxic potential of *S. uberis* for endometrial uterine cells. However, further studies will be necessary to full decipher the factors contribution to the negative impact of *S. uberis* on endometrial epithelial cells.

The effect of *S. uberis* on cellular viability varied among the different strains but no clear link could be found between severity of cellular damage and the presence of distinct *S. uberis* virulence genes. Notably, the prevalence of certain virulence genes was higher in *S. uberis* strains isolated from bovine uteri in frame of the current study than the prevalence reported from mastitis associated *S. uberis* strains. For instance, a prevalence of the hyaluron capsule genes *hasA* and *hasB* of 74% and 66%, and a prevalence of the adhesion molecule gene *sua* was found in 79%–83% in *S. uberis* strains originating from the bovine udder (Reinoso et al., 2011; Reinoso, 2017; Fessia et al., 2019), whereas we detected *hasA* and *hasB* in all strains and *sua* in 93% of the strains originating from bovine uteri. In order to gain further insights of potential factors contributing to the reduced viability of cells infected with *S. uberis*, endometrial epithelial cells were challenged with bacterial culture supernatants. As expected, supernatants of *S. uberis* strains had almost no cytotoxic effect, suggesting that the reduced viability of endometrial epithelial cells would rather be attributed to bacterial cells and structures and/or cell bound factors than to secreted substances. These results are in line with recent findings from mastitis research, showing that surface proteins of *S. uberis* are associated with bovine intramammary infections (Kerro Dego et al., 2018).

Our hypothesis regarding *S. uberis* as an opportunistic pathogen contributing to bovine endometritis is further fostered by the results from *in vitro* mRNA expression studies, which revealed that selected pro-inflammatory genes are upregulated in bovine endometrial cells in a time and concentration dependent (MOI) manner (see Fig. 3). Infection of the primary endometrial epithelial cells resulted in a significant upregulation of mRNA expression of the interleukin *IL1A* and chemokine *CXCL8*, which is in line with results from *S. uberis in vitro* infection studies using primary mammary epithelial cells (Wellnitz et al., 2012). Similar to the study from Wellnitz et al. (2012) we observed strain specific immune responses. The upregulation of *IL1A* and *CXCL8* mRNA in response to *S. uberis* observed in our current study was higher than the effects reported previously for defined uterine pathogens, such as *T. pyogenes* (Ibrahim et al., 2017), using the same cell culture model and similar to the one reported for other potential uterine pathogens, such as *B. pumilus* (Gärtner et al., 2016). Both, the cytokine IL1A and the chemokine CXCL8 play an important role in the inflammatory process linked to uterine diseases. As shown previously, expression of *IL1A*, as well as *CXCL8* is upregulated in cows suffering from postpartum endometritis (Gabler et al., 2009, 2010), suggesting that opportunistic pathogens, such as *S. uberis* and *B. pumilus*, may act as mediator of inflammatory host response. In addition to the above, *S. uberis* infection also led to an increase of *PTGS2*, and *CXCL1/2* mRNA expression, although the effect on the latter two genes was not as pronounced as the effects observed on the aforementioned genes *IL1A* and *CXCL8*. PTGS2 is a key enzyme in prostaglandin production (Gabler et al., 2010) while CXCL1/2, which has been recently linked to subclinical endometritis (Peter et al., 2015), is a chemoattractant of neutrophils. Since CXCL8 and CXCL1/2 are both known for their potential to recruit neutrophils, it is tempting to speculate that neutrophils may play a crucial role in immune response to cope with *S. uberis* infections. However, further analyzes, which are clearly beyond the scope of the current study, will be needed to fully understand the host and bacterial factors contributing to the strain specific response of pro-inflammatory marker genes. Taken together, these results show that *S. uberis* reduces endometrial cell viability under *in vitro* conditions and has the potential to provoke an inflammatory response in bovine endometrial cells. Our work presents a first step towards the understanding of the interaction *S. uberis* and the bovine endometrium. Further research should investigate the molecular mechanism of pathogenicity and immune response of the endometrium, which is expected to be, similar to the interaction of *S. uberis* and mammary epithelial cells (Günther et al., 2016; Li et al., 2018), a rather complex multifactorial process.

5. Conclusions

In conclusion, the present study provides first information regarding the effect of *S. uberis* strains on bovine endometrial cells in a primary cell culture model. Our study revealed that *S. uberis* could have a pathogenic effect on endometrial cells, similarly to other uterine pathogens, such as *T. pyogenes*. First evidence is provided that *S. uberis* strains isolated from the bovine uterus provoke an upregulation of transcription of genes involved in the inflammation pathways, such as the interleukin gene *IL1A* and the chemokine genes *CXCL8* and *CXCL1/2* as well as *PTGS2* genes, *in vitro*. Thus, *S. uberis* must be considered as a potential pathogen for bovine endometritis.

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Author contribution

PB: Contributed to the concept, designed and performed the experiments, conducted the data analysis, and wrote the manuscript; CG:

Contributed to the concept, helped performing the cell culture, analyzing the qPCR data, and edited the manuscript; KW: Performed the sampling and first isolation of strains, and edited the manuscript; MD: Conceived and contributed to the concept, headed the sampling and edited the manuscript; ME-S: Conceived the concept, supervised the design of experiments, the data analysis, and wrote the manuscript.

Declaration of Competing Interest

The authors declare that the research was performed without any commercial or financial affiliation that could be deemed as a potential conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi: https://doi.org/10.1016/j.vetmic.2020.108710.

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