Aus dem Institut für Veterinär-Biochemie
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Studies of the development of endometritis in cattle on a cellular basis

Inaugural-Dissertation
zur Erlangung des Grades eines
Doctor of Philosophy (PhD) in Biomedical Sciences
im Dahlem Research School (DRS) Programm
an der
Freien Universität Berlin

vorgelegt von
Mohammad Abdelwahab Ali Ibrahim
Tierarzt
aus Alexandria, Ägypten

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*For the soul of my brother **Ayman***

*For my **Mom** and my **Dad***

*For **Ahmed**, my brother and his little two sweeties **Renad** and **Retan***

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List of abbreviations

16S rRNA	16S ribosomal ribonucleic acid
AMP	Antimicrobial peptides
B. pumilus	*Bacillus pumilus*
B-cells	B lymphocytes
BPI	Bactericidal permeability increasing protein
cbpA	Collagen-binding protein
$CD4^+$	T helper lymphocyte cell bearing CD4 receptor
$CD8^+$	T cytotoxic lymphocyte cell bearing CD8 receptor
CD14	Cluster of differentiation antigen 14
CD45	Leukocyte-common antigen
CE	Clinical endometritis
CLR	C-type lectin receptors
CXCL	Chemokines (C-X-C motif) ligands
CXCR2	C-X-C chemokine receptor 2
DAMP	Damage-associated molecular patterns
DEFB	Beta-defensin
E. coli	*Escherichia coli*
e.g.	For example
EnPEC	Endometrial pathogenic *Escherichia coli*
ExPEC	Extra-intestinal pathogenic *Escherichia coli*
F. necrophorum	*Fusobacterium necrophorum*
fimA	Fimbrial protein subunit A
fimC	Fimbrial protein subunit C
fimE	Fimbrial protein subunit E
fimG	Fimbrial protein subunit G
fimH	Fimbrial protein subunit H
FRT	Female reproductive tract
FTIR	Fourier transform infrared
HI	Heat-inactivated
i.u.	Intrauterine
IL	Interleukin
IR	Infrared

L	Liters
LAP	Lingual antimicrobial peptide
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
LX	Lipoxins
MAPK	Mitogen-activated protein kinase
MD-2	Lymphocyte antigen 96
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation primary response gene 88
nanH	Neuraminidase H cell wall-bound protein
nanP	Neuraminidase P cell wall-bound protein
NF-κB	Nuclear factor kappa B
NK cells	Natural killer cells
NLR	Nucleotide-binding oligomerization domain like receptors
NOD	Nucleotide-binding oligomerization domain
NSAIDs	Nonsteroidal anti-inflammatory drugs
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
PGES	Prostaglandin E Synthase
$PGF_{2\alpha}$	Prostaglandin F_{2alpha}
PGI_2	Prostacyclin
PGN	Peptidoglycan
PLO	Pyolysin
PMN	Polymorphonuclear cells
pp	Postpartum
PRR	Pattern recognition receptors
PTGS	Prostaglandin-endoperoxide synthase
RIG	Retinoic acid-inducible gene
RLR	Retinoic acid-inducible gene-I-like receptor
SCE	Subclinical endometritis

siRNA	Short (or small) interfering ribonucleic acid
T. pyogenes	*Trueperella pyogenes*
TAP	Tracheal antimicrobial peptide
T-cells	T lymphocytes
TLR	Toll-like receptor
TNFA	Tumor necrosis factor alpha
VDS	Vaginal discharge score

1. Introduction

It is important to sustain food resources to fulfil the needs of the fast-growing human population. In this context, dairy cows make an important contribution to global food sustainability by utilising low quality proteins found in plants and providing high quality proteins in meat and milk, and their derivatives (Bradford, 1999). To meet the growing global demand, the productivity of dairy cows has been increased in recent decades through improvements in management and nutrition, as well as intensive genetic selection (VanRaden, 2004; Hayes et al., 2009). Adoption of these procedures has shown promising results, as the productivity of the animals has doubled compared with 50 years ago. In Germany for example, the average milk yield per cow per year was about 7543 litres (L) in 2014 compared with 3109 L in 1961 (FAO, 2016). However, overall milk production in Germany has improved to a lesser extent, increasing from about 24000 million L in 1961 to 32000 million L in 2014. This is attributed to a decrease in the number of producing dairy animals from 7.9 million in 1961 to 4.2 million in 2014 (FAO, 2016). This sharp decrease can be attributed to many factors, one of these factors is the decrease of reproductive efficiency of highly producing dairy animals (Veerkamp et al., 2003; Walsh et al., 2011). The subfertility of highly producing dairy cows is multifactorial and cannot be associated with one particular cause (Lucy, 2001). The decrease in fertility can be attributed to management, to reproductive impairment such as: uterine infections; impairment of oestrus behavioural expression and delay of ovarian resumption; decreases in oocyte and embryo quality, and other diseases such as hypocalcaemia; ketosis and mastitis (Lucy, 2001; Pryce et al., 2004; Lucy, 2007; Walsh et al., 2011).

Postpartum uterine diseases caused by uterine bacterial infections are a major factor in the incidence of subfertility. They lead to reductions in reproductive performance and in the profit potential of dairy farms (LeBlanc et al., 2002a). Economic losses due to postpartum uterine diseases are estimated to be more than €2 billion per annum for the United States and the European Union (Drillich et al., 2001; Sheldon et al., 2009a). Moreover, reproductive status is considered the single most important reason for culling decisions on dairy farms (Gröhn et al., 2003). This high negative impact of uterine diseases is connected to their high incidence among postpartum cows (Dubuc et al., 2011; LeBlanc, 2014), as about 50% of cows develop postpartum uterine diseases (Sheldon et al., 2009a). However, under the same conditions, some cows still undergo a normal involution process. This raises the question of why some cows develop postpartum uterine diseases, while other cows are able to overcome postpartum uterine infections and progress through normal involution. Thus, attention in the last few years has been directed to the role of the immune system, and in particular focussed on the role of bacteria and their virulence factors in the establishment of uterine infections and postpartum uterine diseases. Better understanding of these processes will aid the development of better prevention strategies and treatments. Therefore, the overall goal of this study was to provide a better understanding of the host-pathogen interactions inside the bovine uterus, and factors affecting these interactions.

2. Overview of literature

2.1. Classification of postpartum uterine diseases

Uterine diseases in cattle range from severe clinical diseases to subclinical chronic diseases (LeBlanc, 2014). Postpartum uterine diseases are commonly manifested in the form of metritis and endometritis. These diseases are defined by their severity, their pathological and clinical characteristics, and their negative effects on subsequent reproductive performance (Sheldon et al., 2009a).

2.1.1. Puerperal metritis and clinical metritis

Puerperal metritis occurs within 21 days after calving and is typified by the presence of a watery and foetid uterine discharge of red-brown coloration with distended uterus, accompanied by systemic signs (reduced milk yield and symptoms of toxaemia), and elevated body temperature >39.5 °C. Animals showing the same signs within the first 21 days postpartum (pp) without elevation of body temperature, toxaemia, or decreased milk yield are considered to be suffering from clinical metritis (Sheldon et al., 2006). Risk factors associated with metritis include stillbirth, twins, dystocia or retained placenta.

Puerperal metritis is found in about 20% of animals (Drillich et al., 2001; Benzaquen et al., 2007), and the incidence of clinical metritis has been reported as 35–50% (Zwald et al., 2004). The economic losses caused by each case of metritis amount to about 350 US$ (Drillich et al., 2001; Sheldon et al., 2009a), resulting from the costs of antibiotic treatment, decreased reproductive performance, decreased milk productivity (Huzzey et al., 2007; Dubuc et al., 2011; Wittrock et al., 2011; Giuliodori et al., 2013), and increased culling of the animals (Linden et al., 2009).

The treatment of metritis has been based mainly on the administration of systemic antibiotics such as ceftiofur and penicillin. In addition to antibiotics, nonsteroidal anti-inflammatory drugs (NSAIDs) or prostaglandin F_{2alpha} ($PGF_{2\alpha}$) have been reported to be beneficial in the treatment of puerperal metritis (Drillich et al., 2007; McLaughlin et al., 2013; Sannmann et al., 2013; Lima et al., 2014).

The economic cost of a single case of metritis is about 292 € (Drillich et al., 2001). Europe has about 25 million cows, while the United States has about 8.5 million cows (Ataide Dias et al., 2007; USDAERS, 2009). Therefore, with a conservative incidence rate of metritis (~20%), the calculated annual cost of metritis will be about 1.4 € billion in Europe, and 650 € million in the United States. Moreover, the costs of clinical and subclinical endometritis are representing an additional economic burden on the dairy industry.

2.1.2. Clinical endometritis

Endometritis is the inflammation of the inner layer of the uterus (endometrium). Diagnosis of endometritis in cattle has been based on the vaginal discharge score (VDS) (Sheldon et al., 2006). Clinical endometritis (CE) is not accompanied by systemic signs and is typified by the presence of a purulent uterine discharge after 21 days pp or mucopurulent uterine discharge after 26 days pp (Sheldon et al., 2006). Diagnosis of endometritis before day 21 pp can be confounded in animals that exhibit spontaneous resolution of bacterial contamination and by the variations in the appearance of normal lochia at this stage (Sheldon et al., 2006).

The incidence of CE is up to about 20% in dairy cattle (LeBlanc et al., 2002a). Endometritis is one of the main causes of decreased fertility in high-producing dairy cows (Sheldon et al., 2009a). The unfavourable effects of endometritis are mainly associated with reproductive impairment (Gilbert et al., 2005; Dubuc et al., 2010). Cows with CE are expected to have delayed resumption of ovarian activity after calving or extended postpartum luteal phases compared with normal cows (Opsomer et al., 2000). Endometritis reduces the conception rate by about 20% (Cerri et al., 2009; Galvão et al., 2009a; Dubuc et al., 2010; Machado et al., 2015), and increases embryonic mortality (Lima et al., 2013; Machado et al., 2015). Even after treatment for CE and resolution of the clinical signs, a subclinical form of endometritis may persist (Sheldon et al., 2009b). Therefore, treated cows are less fertile than cows that have not previously suffered postpartum clinical uterine disease.

The principle of CE treatment is based on killing uterine pathogens, removing of necrotic materials, hastening uterine involution, and inducing the estrous cycle. Treatments involve the use of systemic or intrauterine (i.u.) antibiotics, either solely or combined with $PGF_{2\alpha}$ or NSAIDs (Drillich et al., 2005; Galvão et al., 2009b; Sheldon et al., 2009b; Kaufmann et al., 2010; Lefebvre and Stock, 2012). Although the effects of different treatments and their impact on reproductive performance are heterogenous, a consistent finding is that cows treated with an i.u. infusion of cephapirin were able to eliminate the inflammation and had improved performance compared with cows treated with $PGF_{2\alpha}$ or with untreated cows (LeBlanc et al., 2002b; Lefebvre and Stock, 2012). The response of CE to treatment depends on the severity of the initial inflammation — in a study by McDougall (2001), the cure rate ranged between 40% and 72% two weeks after treatment. Spontaneous cure of CE without treatment was evident in approximately 33% of affected cows.

2.1.3. Subclinical endometritis

Subclinical endometritis (SCE) is the inflammation of the endometrium after day 21 pp without signs of CE. SCE is diagnosed by counting the percentage of polymorphonuclear cells (PMN) relative to the total cell count present in endometrial cytological samples obtained by cytobrushing (Kasimanickam et al., 2004; Kasimanickam et al., 2005a), low-volume uterine lavage (Gilbert, et al. 2005), or histology from biopsy samples (Bonnett et al., 1993). Several cut-off points for the percentage of PMN cells have been used, depending on the time point of sample collection in the postpartum period. The different cut-offs are associated with non-pregnancy at 100, 100–200 or >200 days pp (Kasimanickam et al., 2004). A lower threshold of PMN (≥ 5%) has also been used as a cut-off point to diagnose SCE (Gilbert et al., 2005; Gabler et al., 2010; Madoz et al., 2013). PMN percentages above this threshold have been associated with lower pregnancy rates. The recorded incidence of SCE depends on the cut-off threshold used for diagnosis, and thus has a wide range of values, occurring in 35–75% of animals (LeBlanc et al., 2002a; Gilbert et al., 2005; Sheldon et al., 2006).

Cows with SCE have more days open and have lower conception rates compared with normal cows. Therefore, they are more likely to be culled (Kasimanickam et al., 2004). Although SCE is not accompanied by systemic symptoms like metritis, it may affect milk production in indirect ways, such as via an increase in the release of cytokines including chemokines into the blood that may cause a reduce in the usual synthesis of the liver proteins, and eventually affect the feed intake and metabolism (Bertoni et al., 2008; Kasimanickam et al., 2013; Trevisi et al., 2015).

Treatment of cows with SCE depends on induction of the estrous cycle using $PGF_{2\alpha}$ and may involve the use of antibiotics and/or NSAIDs (Kasimanickam et al., 2005b; Galvão et al., 2009a; Priest et al., 2013). Whether SCE should be treated or not is still under debate. Although some studies have found that treatment has no or little effect on fertility of cows with SCE (Galvão et al., 2009a; Lima et al., 2013), some other studies have reported beneficial effects of the treatment (Kasimanickam et al., 2005b). This inconsistency between studies may reflect a need for alternative safe and effective treatments.

2.2. Bacteria associated with uterine diseases

After parturition, approaching 100% of dairy cows have uterine bacterial infections (Földi et al., 2006; Santos and Bicalho, 2012). The most common bacterial species associated with the incidence of endometritis are *Escherichia coli*, *Trueperella pyogenes*, *Fusobacterium necrophorum*, and *Prevotella* species (Sheldon et al., 2002; Williams et al., 2005; Westermann et al., 2010). These bacteria have been isolated and identified from uterine samples collected from diseased and healthy cows, based on standard aerobic and anaerobic cultivation techniques. However, these techniques likely underestimate the population of unculturable bacteria and their association with uterine diseases. Thus, identification of uterine bacteria using culture-independent techniques such as molecular and sequencing techniques, has recently been developed (Santos et al., 2011; Machado et al., 2012; Santos and Bicalho, 2012; Jeon et al., 2015; Knudsen et al., 2016). Some studies using culture-independent methods found that *E. coli*, *T. pyogenes*, and other bacteria identified by culture-dependent methods were associated with uterine diseases. However, other studies showed that other *Fusobacterium* species, *Bacteroides* species, and *Firmicutes* species but not *E. coli* or *T. pyogenes* were associated with metritis (Santos et al., 2011; Jeon et al., 2015; Jeon et al., 2016). Although the results of these studies are inconsistent, all studies agree that anaerobic bacteria are more abundant in inflamed uteri compared with healthy uteri (Machado et al., 2012; Santos and Bicalho, 2012; Jeon et al., 2015; Wagener et al., 2015; Knudsen et al., 2016). This is may be because the postpartum uterus has a microaerophilic environment resulting from tissue damage and necrosis that probably reduces the oxygen tension. In addition, the endometrium provides nutrients that aid bacterial growth and proliferation (Carneiro et al., 2016).

It has been reported when *E. coli* or *T. pyogenes* were infused into the uterus of healthy cows, signs of endometritis were evident within few days (Del Vecchio et al., 1992; Amos et al., 2014; Lima et al., 2015). Furthermore, an experimental vaccine containing inactivated bacterial components of *E. coli*, *F. necrophorum* and *T. pyogenes* reduced puerperal metritis in dairy cows (Machado et al., 2014). This indicates that *E. coli* and *T. pyogenes* are playing important roles in the development of postpartum uterine diseases in dairy cattle.

Besides *E. coli* and *T. pyogenes*, it is important to consider other bacterial species that may be associated with uterine diseases. It has been reported that alpha-hemolytic *Streptococcus* was strongly associated with reduced reproductive performance (Sens and Heuwieser, 2013). The anaerobes *Prevotella* species and *F. necrophorum* have been reported also to be associated with metritis and endometritis in cattle (Dohmen et al., 2000; Bicalho et al., 2012). Furthermore, a broad variety of other bacteria have been isolated from the uterus of cows with postpartum uterine diseases like *Bacillus* spp., especially *B. pumilus* and *B. licheniformis* (Wagener et al., 2015). However, there is insufficient detailed information concerning their

roles in the development of uterine diseases. Therefore, these bacteria have been classified as potential uterine pathogenic bacteria (Williams et al., 2005; Carneiro et al., 2016).

2.2.1. *Escherichia coli*

E. coli, a Gram-negative, non-spore forming, facultative anaerobic bacterium, has the characteristic of being both a widespread commensal gut species of vertebrates as well as a versatile pathogen (Tenaillon et al., 2010). Most *E. coli* serotypes are harmless; however, some serotypes have been implicated in a wide range of infections that affect animals and humans, including intestinal and extra-intestinal infections (Croxen and Finlay, 2010). The pathogenicity of *E. coli* strains is attributed to their ability to attach to host epithelial cells (Torres et al., 2005), flagellar motility (Lane et al., 2007), and the presence of toxins such as heat stable and labile toxins, and lipopolysaccharide (LPS) (Wolf et al., 1997). Host cells can recognise LPS via Toll-like receptor (TLR) 4, Cluster of differentiation antigen (CD) 14 and the Lymphocyte antigen 96 (MD-2) receptor complex, which leads to an inflammatory response typified by the release of cytokines including chemokines (Herath et al., 2006; Takeuchi and Akira, 2010). It has been presumed that *E. coli* is the main pathogen that initiates postpartum uterine diseases (Williams et al., 2007). It has also been suggested that the presence of *E. coli* during the first week after parturition might support subsequent infections by other pathogens like *T. pyogenes* and *F. necrophorum* (Dohmen et al., 2000; Williams et al., 2007; Bicalho et al., 2010; Sheldon et al., 2010; Bicalho et al., 2012). However, intrauterine presence of *E. coli* after the first week postpartum does not predispose cows to uterine diseases (Bicalho et al., 2012; Machado et al., 2012; Sens and Heuwieser, 2013).

There are a wide variety of *E. coli* strains that are found in the environment and shed in cattle faeces (Bettelheim et al., 2005; Houser et al., 2008). Therefore, it had been assumed that faecal *E. coli* could contaminate the endometrium to cause uterine diseases. However, it was subsequently observed that the *E. coli* associated with uterine diseases is an endometrial-specific strain, which was therefore named endometrial pathogenic *E. coli* (EnPEC) (Sheldon et al., 2010). EnPEC was found to be phylogenetically distinct from the common extra-intestinal pathogenic *E. coli* (ExPEC), and more closely related to human intestinal pathogens, as analysis of EnPEC revealed a lack of virulence factors compared with pathogenic enteric *E. coli* and ExPEC (Sheldon et al., 2010). EnPEC has a greater ability to attach to and invade endometrial cells compared with other strains of *E. coli*. It has been reported that EnPEC possesses a gene encoding the fimbrial protein subunit H (fimH) adhesion factor that helps EnPEC to adhere and colonise endometrial cells (Bicalho et al., 2010; Sheldon et al., 2010; Bicalho et al., 2012). EnPEC could also take advantage of cellular microfilaments and microtubules to invade endometrial cells (Sheldon et al., 2010). In addition, like all Gram-negative bacteria, EnPEC possess LPS, which induces an endometrial inflammatory response, hence exaggerating the uterine pathology.

2.2.2. *Trueperella pyogenes*

T. pyogenes is a Gram-positive, non-motile, non-spore forming, short, rod-shaped bacterium (Jost and Billington, 2005). *T. pyogenes* has undergone several name changes from *Bacillus pyogenes* (Glage, 1903) to *Corynebacterium pyogenes* (Eberson, 1918), to *Actinomyces pyogenes* (Reddy et al., 1982), to *Arcanobacterium pyogenes* (Pascual Ramos et al., 1997), and recently *T. pyogenes* because it exhibits the characteristics of the genus *Trueperella* (Yassin et al., 2011).

T. pyogenes is equipped with several virulence factors that are important for its pathogenicity. Pyolysin (PLO) is the primary virulence factor of *T. pyogenes* and might account for the severe damage and pathology caused by *T. pyogenes* infections (Jost and Billington, 2005; Amos et al., 2014). PLO is a potent cholesterol-dependent cytolysin, secreted by *T. pyogenes* as an exotoxin, that inserts into cholesterol-rich domains in the membranes of host cells and aggregates to form pores, leading to the osmotic death of the cell.

Endometrial epithelial cells are more resistant to PLO than stromal cells, because of their lower cholesterol content (Amos et al., 2014). Therefore, it has been suggested that *T. pyogenes* could cause uterine diseases once the epithelial layer is denuded after parturition as a result of a dystocia or retained placenta, and/or a previous intrauterine infection (Dohmen et al., 2000; Bicalho et al., 2012; Amos et al., 2014). The PLO gene is conserved among different isolates of *T. pyogenes* obtained from different farms in different countries (Silva et al., 2008; Santos et al., 2010; Amos et al., 2014), which may reflect the importance of the gene. In addition to PLO, *T. pyogenes* possess several cell wall endotoxins, which are involved in adherence to and colonisation of host mucosal surfaces. For example: fimbrial protein subunits, which are essential for the biogenesis of fimbriae and for the attachment of *T. pyogenes* to mucosal surfaces; neuraminidases (nan) such as nanH and nanP, which split sialic acids from host molecules, reducing mucus viscosity for increased adhesion; and collagen-binding protein (cbpA), which binds to collagens of host cells (Jost et al., 2001; Jost et al., 2002; Esmay et al., 2003; Pietrocola et al., 2007). Moreover, as in all bacteria, the cell wall of *T. pyogenes* possess lipoteichoic acid (LTA), which induces pro-inflammatory responses in endometrial cells (Davies et al., 2008), hence exaggerating uterine pathology. *T. pyogenes* elicits an inflammatory response in bovine endometrium *in vivo*, and in bovine endometrial explants and cells *in vitro*, increasing the expression of pro-inflammatory mediators such as interleukin (IL) 1B, IL6, IL8 and $PGF_{2\alpha}$ (Miller et al., 2007; Amos et al., 2014; Lima et al., 2015).

Infection with *T. pyogenes* is associated with severe forms of postpartum uterine inflammation (Bonnett et al., 1991; Prunner et al., 2014; Lima et al., 2015). Although *T. pyogenes* is an important bacterium associated with severe endometritis, it has also been isolated from cows with normal puerperium (Silva et al., 2008; Bicalho et al., 2012). Initial attempts to understand its pathogenicity focused on identifying differences between *T. pyogenes* strains isolated from cows with a normal puerperium or clinical forms of uterine inflammation. Silva et al. (2008) found that none of the identified virulence genes were linked to the presence of clinical metritis. Other studies reported that the gene encoding the virulence factor fimbrial protein subunit A (fimA) occurs more frequently in *T. pyogenes* strains isolated from cows with CE (Bicalho et al., 2012), or metritis (Santos et al., 2010) compared with strains found in the uterus of cows with a normal puerperium.

2.2.3. *Bacillus pumilus*

B. pumilus is a Gram-positive, aerobic, rod-shaped endospore-forming bacterium (Priest, 1993), which belongs to the *B. subtilis* group (Fritze, 2004). This bacterium is a ubiquitous soil organism, which may suggest it can contaminate the uterus after parturition. The cell wall of *B. pumilus*, as in all Gram-positive bacteria, comprises peptidoglycan covered by LTA, which contains polyglycosyl phosphates with mono- and disaccharides, which help in adhesion to host cells (Parvathi et al., 2009). Some *B. pumilus* strains have beneficial effects such as the production of antimicrobial compounds against some pathogenic bacteria and fungi. They

reduce metal toxicity (Myresiotis et al., 2014; Saranya et al., 2014; Tiwari et al., 2014), and have been used as probiotics for animals (Larsen et al., 2014). However, other *B. pumilus* strains have been associated with diseases in human and animals. For example, a toxinogenic *B. pumilus* strain isolated from mastitic milk was associated with foodborne disease in human (Nieminen et al., 2007). Two isolates of *B. pumilus* have also been associated with severe sepsis in neonatal infants (Kimouli et al., 2012).

The presence of *Bacillus* species has been reported in the uterus of postpartum cows (Hussain et al., 1990; Bonnett et al., 1991). Several *Bacillus* spp. were isolated from cows with clear or purulent vaginal discharge (Wagener et al., 2015). Some *Bacillus* species, such as *B. licheniformis* have been repeatedly isolated from the bovine uterus, with or without endometritis (Williams et al., 2007). Moreover, *B. pumilus* has been isolated from uteri of normal fertile and repeat-breeder cows (Messier et al., 1984). In a study during the first four weeks after calving, *Bacillus* spp. was among the frequently isolated genera with a prevalence of 9.3%. Approximately half of the *Bacillus* spp. isolates belonged to the species *B. pumilus* (55.5%) and 22.5% to the species *B. licheniformis* (Wagener et al., 2015). In an *in vitro* study, *B. pumilus* caused the death of bovine endometrial epithelial cells and increased the mRNA expression of pro-inflammatory factors (Gärtner et al., 2016). Therefore, *B. pumilus* might be a potentially pathogenic bacterium for endometrial cells.

2.3. Uterine innate immunity

The task of the host immune system is to counter a threat (e.g. a microbial insult) and return tissues to their homeostatic state (Odegaard and Chawla, 2013). The immune response falls into two broad classes: the innate immune response and the adaptive immune response. The latter is composed of highly specific cells and process that eliminate a particular pathogen and usually involves lymphocytes and the production of antibodies. On the other hand, the innate immune response is composed of nonspecific cells and processes to eliminate broad classes of pathogens (Hoebe et al., 2004).

Innate immune responses predominate in the uterus, in which the endometrium plays a pivotal role. The uterine lining epithelial cells form a resistant barrier to chemical, mechanical, and microbial agents (Hjelm et al., 2010; Blaskewicz et al., 2011). Moreover, the endometrial and immune cells can recognise the vast majority of pathogens and initiate a series of events that eventually lead to the elimination of insulting pathogens (Herath et al., 2006; Sheldon and Roberts, 2010; Cronin et al., 2012; Sheldon et al., 2014; Turner et al., 2014).

Under normal circumstances, dairy cows have a robust immune response that is able to eliminate most threats. However, they suffer from reduced immunity for several weeks around calving, reaching its lowest point during the first week after parturition (Singh et al., 2008). The impaired immune function in transition cows might be attributed to metabolic stress and hormonal fluctuations. Metabolic stress could be associated with the peripartum drop in nutrient intake, the breakdown of body fat and protein, and a negative energy balance (Lucy, 2007; LeBlanc, 2014). During late gestation, there are dramatic changes in oestrogen and progesterone concentrations, and at calving there is a massive transient increase in cortisol, which may contribute to peripartum immunosuppression (Goff and Horst, 1997; Ingvartsen, 2006). Lactation also worsens the periparturient immunosuppression (Detilleux et al., 1995). Impairment of uterine innate immunity predisposes cows to reproductive diseases such as retained placenta, metritis, or endometritis (Gilbert et al., 1993; Hammon et al., 2006).

Therefore, it is important to understand the key elements of uterine innate immunity and what may be the factors affecting them.

2.3.1. Recognition of uterine bacteria

A robust innate immune response relies on efficient recognition of threats. Mucosal epithelial and immune cells possess pattern recognition receptors (PRR), which recognise threatening signals in the form of pathogen-associated molecular patterns (PAMP) or damage-associated molecular patterns (DAMP) (Tang et al., 2012). PAMP are conserved microbial domains such as proteins, lipids, lipoproteins, and nucleic acids. DAMP or alarmins are products derived from the host that signal tissue and cell damage.

The four most recognised classes of PRR include TLR, nucleotide-binding oligomerisation domain (NOD)-like receptors (NLR), retinoic acid-inducible gene (RIG)-I-like receptors (RLR) and C-type lectin receptors (CLR) (Takeuchi and Akira, 2010; Moresco et al., 2011).

TLR possess the ability to recognise a wide range of microbial ligands. The lipopeptides found in all bacteria are recognised by heterodimers formed by TLR1, TLR2, and TLR6. LPS found in all Gram-negative bacteria is recognised by TLR4, CD14 and the MD-2 complex. Nucleic acids of pathogens are recognised by TLR3, TLR7, TLR8, and TLR9, and flagellin is recognised via TLR5 (Jin and Lee, 2008; Takeuchi and Akira, 2010).

When microbial ligands bind to TLR, the TLR undergo conformational changes leading to the formation of homodimers or heterodimers (TLR1/TLR2 and TLR2/TLR6). These transformations trigger intracellular signalling pathways such as the Myeloid differentiation primary response gene 88 (MyD88)-dependent intracellular signalling pathway, which activates the Nuclear Factor Kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) cascades to induce the expression of genes encoding pro-inflammatory mediators (Lamkanfi and Dixit, 2014; Philpott et al., 2014; Sheldon et al., 2017).

In vivo and *in vitro* studies have shown considerable evidence for the presence of TLR in the bovine endometrium. The bovine endometrium expresses TLR1 through TLR10 *in vivo* (Davies et al., 2008; Chapwanya et al., 2012), whereas endometrial epithelial cells express TLR1 through TLR7 and TLR9. Endometrial stromal cells express TLR1 through TLR4, TLR6, TLR7, TLR9, and TLR10 (Davies et al., 2008). In addition, using short interfering RNA (siRNA) and other biochemical inhibitors, the functional activities of TLR such as TLR1, TLR2, TLR4 and TLR6 and their key pathways have been identified in bovine endometrial cells (Cronin et al., 2012; Turner et al., 2014). Moreover, endometrial tissues and cells express different pro-inflammatory mediators and antimicrobial peptides (AMP) upon stimulation with different PAMP (Sheldon and Roberts, 2010; Borges et al., 2012; Healy et al., 2015). In the human endometrium, TLR expression is regulated in part by the stage of the estrous cycle (Aflatoonian et al., 2007). However, this regulation was not evident in the bovine uterus (Davies et al., 2008).

2.3.2. Endometrial inflammatory response to threats and its regulation

Generally, inflammation is the main response to the activation of TLR by PAMP or DAMP (Sheldon et al., 2017). This inflammatory response is composed of the synthesis and release of pro-inflammatory molecules (such as cytokines including chemokines), other mediators (such as prostaglandins), and AMP, and the migration of macrophages and neutrophils to the site of infection or damage and their activation to resolve the insult to the tissue (Zerbe et al., 2003; Beutler, 2009; Takeuchi and Akira, 2010; Moresco et al., 2011; Oeckinghaus et al.,

2011). In the same context, the components of the inflammatory response were evident in the bovine uterus using *in vivo* and *in vitro* studies. These components are regulated by many factors including nutrition, hormonal changes, and the severity of the insult to endometrial tissues and cells (Sheldon et al., 2017).

2.3.2.1. Pro-inflammatory mediators

Cytokines are protein mediator molecules that are released by activated sentinel cells. They diffuse from their site of release and bind to receptors on nearby cells to trigger their responses. Sentinel cells synthesise and secrete three major cytokines — tumour necrosis factor A (TNFA), IL1, and IL6. TNFA is produced in the very early stages of inflammation, followed by waves of IL1, then IL6 (Tizard, 2013).

Chemokines are a family of a small chemotactic cytokines. They coordinate the migration of immune cells to sites of inflammation or microbial invasion. Chemokines are produced by different immune and epithelial cells in infected or damaged tissues (Charo and Ransohoff 2006). The chemokines (C-X-C motif) ligands 1 (CXCL1), CXCL2, CXCL3, CXCL5 and CXCL8, which is known as IL8, are strong chemo-attractants that all bind to chemokine receptor 2 (CXCR2) and stimulate the recruitment and activation of neutrophils (Thelen, 2001; Moser et al., 2004).

Prostaglandins play a significant role in reproductive tissue homeostasis and in inflammatory responses (Kaneko and Kawakami, 2009; MacKintosh et al., 2013). Prostaglandins are formed when arachidonic acid is released from the plasma membrane by phospholipases, and converted by prostaglandin-endoperoxide synthases (PTGS) into prostanoids such as prostaglandins, thromboxanes and prostacyclins (Ricciotti and FitzGerald, 2011). There are four principal bioactive prostaglandins generated *in vivo*, which are prostacyclin (PGI_2), PGE_2, PGD_2, and $PGF_{2\alpha}$ (Tilley et al., 2001; Smyth et al., 2009). Under homeostatic conditions, the production of prostaglandins is very low, but in acute inflammation higher expression of PTGS2 is induced leading to increase production of prostaglandins immediately prior to the recruitment of leukocytes and the infiltration of immune cells (Ricciotti and FitzGerald, 2011).

The endometrial tissue is distinct from other body tissues because uterine innate immunity undergoes fluctuations during the oestrous cycle under the control of steroidal sex hormones such as progesterone and oestrogen (Lewis, 2004). This may be critical for the control of different physiological processes needed for successful reproduction. For example, several pro-inflammatory factors under the influence of steroidal hormones are involved in the regulation of luteal function (Koets et al., 1998; Goff, 2004; Schams and Berisha, 2004; Skarzynski et al., 2005; Berisha et al., 2010). During phases of progesterone dominance, several chemokines and prostaglandin synthetases have also been found to be regulated in the endometrium of cows during the implantation window, which may help in promoting vascularisation and immune cell distribution, necessary for the implantation and growth of the semi-allogeneic embryo (Arosh et al., 2003; Emond et al., 2004; Imakawa et al., 2006; Mansouri-Attia et al., 2009; Rekik et al., 2011; Spencer et al., 2013). Pro-inflammatory factors that are highly expressed in the endometrium around ovulation such as CXCL5, IL1B and IL8 (Fischer et al., 2010; Gabler et al., 2009), or found in the seminal plasma such as IL6, IL10, and TNFA (Vera et al., 2003; Aloé et al., 2012) may stimulate uterine contractions and the migration of leukocytes to aid sperm transport and the elimination of unhealthy spermatozoa, or counteract any infection that may have taken place during mating, or to prepare the uterus

and to adjust the maternal immunity to facilitate the implantation of a semi-allogeneic embryo (Vera et al., 2003; Parent and Fortier, 2005; Alghamdi et al., 2009; Gabler et al., 2009; Fischer et al., 2010; Aloé et al., 2012)).

Uterine inflammation is characterised by dysregulation of expression of genes of the pro-inflammatory response (**Fig. 1**). The synthesis of several cytokines (TNFA, IL1A, IL1B, and IL6), chemokines (CXCL1/2, CXCL3, CXCL5, and IL8), prostanoids (PGE_2 and $PGF_{2\alpha}$) and enzymes involved in their synthesis (PTGS2 and PTGES) have been shown to be increased in inflamed uterine tissue compared with healthy uterus during postpartum period (Gabler et al., 2009; Fischer et al., 2010; Sheldon et al., 2014; Ledgard et al., 2015; Peter et al., 2015). The dysregulation of these pro-inflammatory factors is supported by the clinical picture of endometritis. For example, the dysfunction of a corpus luteum during endometritis is believed due to the dysregulation of PGE_2:$PGF_{2\alpha}$ ratio, which leads to either shortening or prolongation of life span of corpus luteum (Herath et al., 2009). These *in vivo* findings are also supported by *in vitro* evidence for the functional expression of these pro-inflammatory factors in the endometrial tissue. Endometrial cells or *ex vivo* organ cultures responded to the presence of bacteria associated with uterine diseases or their PAMP, or DAMP, by increased expression of various pro-inflammatory mediators including IL1A, IL1B, IL6, IL8, and PGE_2 (Sheldon et al., 2010; Amos et al., 2014; Healy et al., 2014; 2015; Gärtner et al., 2016).

2.3.2.2. Antimicrobial peptides (AMP)

AMP are widely expressed in various types of tissues and cells in almost all species, which is supporting evidence for their vital role as effective weapons against different pathogens (Zasloff, 2002). AMP prevent and/or reduce infection by killing microorganisms or inhibiting their growth (Ganz, 2003; Selsted and Ouellette, 2005). Beyond their direct antimicrobial effect, AMP also play roles in cellular signalling and differentiation, aiding in the modulation of local and systemic immune responses and tissue homeostasis (**Fig. 1**) (Zasloff, 2002; Ganz, 2003; Selsted and Ouellette, 2005).

AMP are short polypeptides (<100 amino acids), and can have an amphipathic structure i.e. clusters of hydrophobic and cationic amino acids are spatially organised in distinct parts of the molecule, which enable them to interact with anionic moieties of prokaryotic cells such as negatively charged phospholipids of the cell membrane, lipid A of LPS or LTA (Zasloff, 2002; Yeaman and Yount, 2003). Expression of AMP occurs either constitutively, as a prophylactic defence, or is induced in response to potentially threatening stimuli such as physical or microbial insults, or in response to pro-inflammatory cytokines (Ganz, 2003; Goldmann and Medina, 2012). Expression of AMP may be also regulated under influence of steroidal hormones (Cunha et al., 2004).

In the human female reproductive tract (FRT), AMP expression is menstrual cycle-dependent, with higher expression during the secretory and menses phases of the menstrual cycle, when infections are more likely to occur (King et al., 2003; Yanaihara et al., 2004; Kunimi et al., 2006; Shust et al., 2010). This cyclic regulation of AMP expression was not evident in women who used an oral contraceptive pill or progesterone intrauterine release device (Fleming et al., 2003; King et al., 2003).

AMP provide an important chemical barrier to invading pathogens (**Fig. 1**), and detection of AMP expression at different mucosal sites would be expected. In the context of the bovine

uterus, endometrial cells express many AMP including defensins, bactericidal permeability increasing protein (BPI) and S100 proteins (Davies et al., 2008; Wathes et al., 2009; Swangchan-Uthai et al., 2013), which are discussed below.

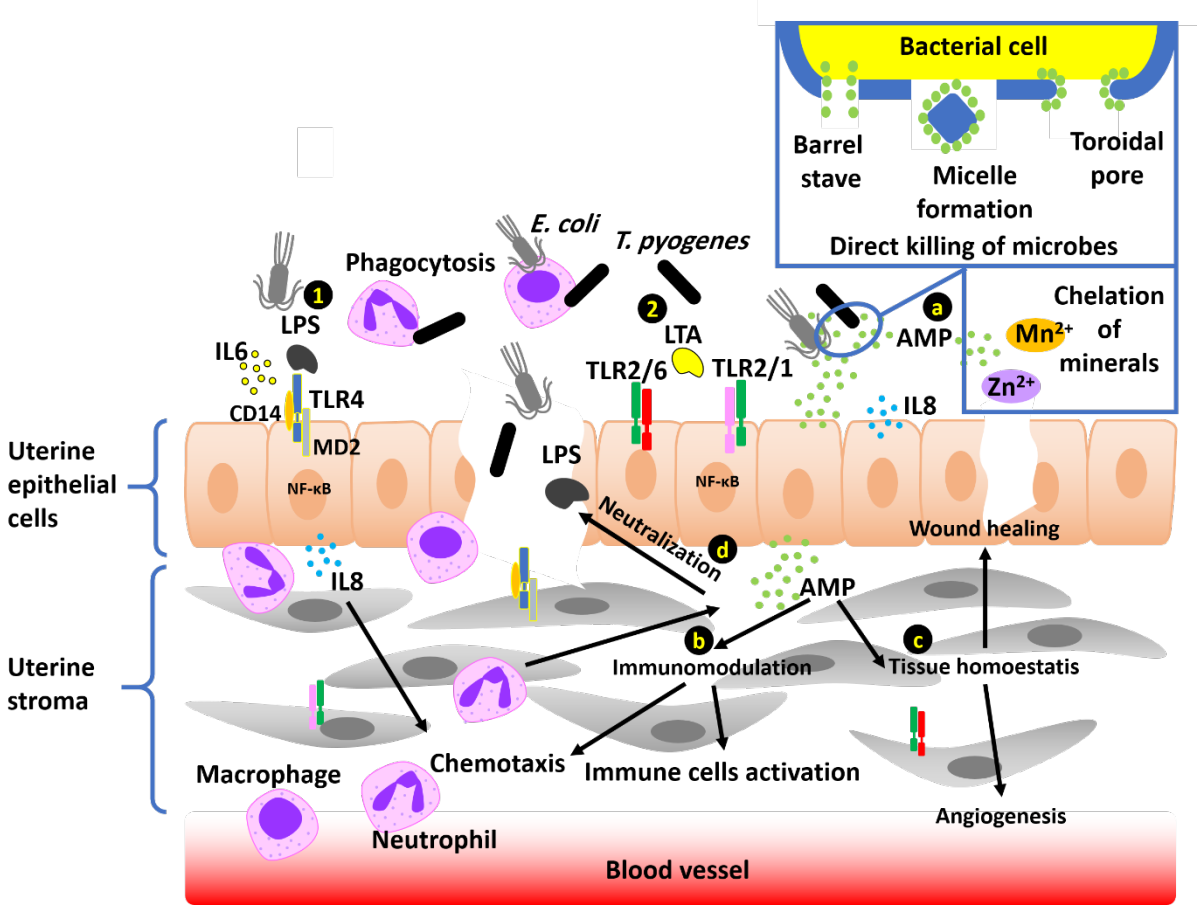

Figure 1. Endometrial cell responses to pathogenic bacteria are typified by the expression of cytokines including chemokines, and antimicrobial peptides, and the migration of immune cells. (1) LPS is recognized by TLR4, CD14 and MD-2 complex, while (2) LTA is recognized by TLR2/TLR1 or the /TLR6 complex. Activation of TLR results in the expression of cytokines such as IL6, chemokines such as IL8, and AMP. AMP has multiple roles; (a) bactericidal: defensins kill the bacteria through disruption of the bacterial cell wall by barrel stave formation, micelle formation, or toroidal pore formation. Meanwhile, the S100A protein family kill bacteria by chelating Zn^{2+} and Mn^{2+} that are essential for bacterial growth; (b) immunomodulation through promotion of chemotaxis and activation of immune cells; (c) tissue homeostasis via the promotion of angiogenesis and wound healing; (d) promotion of anti-inflammatory effects via neutralization of LPS and inhibition of proteases.

2.3.2.2.1. Defensins

Defensins represent one of the most important classes of AMP because they are widely distributed in mammalian epithelial cells and phagocytes (Zasloff, 2002; Ganz, 2003; Selsted and Ouellette, 2005). They are small cationic peptides (<45 amino acids) that contain six cysteines linked by three disulphide bonds (Lehrer and Lu, 2012). These disulphide bonds are essential to maintain the conformational stability and biological activities of defensins. There are many types of defensins but beta-defensins (DEFB) are the only type present in the bovine genome (Meade et al., 2014).

DEFB are expressed in immune cells as well as in the mucosal lining of different organs (Zasloff, 2002; Ganz, 2003; Selsted and Ouellette, 2005). DEFB target pathogens based on their charged cell walls. The positively charged regions within the defensins are attracted to negatively charged microbial membranes, and the hydrophobic domains of the defensin become buried within the interior lipid membrane of the microbe (Ganz, 2003). This disturbs the membrane function and leads to lysis of the microorganism (**Fig. 1**). The host cell walls are not attacked by DEFB, because of their neutral charged cells walls due to presence of cholesterol and lipids (Zasloff 2002). In addition to their antimicrobial effects, DEFB have an immunomodulatory function (**Fig. 1**) and can stimulate adaptive immune responses (Raj and Dentino, 2002; Bowdish et al., 2005; Allaker, 2008).

Bovine tracheal antimicrobial peptide (TAP) was one of the first discovered DEFB (Diamond et al., 1991), and shows a strong association with different inflammatory conditions (Caverly et al., 2003). Further analysis of the genome of *Bos taurus* has revealed the presence of about 57 putative DEFB genes (Diamond et al., 1991). In the context of the bovine uterus, many of the DEFB are expressed in the endometrium, for example the bovine DEFB family including DEFB1-5, lingual AMP (LAP), and TAP (Cormican et al., 2008). Higher expression of these AMP was evident in uterine cells treated with PAMP *in vitro* (Davies et al., 2008), reflecting the functional importance of AMP in the bovine uterus, and supporting the notion that TLR mediate the induction of AMP in response to PAMP (Selsted and Ouellette, 2005).

2.3.2.2.2. Bactericidal/permeability-increasing protein

BPI protein is a cationic antimicrobial peptide expressed in many cell types in humans, rabbits and cows (Levy et al., 2000). BPI is characterised by its LPS neutralising activity, because BPI has a higher affinity for the lipid A component of LPS in Gram-negative bacteria (Gazzano-Santoro et al., 1992; Levy et al., 2000; Ciornei et al., 2002). This interaction is also crucial for its antimicrobial activity (Mannion et al., 1990). After binding to LPS, BPI penetrates into the inner membrane, leading to membrane impairment and eventually death of bacterial cells (Mannion et al., 1990; Elsbach and Weiss, 1998). Constitutive expression of BPI is evident at the mRNA and protein levels in different mucosal surfaces, suggesting a role for BPI in controlling the microflora environment. However, induced higher expression of BPI at the epithelial surface of the intestinal and genital tract has also been observed under the effect of endogenous lipid mediators called lipoxins (LX) (Canny et al., 2002; Canny et al., 2006). LX are related to anti-inflammatory molecules called resolvins, which play a key role in the resolution of inflammation (McMahon and Godson, 2004). Therefore, LX-induced epithelial BPI expression might contribute to the protective effects of these molecules.

One important property of BPI is that its activity against Gram-negative bacteria is not affected by different biological fluids including plasma, serum, whole blood, and milk, suggesting BPI as a target for biopharmaceutical development (Levy et al., 2000; Chockalingam et al., 2007; Canny and Levy, 2008).

2.3.2.2.3. S100 proteins

S100 proteins are calcium-binding multifunctional proteins, and are produced by various cell types such as stimulated neutrophils and monocytes or released by apoptotic cells (Hashemi et al., 2001; Donato et al., 2013). They are involved in different physiological calcium-dependent cellular processes such as motility, contraction, cell growth, differentiation and

structural organisation of membranes (Santamaria-Kisiel et al., 2006). They also have different roles in innate immunity such as modulation of immune cell differentiation and migration (Lagasse and Weissman, 1992; Ryckman et al., 2003; Vogl et al., 2004; McNeill et al., 2007), and promotion of wound healing (Goebeler et al., 1995; Thorey et al., 2001), as well as having anti-inflammatory (Lim et al., 2008), and antimicrobial activities (Sohnle et al., 2000; Zaia et al., 2008).

Members of the S100A protein family such as S100A8 and S100A9 exert their antimicrobial action without directly contacting the pathogen, as they act through chelation of several ions, such as zinc and manganese (**Fig. 1**), that are required for microbial growth (Sohnle et al., 2000; Corbin et al., 2008; Sedaghat and Notopoulos, 2008). S100A8 and S100A9 form a heterodimer called calprotectin, which have a zinc-binding capacity higher than other S100 proteins, therefore it has a higher antimicrobial activity (Striz and Trebichavsky, 2004).

Many of the S100 protein family, including S100A8, S100A9, and S100A12, are associated with various inflammatory diseases such as rheumatoid arthritis, chronic bronchitis, and cystic fibrosis (Frosch et al., 2000; Broome et al., 2003; Foell et al., 2004). It has been suggested that members of S100 protein family can be used as diagnostic markers for various diseases (Sedaghat and Notopoulos, 2008).

Many members of the S100A family are associated with different physiological and inflammatory conditions in the FRT of human, cows, and mice (Passey et al., 1999; Espinoza et al., 2003; Havelock et al., 2005; Kunimi et al., 2006; Zegels et al., 2009; Hanaue et al., 2011) and thus likely play an important role in human and animal reproduction. In the bovine uterus, S100A8 and S100A9 have been detected in the luminal and glandular epithelium, and in the stroma of the endometrium (Swangchan-Uthai et al., 2013). In addition, cows suffering from negative energy balance and chronic postpartum endometritis, show an elevated expression of S100A8 and S100A9, so these proteins could be key components of the endometrial response to postpartum uterine infections (Swangchan-Uthai et al., 2012; Swangchan-Uthai et al., 2013).

2.3.3. Uterine immune cells

The bovine endometrium hosts various populations of immune cells, which play a vital role during embryo implantation or during course of infection (Hansen, 2007; Oliveira and Hansen, 2008; Singh et al., 2008). These immune cells are mainly lymphocytes, and are located in the stroma, the glandular and luminal epithelium. The lymphocyte population is composed of B lymphocytes (B-cells) such as B-B7$^+$ cells, T lymphocytes (T-cells) such as T helper lymphocyte cell bearing CD4 receptor (CD4$^+$cells) and T cytotoxic lymphocyte cell bearing CD8 receptor (CD8$^+$ cells) and natural killer (NK) cells (CD335$^+$ cells) in both pregnant and cyclic endometrium (Leung et al., 2000; Mansouri-Attia et al., 2012; Oliveira et al., 2013). The distribution and number of the NK cell population is affected by pregnancy from around day 16, which implies the beneficial and important role of these cells in maternal recognition and establishment of pregnancy (Oliveira et al., 2013). Also, there are some reports that intrauterine infusion of peripheral blood mononuclear cells (PBMC) improves pregnancy outcomes in humans and cows by inducing of a beneficial inflammatory response in the uterus that facilitates embryo implantation (Yoshioka et al., 2006; Ideta et al., 2010a; Ideta et al., 2010b).

During the postpartum period, the elimination of uterine bacterial infection involves recruitment of professional phagocytic immune cells such as PMN and macrophages, constituting a typical inflammatory response (LeBlanc, 2014; Sheldon et al., 2014; Sheldon et al., 2017). These cells adhere, attach, digest, and eliminate insulting bacteria (Singh et al., 2008). Therefore, during the first three weeks after parturition, the proportion of neutrophils remains high in the endometrial tissue as part of a physiological inflammatory process associated with uterine clearance and remodelling (Gilbert and Santos, 2016). Thus, the proportion of PMN in uterine cytological samples beyond the first three weeks after parturition has been used to reflect the uterine health status. Cows with normal puerperium should have a robust but controlled inflammatory response. However, cows suffering from postpartum uterine diseases will have an uncontrolled and persistent inflammatory response, which is evidenced by the persistent high expression of inflammatory genes and infiltration of immune cells into endometrial tissues beyond the first three weeks after parturition (Gabler et al., 2010; Chapwanya et al., 2012; Peter et al., 2015). Cows that develop metritis or endometritis are significantly more likely to have PMN with decreased killing power, and PMN with a lower glycogen content compared with healthy cows (Cai et al., 1994; Mateus et al., 2002a; Hammon et al., 2006; Galvão et al., 2010). This may indicate that cows that develop endometritis have less efficient immune cells, leading to the recruitment of greater numbers of cells and hence, the development of a persistent uterine inflammation.

3. Aim of study

The philosophy behind the current study is to fill gaps in our current understanding of the innate uterine immune response under different conditions by using different models spanning the use of an *ex-vivo* model during the estrous cycle, an *in vivo* model during the postpartum period, and *in vitro* models of sentential cells involved in uterine immunity (uterine epithelial cells and/or immune cells) under defined conditions. These conditions include co-culture with pathogenic bacteria (*T. pyogenes*) or potentially pathogenic bacteria (*B. pumilus*) associated with uterine diseases. A further aim was to investigate factors associated with the virulence and pathogenicity of *T. pyogenes*, a common bacterium associated with severe form of endometritis.

The current study has two main hypotheses. First, AMP as an important component of the uterine innate immunity play an important role in the bovine uterus in protection against bacterial infections, which likely occurs during distinct stages of the estrous cycle or during the puerperium. Second, the pathogenicity of *T. pyogenes* can be attributed to certain strain characteristics.

Therefore, objectives of the current study were:

First, to evaluate the mRNA expression of AMP in uterine epithelial cells collected: (a) at different stages of the estrous cycle; (b) during the postpartum period (Days 24–51) from cows with healthy endometrium, or signs of SCE, or CE; (c) during *in vitro* co-culture of bovine endometrial epithelial cells with *B. pumilus*.

Second, (a) to characterise two different strains of *T. pyogenes*, one isolated from the uterus of a cow developing clinical endometritis (TP2) and a strain from the uterus of a healthy cow (TP5) in terms of their metabolic profiles, growth rates, and mRNA expression of known virulence factors; (b) to evaluate and compare the mRNA expression of pro-inflammatory factors of bovine endometrial epithelial cells in the presence of each strain (TP2 and TP5); and (c) to examine the mRNA expression of pro-inflammatory factors in endometrial epithelial cells co-cultured with TP2 in the presence or absence of PBMC.

4. Subsuming the published work

Publication 1: **Increased mRNA expression of selected antimicrobial peptides around ovulation and during inflammatory processes in the bovine endometrium postpartum**

Ibrahim, M., Peter, S., Gärtner, M.A., Michel, G., Jung, M., Einspanier, R., and Gabler, C. (2016). Increased mRNA expression of selected antimicrobial peptides around ovulation and during inflammatory processes in the bovine endometrium postpartum. *Theriogenology* 86**,** 2040-2053.

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Publication 2: **Bovine endometrial epithelial cells scale their pro-inflammatory response *in vitro* to pathogenic *Trueperella pyogenes* isolated from the bovine uterus in a strain-specific manner**

Ibrahim M, Peter S, Wagener K, Drillich M, Ehling-Schulz M, Einspanier R and Gabler C (2017) Bovine Endometrial Epithelial Cells Scale Their Pro-inflammatory Response *In vitro* to Pathogenic *Trueperella pyogenes* Isolated from the Bovine Uterus in a Strain-Specific Manner. *Front. Cell. Infect. Microbiol*. 7:264.

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Bovine Endometrial Epithelial Cells Scale Their Pro-inflammatory Response *In vitro* to Pathogenic *Trueperella pyogenes* Isolated from the Bovine Uterus in a Strain-Specific Manner

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Abstract

Among different bacteria colonizing the bovine uterus, *Trueperella pyogenes* is found to be associated with clinical endometritis (CE). The ability of cows to defend against *T. pyogenes* infections depends on the virulence of invading bacteria and on the host's innate immunity. Therefore, to gain insights into bacterial factors contributing to the interplay of this host pathogen, two strains of *T. pyogenes* were included in this study: one strain (TP2) was isolated from the uterus of a postpartum dairy cow developing CE and a second strain (TP5) was isolated from a uterus of a healthy cow. The two strains were compared in terms of their metabolic fingerprints, growth rate, virulence gene transcription, and effect on bovine endometrial epithelial cells *in vitro*. In addition, the effect of the presence of peripheral blood mononuclear cells (PBMCs) on the response of endometrial epithelial cells was evaluated. TP2, the strain isolated from the diseased cow, showed a higher growth rate, expressed more virulence factors (*cbpA, nanH, fimE*, and *fimG*), and elicited a higher mRNA expression of pro-inflammatory factors (*PTGS2, CXCL3*, and *IL8*) in bovine endometrial epithelial cells compared with TP5, the strain isolated from the healthy cow. The presence of PBMCs amplified the mRNA expression of pro-inflammatory factors (*PTGS2, CXCL3, IL1A, IL6*, and *IL8*) in bovine endometrial epithelial cells co-cultured with live TP2 compared with untreated cells, especially as early as after 4 h. In conclusion, particular strain characteristics of *T. pyogenes* were found to be important for the development of CE. Furthermore, immune cells attracted to the site of infection might also play an important role in up-regulation of the pro-inflammatory response in the bovine uterus and thus significantly contribute to the host-pathogen interaction.

Keywords: *Trueperella pyogenes*, endometritis, bovine endometrial cells, immune cells, FTIR spectroscopy

Introduction

The subfertility of high-producing dairy cows represents a major obstacle to the profitability and sustainability of the dairy industry. Among the most common reasons for subfertility are postpartum uterine diseases caused by uterine bacterial infections after parturition (Sheldon et al., 2009). During the postpartum period, up to 50% of all dairy cows develop uterine inflammatory diseases, such as metritis, and clinical or subclinical endometritis (Sheldon et al., 2009). The incidence of uterine inflammation is associated with the presence of certain bacterial species in the uterus, such as *Escherichia coli, Trueperella pyogenes, Fusobacterium* spp., and *Bacteroides* spp. (Williams et al., 2005; Sheldon et al., 2010; Machado et al., 2012; Wagener et al., 2015). In particular, *T. pyogenes* has been found to be associated with chronic and severe forms of endometritis (Bonnett et al., 1991; Wagener et al., 2014b).

T. pyogenes is a gram-positive opportunistic bacterium that acts as a primary uterine pathogen (Amos et al., 2014; Lima et al., 2015). The pathogenicity of *T. pyogenes* is attributed to pyolysin (PLO), which causes cytolysis of host cells (Jost et al., 1999; Amos et al., 2014). Moreover, *T. pyogenes* expresses a number of known and putative virulence factors, which may be involved in adhesion to host cells (Jost and Billington, 2005). Furthermore, *T. pyogenes* could trigger a pro-inflammatory response within the uterus, with transmigration of neutrophils and evidence of mucopurulent discharge (Amos et al., 2014; Lima et al., 2015). The establishment of uterine bacterial infections depends on the pathogenic potential of invading bacteria and the local uterine immune response. Endometrial epithelial cells, as the first line of defense, can initiate an immune reaction by increased synthesis of different cytokines [interleukin 1A (*IL1A*), *IL6, IL8*, and CXC ligand 3 (*CXCL3*)] and prostaglandin endoperoxide synthase 2 (*PTGS2*; Amos et al., 2014; Turner et al., 2014; Gärtner et al., 2016), leading to an influx of immune cells to the site of infection (Takeuchi and Akira, 2010). Although *T. pyogenes* is associated with severe endometritis, it has also been isolated from cows without signs of uterine disease at puerperium (Silva et al., 2008; Santos et al., 2010; Wagener et al., 2015).

The effect of uterine pathogens on endometrial epithelial cells has been studied extensively using *in vitro* models (Davies et al., 2008; MacKintosh et al., 2013; Gärtner et al., 2016). These models have used epithelial cells and/or stromal cells. However, this may not reflect the complex host-pathogen interactions *in vivo*, which involve further interactions between endometrial epithelial cells and immune cells. In addition, endometrial explants have been used, but in this model it is not possible to discriminate between the distinct influences of each cell type (Borges et al., 2012).

Therefore, the aim of this study was to reveal the mechanisms of host-pathogen interactions in the bovine endometrium *in vitro* that may be associated with the establishment of uterine diseases. The objectives were to characterize a *T. pyogenes* strain isolated from the uterus of a cow developing clinical endometritis (CE) and a strain from a healthy cow to assess the importance of strain-specific factors for the development of bovine CE. Further objectives were to examine the viability and mRNA expression of pro-inflammatory factors of bovine endometrial epithelial cells in the presence of (1) two different strains of *T. pyogenes*, including live bacteria, and bacterial endo- and exo-toxins, and (2) peripheral blood mononuclear cells (PBMCs) in an *in vitro* model extending the epithelial cell co-culture system with *T. pyogenes*. PBMCs were chosen to mimic some aspects of a chronic uterine inflammation caused by *T. pyogenes* infection that involves the infiltration of lymphocytes. In addition, PBMCs were

evaluated for their response in the presence of *T. pyogenes* concerning the viability and mRNA expression of selected pro-inflammatory factors.

Materials and Methods

All animal experimental procedures were carried out in accordance with the European Community Directive 86/609/EEC and were approved by the Ethics Committee of the University of Veterinary Medicine Vienna (08/01/97/2011; date of approval February 1, 2011) and the responsible state veterinarian in Schleswig-Flensburg (Schleswig-Holstein, Germany). As only abattoir waste was used for the cell culture experiments, there was no need to adhere to institutional or national research council guidelines.

Cultivation and Preparation of Bacteria

Intrauterine bacteriological samples were collected using the cytobrush technique from Holstein-Friesian cows on a commercial dairy farm in Schleswig-Holstein (Germany), as described previously (Wagener et al., 2014b, 2015). The strains of *T. pyogenes* used in this study were isolated from cows on day 15 postpartum (pp). One *T. pyogenes* strain (TP2) was isolated from a cow that showed vaginal discharge with more than 50% pus on day 21 pp and was classified accordingly as a cow developing CE. Another *T. pyogenes* strain (TP5) was isolated from a cow that showed clear vaginal discharge on day 21 pp and was classified accordingly as a healthy cow. The bacteria were cultivated in 5 ml brain heart infusion (BHI) broth (Fluka, Steinheim, Germany), supplemented with 5% heat-inactivated (HI) fetal calf serum (FCS; Biochrom, Berlin, Germany) at 37°C for 48 h under aerobic conditions. The bacteria were harvested by centrifugation at 2,000 × g for 10 min and washed once with Dulbecco's phosphate-buffered saline (PBS; Sigma-Aldrich, Deisenhofen, Germany). Both strains (TP2, TP5) were stored in aliquots containing 80% (v/v) bacteria suspended in PBS and 20% (v/v) glycerol at −80°C until further use. Some aliquots were thawed and plate counting was performed on sheep blood agar (Oxoid, Hampshire, United Kingdom) to determine the number of colony-forming units (CFU)/ml.

Heat-inactivated (HI) bacteria and bacteria-free filtrates (BFF) of both *T. pyogenes* strains (TP2, TP5) were prepared by enriching them from a glycerol stock in BHI supplemented with 5% FCS for 48 h at 37°C. Bacteria were harvested by centrifugation at 2,000 × g, washed with PBS and incubated in epithelial cell culture medium [Dulbecco's Modified Eagle Medium (DMEM)/Ham's F-12 medium containing 10% FCS (both Biochrom)] at 37°C and 5% CO_2 until the medium changed color to yellow. To heat inactivate the bacteria, a portion of this bacterial suspension was incubated at 63°C for 30 min, centrifuged at 2,000 × g for 10 min, washed once with PBS, re-suspended in PBS, and stored in aliquots at −80°C until further use. To obtain the BFF, another portion of the bacterial suspension was centrifuged at 2,000 × g for 10 min. The supernatant was filtered through a 0.22 µm filter (Rotilabo syringe filters, Carl Roth, Karlsruhe, Germany) and stored in aliquots at −80°C until further use. Aliquots of the bacterial suspension were used for plate counting on sheep blood agar (Oxoid) to determine the number of CFU/ml for HI bacteria or equivalents to BFF. A sample of the prepared HI bacteria and BFF was cultured on sheep blood agar plates and incubated at 37°C for 48 h. Absence of bacterial colonies indicated complete inactivation and absence of any live bacteria.

Bacterial Growth Measurements

Both *T. pyogenes* strains (TP2 and TP5), reactivated from glycerol stocks, were pre-grown in 5 ml BHI broth supplemented with 5% HI FCS at 37°C under aerobic conditions for 48 h. The optical density of each bacterial suspension was measured at the wavelength of 600 nm (OD_{600}) and bacteria were diluted into 50 ml BHI broth supplemented with 5% HI FCS to obtain a starting OD_{600} of 0.05, which was confirmed by OD_{600} measurement. Bacterial cultures were incubated at 37°C under aerobic conditions and OD_{600} was measured every 2 h over a period of 24 h. The experiment was repeated independently five times.

In addition, samples from three of the independent growth experiments were taken after 6, 12, and 24 h for transcriptional analyses of selected virulence genes, as described in detail below. Cells were harvested by centrifugation at 2,000 × g for 10 min and washed once with PBS. The resulting bacterial cell pellets were stored at −80°C until further use.

Fourier Transform Infrared (FTIR) Spectroscopy Analysis

FTIR spectroscopy was employed to generate bacterial metabolic fingerprints, as described previously (Ehling-Schulz et al., 2005). Briefly, bacteria were grown as lawns on tryptic soy agar (Oxoid) for 24 h at 30°C. FTIR measurements were carried out using a Tensor 27 FTIR spectrometer (Bruker Optics, Billerica, MA, USA) coupled to a HTS-XT microplate adapter. Data analysis of FTIR spectra was performed using the OPUS software (version 5.5; Bruker Optics). First derivatives of the original spectra were calculated and spectral windows of 3,030 to 2,830, 1,350 to 1,200, and 900 to 700 cm^{-1} were used with a weight factor of 1 and a repro-level 30 for the cluster analysis. Hierarchical cluster analysis (HCA) was performed as described previously (Wagener et al., 2014a). Dendrograms were calculated using Ward's algorithm and *Streptococcus uberis* as an outgroup. To identify the spectral regions with the most significant differences between TP2 and TP5, normalized average spectra of the three independent FTIR measurements were calculated for both strains, and the average spectra of TP2 and TP5 were compared.

Screening for the Presence of Selected Virulence Genes in the Genome of Different *T. pyogenes* Strains

Bacterial genomic DNA was extracted using the RTP Bacteria DNA Mini Kit (Stratec, Berlin, Germany) according to the manufacturer's instructions. For this purpose, cell pellets obtained from the growth curve experiments of the two different *T. pyogenes* strains were used. DNA yield was estimated by spectrophotometry at a wavelength of 260 nm.

A conventional PCR was carried out, as described previously (Gärtner et al., 2016), using 150 ng of genomic bacterial DNA. Primer pairs were designed using Primer-BLAST (Ye et al., 2012) and synthesized with Eurofins Genomics (Ebersberg, Germany). The amplification was performed using cycling conditions as follows: 10 min at 95°C, 40 cycles of 15 s at 95°C, 30 s at the specific annealing temperature for each primer pair (Table 1) and 30 s at 72°C, followed by a final extension step at 72°C for 4 min. PCR without DNA served as a negative control. The identity of each amplicon was further confirmed by DNA sequencing (GATC Biotech, Konstanz, Germany). Based on sequence data, specific primers for *fimA, fimC, fimE*, and *fimG* (Table 1) were designed for the subsequent quantitative PCR.

Table 1. Selected known and putative *T. pyogenes* virulence gene transcripts, primer sequences, and annealing temperatures used for PCR/qPCR with expected amplicon size.

Gene symbol	Accession no./ reference	Primer sequence (5' ➔ 3')	Annealing temperature	Amplicon size (bp)
plo	AB027461.1	**F:** AAG TAT CCT GAC CAT GCT GC **R:** GCC GAA AAC GCT ATG TGG AG	60 °C	228
cbpA	AY223543.1	**F:** TAC TGT TCG TCC AAC TCG CAT C **R:** TGC CCG GCT TGA TAT AAC CTT C	60 °C	110
nanH	AF298154.1	**F:** CAC GGA CGT GAA GAG CTT TG **R:** ACT TCA ACC TTC GGC TCT GG	63°C	243
nanP	AY045771.1	**F:** GAT GAC GCA AAC AAG ACG CC **R:** GTC AGC ACA AAA CCA GCC AG	60 °C	152
fimA	(Silva et al. 2008)	**F:** CAC TAC GCT CAC CAT TCA CAA G **R:** GCT GTA ATC CGC TTT GTC TGT G	57°C	605
fimA-Q	this study	**F:** CCG TTC CTC GTT ACC CTT CC **R:** CAG GTA ATC TCA GCA CCG GG	60 °C	152
fimC	(Silva et al. 2008)	**F:** TGT CGA AGG TGA CGT TCT TCG **R:** CAA GGT CAC CGA GAC TGC TGG	60 °C	843
fimC-Q	this study	**F:** GCC GTT CGC TTC ACA CTT AC **R:** ATG GCA AAA CCA AAG ACG CC	60 °C	137
fimE	(Silva et al. 2008)	**F:** GCC CAG GAC CGA GAG CGA GGG C **R:** GCC TTC ACA AAT AAC AGC AAC C	55°C	775
fimE-Q	this study	**F:** CGC CCG TTC TTC TTT GCT TC **R:** TGC CTC GTT GAG ACC AAG TC	60 °C	143
fimG	(Silva et al. 2008)	**F:** ACG CTT CAG AAG GTC ACC AGG **R:** ATC TTG ATC TGC CCC CAT GCG	57°C	929
fimG-Q	this study	**F:** GTA GCC GGA GTT GAG GAA GG **R:** ATC CTC GCT CTC TTG CTG TG	60 °C	157
16S rRNA	NR_044858.1	**F:** AAG ACC GGG GCT TAA CTT CG **R:** AGT AAC CTG CCT TCG CCA TC	60 °C	133
smc	U84782.2	**F:** ATG ATC ACA CTC CCG CAA CC **R:** GGG TTG ATC TTG CCC AAA CG	60 °C	125

Primary Bovine Endometrial Epithelial Cell Culture

Bovine endometrial epithelial cells were isolated and cultured as described previously (Betts and Hansen, 1992; Gärtner et al., 2015). Briefly, apparent healthy uteri from non-pregnant cows were obtained from a local abattoir. Small pieces of the endometrium were cut from various loci in the intercaruncular regions. The tissue mass was chopped very finely before being digested in an enzyme solution composed of 150 U/ml collagenase (Sigma-Aldrich), 150 U/ml hyaluronidase (Sigma-Aldrich), 200 U/ml penicillin (Biochrom), and 200 µg/ml streptomycin (Biochrom) in Hank's Balanced Salt Solution (Biochrom) for 2 h at 37°C. After centrifugation, the cell pellet obtained was washed in epithelial cell culture medium (DMEM/Ham's F-12 medium containing 10% FCS, 55 µg/ml gentamicin, and 1.4 µg/ml amphotericin B; all from Biochrom). Then, the cells were seeded in 25 cm^2 flasks (Corning, Corning, NY, USA) and incubated in a humidified incubator at 37°C and 5% CO_2 for 18 h to allow selective attachment of stromal cells. After this time, the medium containing unattached cells was re-seeded to obtain a pure epithelial cell population (Sheldon et al., 2010).

In the first passage, endometrial epithelial cells were cultured until they reached more than 80% confluence. At that time, they were seeded into 24-well plates at a density of 1×10^5 cells in 0.5 ml culture medium or in 6-well plates at a density of 3×10^5 cells in 3 ml culture medium.

After the endometrial epithelial cells reached confluence in the second passage, they were prepared for co-culture experiments by removing the medium and washing twice with PBS.

Immunocytochemistry against pan-keratins was performed as described previously (Miessen et al., 2012). After the second passage, a 100% pure epithelial cell population was observed.

PBMC Isolation and Culturing

Whole cow blood collected at the slaughterhouse was used for PBMC isolation by density gradient centrifugation using Ficoll-Paque plus (GE Healthcare, Uppsala, Sweden). The isolated PBMCs were pre-incubated with epithelial cell culture medium at a density of 5×10^6 cells/ml for 24 h in a humidified incubator at 37°C and 5% CO_2 to adjust the cells to culture conditions.

After the 24 h incubation, the PBMCs were washed twice with PBS and suspended in antibiotic-free epithelial cell culture medium before co-culturing with endometrial epithelial cells and/or *T. pyogenes*.

Co-culture Experiments

In experiment 1, endometrial epithelial cells (*n* = 5 cows) were co-cultured in passage 2 in 24-well plates with bacterial strain TP2 or TP5 in the form of live bacteria at a multiplicity of infection of 1 (MOI = 1), HI bacteria at MOI = 1, or BFF equivalent to MOI = 1.

In experiment 2, endometrial epithelial cells were co-cultured (*n* = 5 cows) in 6-well plates with live TP2 at MOI = 1 and/or heterogeneous PBMCs at a ratio of 1:1 to endometrial epithelial cells.

Different treatments were applied using antibiotic-free epithelial cell culture medium. Epithelial cells cultured with epithelial cell culture medium without antibiotics served as controls. After the indicated time points in Figures 5, 6, the medium was removed, and the epithelial cells were washed twice with PBS and lysed with RLT lysis buffer (Qiagen, Hilden, Germany).

Control epithelial cells were also lysed at 0 h. Cell lysates were stored at −80°C until further use. In addition, the viability of the epithelial cells (*n* = 3) was monitored in 24-well plates for all treatments and corresponding controls by Trypan blue staining up to 72 h of co-culture, as described previously (Gärtner et al., 2016). Different optical fields were considered, pictures were taken and analyzed using ImageJ version 1.51 (National Institutes of Health, USA). The number of viable (unstained) cells was calculated as the percentage of the total number of cells.

In experiment 3, the hypothesis that PBMCs can influence the growth of *T. pyogenes* in lower concentrations was tested. PBMCs (*n* = 3 cows) were seeded after pre-incubation in 6-well plates at a density of 1×10^7 cells in antibiotic-free epithelial cell culture medium and co-cultured with TP2 at MOI = 0.1. After 2, 4, and 6 h, PBMCs were harvested by centrifugation at 850 × g for 5 min and washed once with PBS. The cell pellets were stored at −80°C until use. In addition, the viability of the PBMCs (*n* = 3) co-cultured with TP2 at two different MOI (0.1 and 1) was monitored up to 48 h using a Neubauer chamber and Trypan blue exclusion.

Total RNA Extraction and Reverse Transcription

Total RNA was isolated from lysed co-cultured endometrial epithelial cells, as described previously (Gärtner et al., 2016). Lysed PBMCs were subjected to total RNA extraction using the InviTrap Spin Cell RNA Mini Kit (Stratec) according to the manufacturer's instructions.

Bacterial total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's protocol with some modifications. Briefly, a frozen bacterial cell pellet was suspended in 100 µl 10 mM Tris-HCl—1 mM EDTA buffer (pH 8). Then, 500 µl lysis buffer RLT (Qiagen) containing 1% of 2-mercaptoethanol (Carl Roth) was added to the cell suspension and this was transferred in its entirety to a 2-ml screw cap microtube (Sarstedt, Nümbrecht, Germany) containing 300 mg of 0.1 mm silica spheres (Lysing Matrix B; MP Biomedicals, Eschwege, Germany). The bacterial cells were lysed by shaking the tube using a Fastprep machine (FP120, Savant Instruments, New York, USA) at maximum speed for 30 s for 4 cycles with 2 min cooling on ice between consecutive cycles. The cell debris was removed by centrifugation at 11,000 × g for 1 min and the supernatant containing total RNA was loaded on an RNeasy spin column.

The total RNA yield from eukaryotic and bacterial cells was estimated spectrophotometrically at 260 nm. RNA integrity and quality was verified using an RNA 6000 Nano LabChip kit on an Agilent 2100 Bioanalyzer (both Agilent Technologies, Waldbronn, Germany).

Reverse transcription (RT) was performed as described previously (Odau et al., 2006). Briefly, 150 ng total RNA from eukaryotic or bacterial cells was treated with DNAse I (Fermentas, St. Leon-Roth, Germany) in a first step to remove any genomic DNA contamination (Huang et al., 1996). In the second step, first-strand cDNA was synthesized from total RNA using 2.5 µM random hexamers, 0.66 mM dNTPs, 1 × RT buffer and 200 U RevertAid reverse transcriptase (all from Fermentas) in a total volume of 60 µl. Each sample was stored as 20 µl aliquots at −20°C until further use. Reactions without reverse transcriptase were run in parallel to the RT to confirm the absence of any genomic DNA or contamination.

Quantitative PCR (qPCR)

qPCR was carried out as described previously (Odau et al., 2006) following the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines (Bustin et al., 2009). Briefly, 1 µl cDNA was amplified in the presence of 1x SensiMix Low-ROX (Bioline, Luckenwalde, Germany) and 0.4 µM of each primer (Tables 1, 2) in a total volume of 10 µl using the Rotor Gene 3000 (Corbett Research, Mortlake, Australia).

Table 2. Selected bovine gene transcripts, primer sequences, and annealing temperatures used for quantitative PCR with expected amplicon size.

Gene symbol	Accession no./ reference	Primer sequence (5' ➔ 3')	Annealing temperature	Amplicon size (bp)
18S rRNA	(Odau et al. 2006)	**F:** GAG AAA CGG CTA CCA CAT CCA A **R:** GAC ACT CAG CTA AGA GCA TCG A	61°C	337
ACTB	(Gärtner et al. 2015)	**F:** CGG TGC CCA TCT ATG AGG **R:** GAT GGT GAT GAC CTG CCC	58°C	266
GAPDH	(Gärtner et al. 2015)	**F:** CCC AGA AGA CTG TGG ATG G **R:** AGT CGC AGG AGA CAA CCT G	62°C	306
SDHA	(Gärtner et al. 2015)	**F:** GGG AGG ACT TCA AGG AGA GG **R:** CTC CTC AGT AGG AGC GGA TG	60 °C	219
CXCL3	(Gärtner et al. 2016)	**F:** GCC ATT GCC TGC AAA CTT **R:** TGC TGC CCT TGT TTA GCA	56°C	189
IL8	(Fischer et al. 2010)	**F:** CGA TGC CAA TGC ATA AAA AC **R:** CTT TTC CTT GGG GTT TAG GC	56 °C	153
IL1A	(Gabler et al. 2009)	**F:** TCA TCC ACC AGG AAT GCA TC **R:** AGC CAT GCT TTT CCC AGA AG	59°C	300
IL6	(Konnai et al. 2003)	**F:** TCC AGA ACG AGT ATG AGG **R:** CAT CCG AAT AGC TCT CAG	56°C	236
PTGS2	(Odau et al. 2006)	**F:** CTC TTC CTC CTG TGC CTG AT **R:** CTG AGT ATC TTT GAC TGT GGG AG	60 °C	359

The qPCR was performed with the following temperature profile: an initial denaturation step at 95°C for 10 min, followed by a cycling step (45 cycles, each cycle consisting of 15 s denaturation at 95°C, 20 s annealing at temperature specified for each gene (Tables 1, 2), and 30 s extension at 72°C), followed by a melting curve program (temperature ramp starting from 50 to 99°C with continuous fluorescence monitoring), and a final cooling step at 40°C for 1 min.

To quantify the content of each expressed gene of interest, a serial dilution of PCR products with a known concentration of the target gene was amplified simultaneously with the samples, generating a standard curve. In relation to the standard curve generated, concentrations of target genes were calculated using the Rotor Gene 6.1 software (Corbett Research). The specificity of amplification of target genes was confirmed by melting point analysis using the Rotor Gene 6.1 software (Corbett Research) and by sequencing (GATC Biotech) of the obtained amplicons, which showed a 100% homology to the published sequences or to the bacterial sequences already obtained in the study.

Statistical Analysis

To normalize the qPCR data obtained, the mRNA expression values of pro-inflammatory factors generated in mammalian cells were divided by an accurate normalization factor. This factor was calculated using geNorm (Vandesompele et al., 2002) for the most stable expressed reference genes in the endometrial epithelial cells [18S ribosomal RNA (*18S rRNA*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and succinate dehydrogenase complex, subunit A (*SDHA*)], in PBMCs [*18S rRNA*, beta actin (*ACTB*), and *SDHA*], and in bacteria [*16S rRNA* and chromosome segregation protein (*smc*)].

Due to the large number of samples of endometrial epithelial cells co-cultured with different strains of *T. pyogenes*, the target genes were amplified in more than one run. Therefore, the normalized values obtained from these cells were inter-run calibrated by dividing using an inter-calibration factor, which was calculated by the geometric averaging of the normalized mRNA expression values of 10 inter-run calibrator samples (Hellemans et al., 2007).

The normalized values obtained for pro-inflammatory factor mRNA expression in the endometrial epithelial cells co-cultured with *T. pyogenes* and/or PBMCs, and PBMCs and the normalized mRNA expression values of virulence genes in different *T. pyogenes* strains were log-transformed.

Normalized inter-run calibrated values and normalized log-transformed values in controls and treatments at each time point were scaled relative to the mRNA expression value at 0 h, set equal to one. These relative values were used to generate bar charts, presenting means ± SEM.

An independent *t*-test was undertaken to compare the differences between the growth rate and normalized mRNA expression of the virulence genes of two *T. pyogenes* strains (TP2 vs. TP5) at different time points. One-way repeated measures analysis of variance (ANOVA) with the Bonferroni *post-hoc* test was used to compare the differences between the normalized mRNA expression of virulence factors at three different time points within each *T. pyogenes* strain. The Wilcoxon signed-rank test was used to calculate statistical differences between the treatments with different *T. pyogenes* strains. For this, each treatment was compared with the control at the same time point, and the same treatments of each strain (TP2 vs. TP5) were also compared. General linear model multivariate ANOVA with the Bonferroni *post-hoc* test

was used for multiple comparisons of different treatments with *T. pyogenes* and/or PBMCs. A paired *t*-test was performed to compare the effect of *T. pyogenes* on PBMCs (untreated vs. treated).

All statistical calculations were undertaken using SPSS version 22 (SPSS, Chicago, USA). Values of $P \leq 0.05$ were considered to be significant; *P*-values of 0.05–0.1 were considered to indicate tendencies.

Results

Growth of the Two Different *T. pyogenes* Strains

T. pyogenes strain TP2 grew faster than TP5 during the first hours (Figure 1), reaching a significant difference at 6 h ($P = 0.043$). However, both strains reached their plateau phase of growth after 18 h. In addition, TP2 grew as a homogeneous suspension during the entire 24 h period in contrast to TP5, which grew up to 14 h rather as a cloudy suspension and thereafter as a homogeneous one.

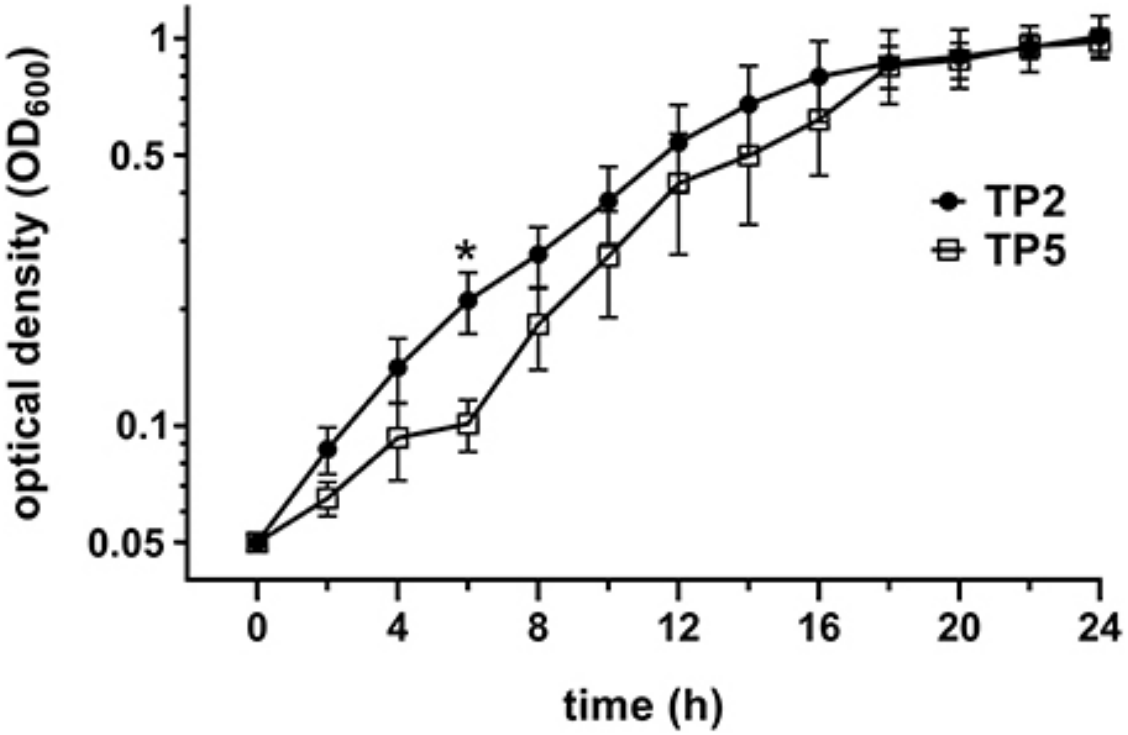

Figure 1. Comparison of standard growth curves of TP2 and TP5. Standard growth curves were generated by measuring OD_{600} by spectrophotometer every 2 h for 24 h. Data are presented as mean ± SEM of OD_{600} readings for 5 different inocula for each strain. Asterisk indicates a significant difference ($P < 0.05$) between the two strains at that time point.

Metabolic Fingerprints of TP2 and TP5

FTIR spectroscopy was used as a high-resolution vibrational spectroscopic technique to generate metabolic fingerprints of the two *T. pyogenes* strains included in the study (Figure 2). FTIR spectral analysis of the average spectra generated from the multiple independent measurements of the strains revealed the most pronounced differences in the metabolic profiles of TP2 and TP5 in the amide/protein region (wavenumber 1,800 to 1,500 cm^{-1}) and

polysaccharide region (wavenumber 1,200 to 900 cm^{-1}). The most prominent differences between the two strains were located at wavenumber 1,658 cm^{-1} (amide I region), 1,544 cm^{-1} (N = O bonds), and in the polysaccharide region at wavenumber 1,090 to 980 cm^{-1}

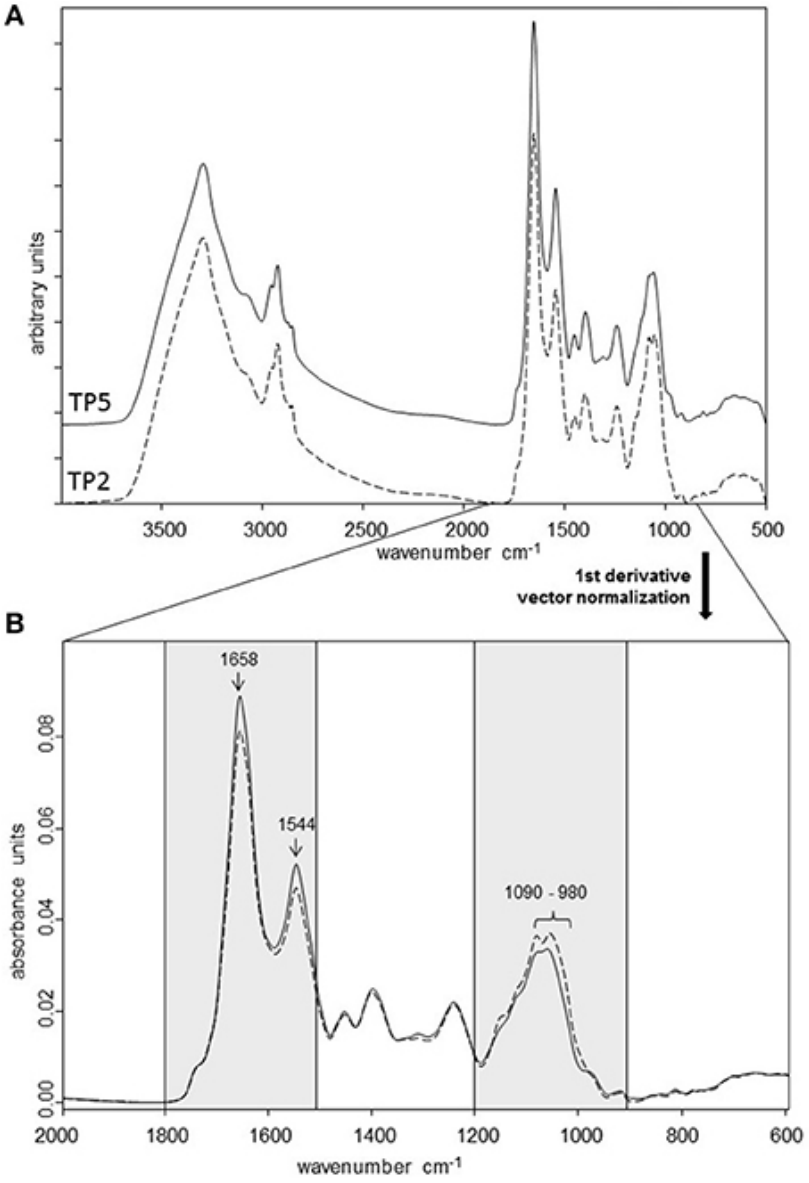

Figure 2. (A) FTIR spectra from TP2 and TP5 recorded in the transmission mode at 4,000 to 500 cm^{-1}. First derivative, normalized average FTIR spectra of three independent measurements of TP2 and TP5. **(B)** Spectra are shown from the spectral range with the most pronounced differences between the two strains (amide/protein region: 1,800 to 1,500 cm^{-1}; polysaccharide region: 1,200 to 900 cm^{-1}).

Presence and mRNA Expression of Virulence Genes

All the virulence genes investigated, except *nanP*, were present in the genome of the two *T. pyogenes* strains (data not shown). The *nanP* gene was only present in the bacterial genomic DNA of TP5, not of TP2.

According to the BLASTN analysis, the partial sequences of all virulence factors showed homologies to the sequences (GenBank accession numbers in parenthesis), as follows: *cbpA* 100% (AY223543.1), *fimA* 98% (CP007003.1 and CP007519.1), *fimC* 99% (CP007003.1 and CP007519.1), *fimE* 96–99% (CP012649.1, CP007003.1, and CP007519.1), *fimG* 98–99% (CP007003.1, CP007519.1, and CP012649.1), *nanH* 93–98% (AF298154.1, CP007003.1, and

CP007519.1), *nanP* 97–100% (CP007003.1, CP012649.1, and AY045771.1), and PLO 97–99% (KJ150329.1, CP007519.1, and AB027461.1).

Different mRNA expression of most virulence genes was noted between the two *T. pyogenes* strains (Figure 3). In detail, transcripts of *PLO* were detected during all time points with no significant differences between the two strains (Figure 3A). However, *PLO* mRNA expression was time-dependent in TP2 but not in TP5. mRNA expression of *PLO* in TP2 was significantly—20- and 7-fold—higher after 24 h compared with 6 and 12 h, respectively.

The *cbpA* gene was present in the genomes of both strains. However, *cbpA* mRNA expression was only detected in TP2, not in TP5 (Figure 3B), and showed a tendency for decreased mRNA expression after 24 h compared with 6 h ($P = 0.057$).

nanH mRNA expression was observed in TP2 and TP5 at all time points (Figure 3C). The transcription level of *nanH* in TP5 was similar during the 24 h cultivation. In contrast, *nanH* mRNA expression in TP2 increased 3-fold from 6 to 12 h, reaching a 4-fold lower level after 24 h. However, this finding failed to reach statistical significance ($P > 0.1$). After 6 and 12 h, *nanH* transcription in TP2 was significantly higher ($P = 0.004$ and 0.014, respectively) compared with TP5. *nanH* mRNA expression was 2-fold higher in TP2 than in TP5 after 24 h, but this difference did not reach statistical significance ($P = 0.068$).

Nearly, the same transcript amounts of *nanP* were detected in TP5 at the selected time points, but none in TP2 not containing this gene in its bacterial genome (Figure 3D).

Transcripts of *fimA* were detected in TP2 and TP5 at all time points investigated, with no significant differences between the two strains (Figure 3E). mRNA expression of *fimC* was observed during the entire cultivation time in both the *T. pyogenes* strains investigated (Figure 3F). A time-dependent *fimC* mRNA expression was noted for TP2, but not for TP5. The amount of *fimC* mRNA in TP2 was lower after 24 h (4-fold) compared with 6 h ($P = 0.027$) and 12 h ($P = 0.057$). Nearly the same *fimC* transcript amounts were detected in TP2 and TP5 after 6 and 12 h. However, mRNA expression of *fimC* after 24 h tended to be about 3-fold higher in TP5 compared with TP2, but failed to reach statistical significance ($P = 0.051$).

Similar to *cbpA*, the gene of *fimE* was present in the genomes of both strains. However, *fimE* transcripts were only detected in TP2, not in TP5 (Figure 3G). In addition, *fimE* mRNA expression was time-dependent and tended to increase ($P = 0.06$) from 6 h to 2-fold higher values at 12 h and decreased about 4-fold ($P = 0.002$) after 24 h compared with 12 h.

Transcripts of *fimG* were detected in TP2 and TP5 (Figure 3H). After 6 h, *fimG* mRNA expression was significantly higher in TP2 compared with TP5. No differential *fimG* mRNA expression was noted between the two strains after 12 and 24 h. The mRNA expression pattern of *fimG* in TP2 decreased with cultivation time, with about 15- and 4-fold lower *fimG* mRNA expression after 24 h compared with 6 and 12 h, respectively.

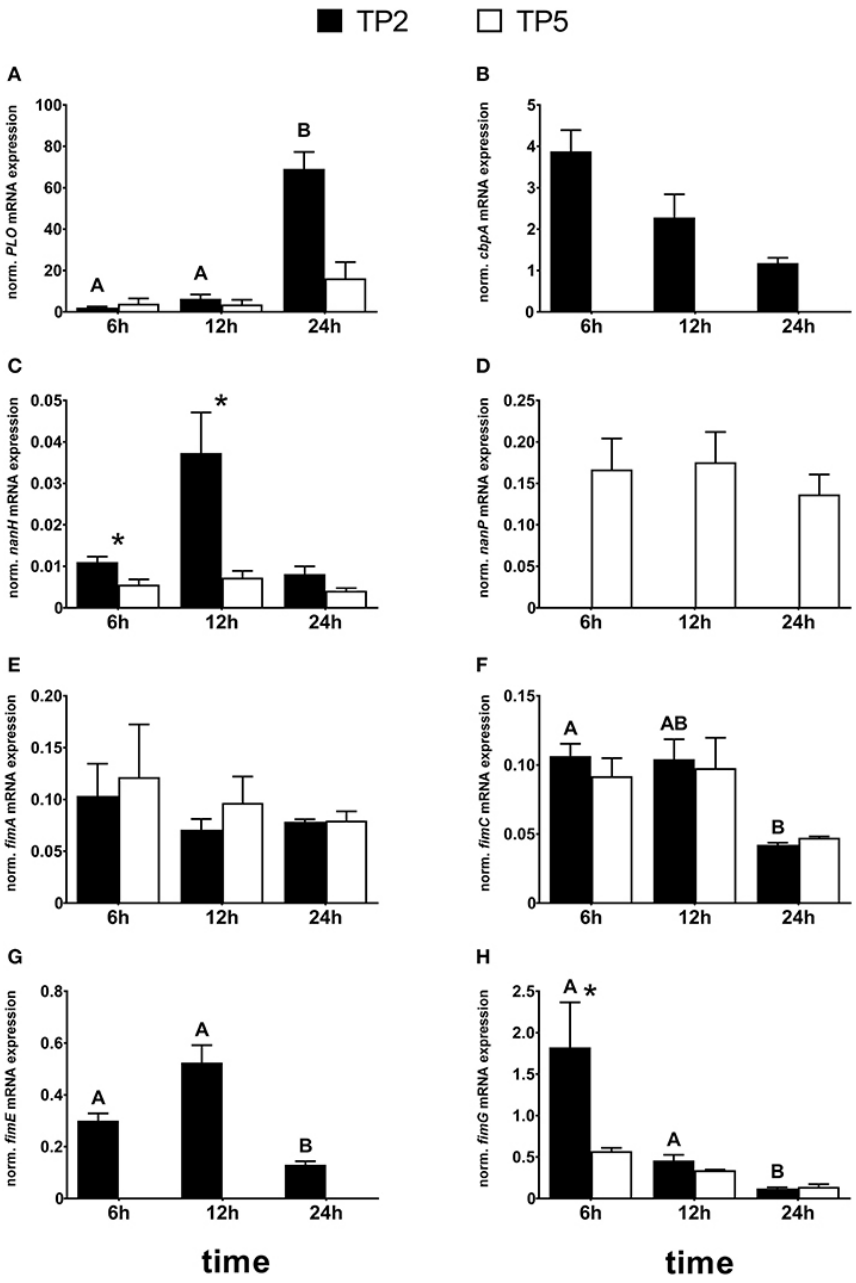

Figure 3. Normalized mRNA expression of the virulence factors: **(A)** *PLO*, **(B)** *cbpA*, **(C)** *nanH*, **(D)** *nanP*, **(E)** *fimA*, **(F)** *fimC*, **(G)** *fimE*, and **(H)** *fimG* in TP2 and TP5 cultured in BHI supplemented with 5% HI FCS up to 24 h. The bars show means ± SEM of mRNA expression values from different inocula (n = 3). Asterisks above the bars indicate significantly different mRNA expression ($P < 0.05$) between the two strains at the same time point. Different capital letters (A,B) above the bars indicate a significant difference within TP2 between different time points ($P < 0.05$).

Viability Assay

The effect of live bacteria, BFF (representing exotoxins), and HI bacteria (representing endotoxins) of both *T. pyogenes* strains on bovine endometrial epithelial cells was evaluated (Figure 4A). Similar cytotoxicity on endometrial epithelial cells was elicited by the live form of each of the two strains of *T. pyogenes*, which caused death in >90% of the cells within 16 h. This was not observed after 8 h, when >95% cells were still viable in treated and control

epithelial cells (data not shown). In contrast, HI bacteria and BFF of the two strains did not elicit a cytotoxic effect on endometrial epithelial cells up to 72 h.

The viability of bovine endometrial epithelial cells co-cultured with the *T. pyogenes* strain TP2 isolated from a cow developing CE in the presence/absence of PBMCs was investigated (Figure 4B). More than 90% of the endometrial epithelial cells were dead after 16 h of co-culture with live TP2 alone, or with TP2 and PBMCs together, compared with the control. The presence of PBMCs did not affect the viability of endometrial epithelial cells compared with the control.

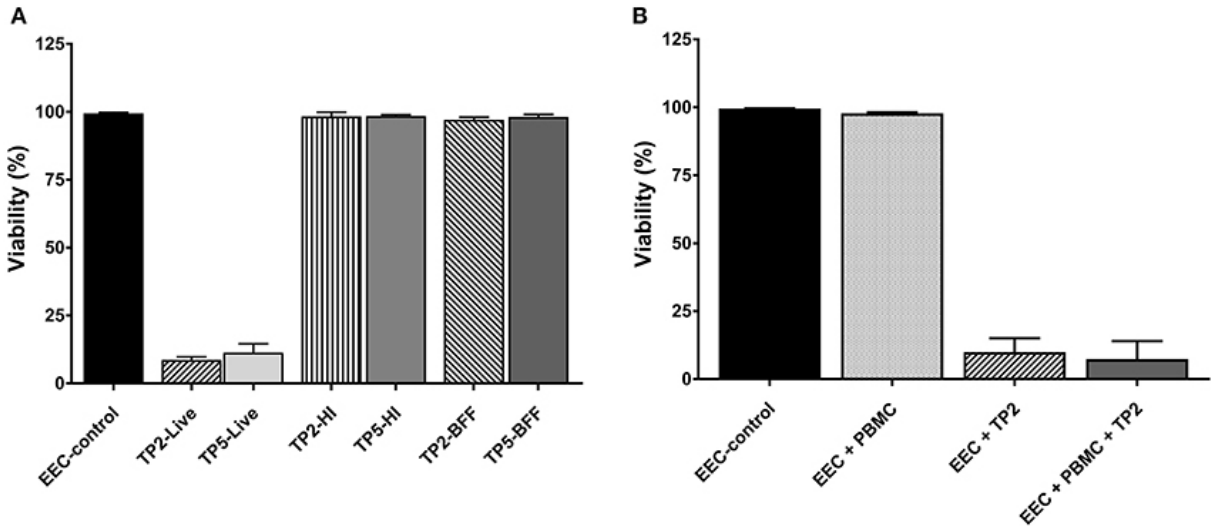

Figure 4. Percentage of viability of endometrial epithelial cells (EEC–control) **(A)** co-cultured with two strains of *T. pyogenes* (TP2 or TP5) in the form of live bacteria at MOI = 1 (−Live), or with heat-inactivated bacteria at MOI equivalent to 1 (−HI), or with bacteria-free filtrate at MOI equivalent to 1 (−BFF), and **(B)** co-cultured with live TP2 at MOI = 1 in the presence or absence of PBMC at a ratio of 1:1.

In addition, the viability of bovine PBMCs co-cultured with *T. pyogenes* strain TP2 at different MOI (0.1 and 1, respectively) was evaluated. After 8 h, the percentage of live PBMCs co-cultured with TP2 recovered at different MOI was lower compared with the percentage of recovered PBMCs incubated with control culture medium (data not shown). After 16 h, 100% of PBMCs were dead in the presence of TP2 at an MOI of 0.1 and 1 compared with control cells (data not shown).

mRNA Expression of Pro-inflammatory Factors in Bovine Endometrial Epithelial Cells Co-cultured with Two Different *T. pyogenes* Strains

The mRNA expression of *PTGS2* in endometrial epithelial cells was influenced significantly by the presence of TP2 but not by TP5 (Figure 5A). In detail, a significantly (3-fold) higher amount of *PTGS2* mRNA was observed in epithelial cells co-cultured with live TP2 after 8 h compared with the control. The *PTGS2* mRNA expression tended to decrease in the presence of live TP5 after 2 h ($P = 0.08$). In addition, the presence of live TP2 resulted in significantly higher *PTGS2* mRNA expression of endometrial epithelial cells, 1.5- and 2-fold after 2 and 8 h respectively, compared with live TP5. Endometrial epithelial cells co-cultured with TP2 BFF after 6 h showed a lower ($P < 0.05$) *PTGS2* mRNA expression compared with untreated cells. Furthermore, transcript amounts of *PTGS2* tended to decrease ($P = 0.08$) in the presence of HI or BFF TP5 after 6 h.

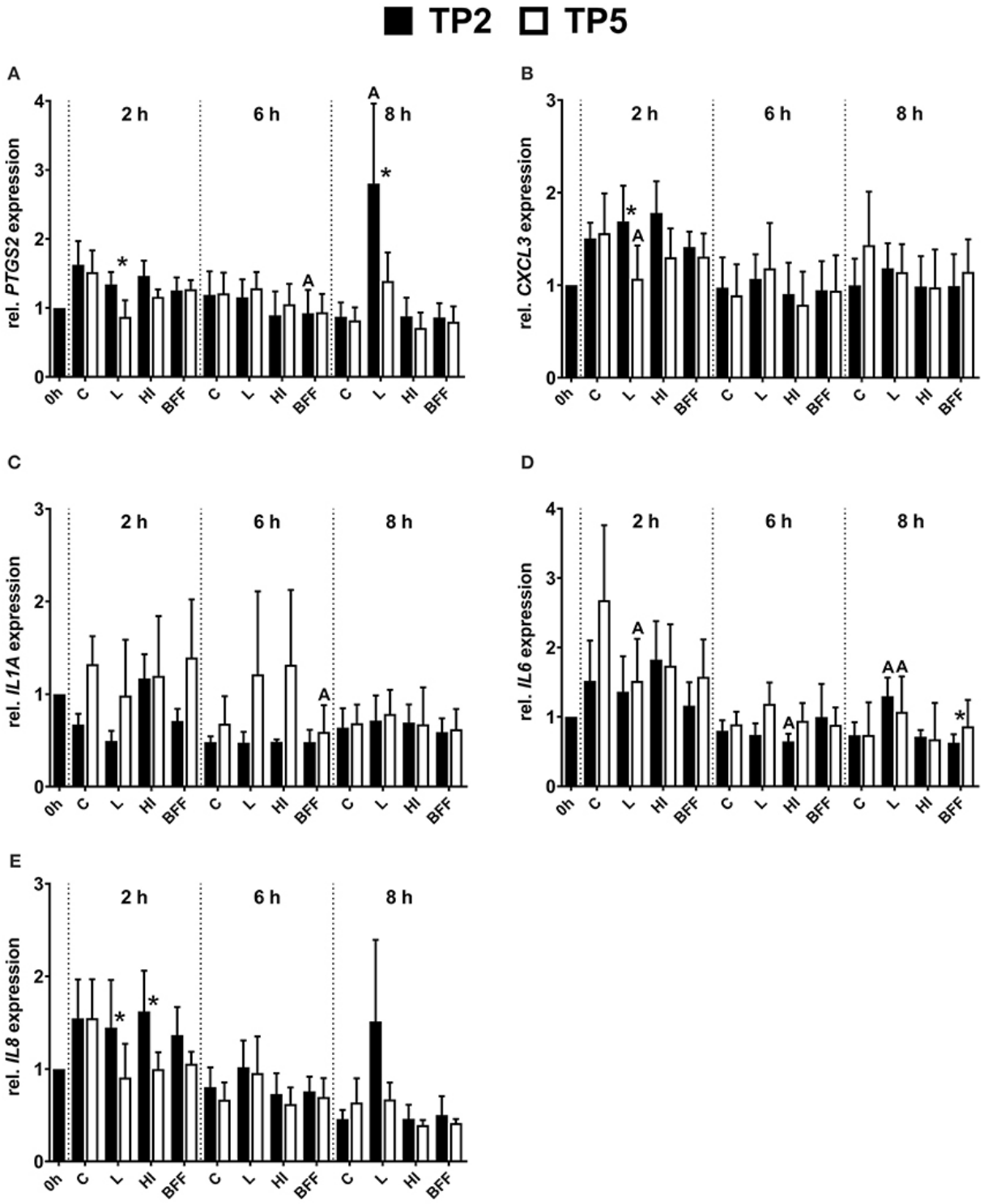

Figure 5. Relative mRNA expression of **(A)** *PTGS2*, **(B)** *CXCL3*, **(C)** *IL1A*, **(D)** *IL6*, and **(E)** *IL8* in bovine endometrial epithelial cells co-cultured with TP2 or TP5 at MOI = 1 up to 8 h in the form of live (L), heat-inactivated (HI), or bacteria-free filtrate (BFF). The bars show means ± SEM of mRNA expression values from different cultures (n = 5 cows). The letters above the bars indicate significantly different mRNA expression (P < 0.05) compared with the control (C; untreated) at the same time point. Asterisks above the bars indicate significantly different mRNA expression (P < 0.05) between TP2 and TP5 at the same time point.

The presence of live TP2 did not affect *CXCL3* mRNA expression, with similar transcript amounts in endometrial epithelial cells compared with non-treated controls during the 8 h time period (Figure 5B). However, in presence of live TP5, significantly lower *CXCL3* mRNA expression was observed compared with controls after 2 h. Therefore, the mRNA expression of *CXCL3* in endometrial epithelial cells 2 h co-cultured with live TP2 was higher ($P < 0.05$) compared with live TP5.

During the 8 h co-culturing period, *IL1A* mRNA expression in endometrial epithelial cells was not significantly affected by the presence of live or HI TP2 and TP5, respectively (Figure 5C). However, lower *IL1A* mRNA expression ($P < 0.05$) was observed after 6 h in the presence of BFF TP5 compared with control cells, but not for BFF TP2.

IL6 showed differences in the mRNA expression pattern in endometrial epithelial cells co-cultured with TP2 or TP5 at different time points (Figure 5D). After 2 h, *IL6* mRNA expression was significantly lower in the presence of live TP5 in comparison with untreated cells, but this was not observed for TP2. Significantly higher mRNA expression of *IL6* was detected after 8 h in the presence of live TP2 and live TP5 compared with control cells. Endometrial epithelial cells co-cultured with HI TP2 showed slightly lower ($P < 0.05$) *IL6* mRNA expression after 6 h compared with untreated cells. In contrast to the other pro-inflammatory factors, a lower mRNA expression of *IL6* in endometrial epithelial cells was observed in the presence of BFF TP2 compared with BFF TP5 after 8 h.

IL8 mRNA expression in endometrial epithelial cells was similar in the presence of TP2 in the form of HI or BFF compared with control cells at each time point during the 8 h co-culture period (Figure 5E). However, *IL8* mRNA expression after 8 h tended to be higher ($P = 0.08$) in epithelial cells in the presence of live TP2 compared with the control. In addition, only a numerical decrease ($P = 0.08$) of *IL8* mRNA expression was observed after 2 h in presence of live TP5 in comparison with untreated cells. Endometrial epithelial cells showed a significantly higher *IL8* mRNA expression in presence of live and HI TP2 compared with live and HI TP5 after 2 h, respectively.

mRNA Expression of Pro-inflammatory Factors in Bovine Endometrial Epithelial Cells Co-cultured with TP2 in the Presence/Absence of PBMCs

To monitor the absence/influence of any contamination with immune cells, mRNA expression of *CD45* was evaluated. Transcripts of *CD45* were not detected in almost all endometrial epithelial cell samples co-cultured with PBMCs. The melting curve analysis showed no or non-specific amplification except for six samples co-cultured with PBMCs, the threshold cycle value of which was >38 (data not shown).

Nearly, the same transcript amounts of *PTGS2* were noted in endometrial epithelial cells co-cultured with PBMCs compared with controls during different time points (Figure 6A). Endometrial epithelial *PTGS2* mRNA expression was 4-fold higher in presence of TP2 alone after 8 h compared with untreated cells controls but failed to reach a significant difference ($P = 0.096$). However, endometrial epithelial cells in the presence of TP2 and PBMCs showed significantly higher *PTGS2* mRNA expression (9-fold) after 8 h compared with controls.

The mRNA expression of *CXCL3* in endometrial epithelial cells was not affected significantly by the presence of PBMCs alone compared with untreated cells throughout the entire time course of co-culturing (Figure 6B). There was significantly higher *CXCL3* mRNA expression in

endometrial epithelial cells co-cultured with TP2 and PBMCs compared with the untreated controls ($P < 0.001$), but not with TP2 alone ($P = 0.17$), after 4 h. However, greatly higher mRNA expression of *CXCL3* (50- and 90-fold) was observed in endometrial epithelial cells after 8 h co-cultured with TP2 compared with untreated cells and cells co-cultured with PBMCs, respectively. This effect was further amplified in endometrial epithelial cells in the presence of TP2 and PBMCs, which showed a 90- to 170-fold increase of *CXCL3* mRNA expression in comparison with controls. There was no significant effect in the presence of TP2 or PBMCs, either alone or combined, on *IL1A* mRNA expression in endometrial epithelial cells after 2 h of co-culture (Figure 6C).

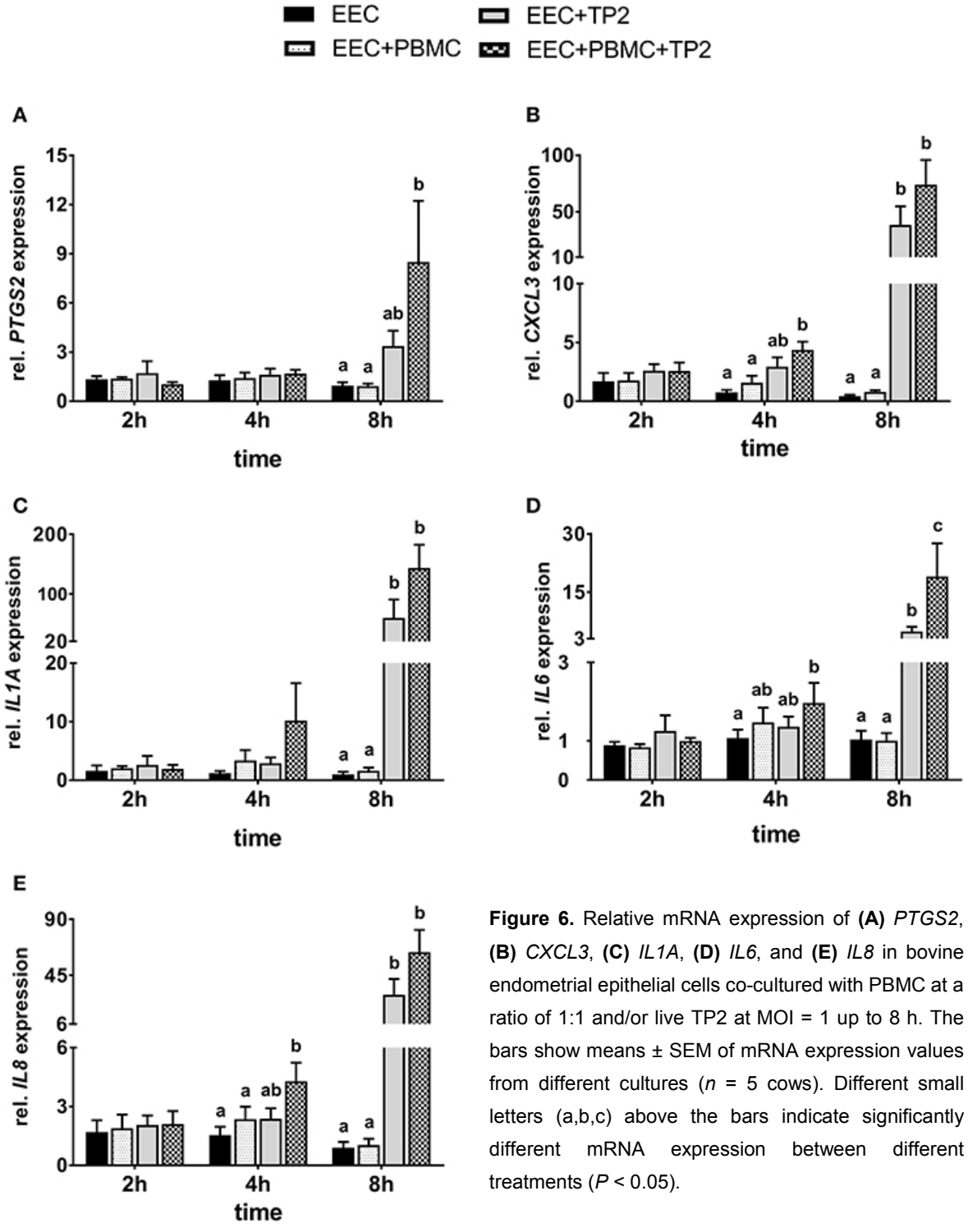

Figure 6. Relative mRNA expression of **(A)** *PTGS2*, **(B)** *CXCL3*, **(C)** *IL1A*, **(D)** *IL6*, and **(E)** *IL8* in bovine endometrial epithelial cells co-cultured with PBMC at a ratio of 1:1 and/or live TP2 at MOI = 1 up to 8 h. The bars show means ± SEM of mRNA expression values from different cultures ($n = 5$ cows). Different small letters (a,b,c) above the bars indicate significantly different mRNA expression between different treatments ($P < 0.05$).

After 4 h, epithelial cells co-cultured with TP2 and PBMCs showed an 8-fold higher *IL1A* mRNA expression compared with untreated cells. This increase failed to reach statistical significance ($P = 0.06$) due to the high variability between the animals. However, mRNA expression of *IL1A* in endometrial epithelial cells after 8 h was significantly higher with TP2 alone (35- and 60-fold), or TP2 and PBMCs (90- and 140-fold), when compared with untreated cells and cells co-cultured with PBMCs, respectively.

The *IL6* mRNA expression pattern was similar in treated endometrial epithelial cells and the controls after 2 h (Figure 6D). Although the mRNA expression of *IL6* did not increase in response to TP2 alone after 4 h, higher mRNA expression in epithelial cells ($P < 0.05$) was observed in the presence of TP2 and PBMCs compared with untreated cells. After 8 h, TP2 alone, or TP2 and PBMCs together induced higher *IL6* mRNA expression in endometrial epithelial cells compared with the controls. In addition, *IL6* mRNA expression in endometrial epithelial cells co-cultured with TP2 and PBMCs was 4-fold higher ($P < 0.05$) in comparison with epithelial cells co-cultured with TP2 alone after 8 h. *IL8* mRNA expression exhibited no differences between treated endometrial epithelial cells and controls after 2 h (Figure 6E).

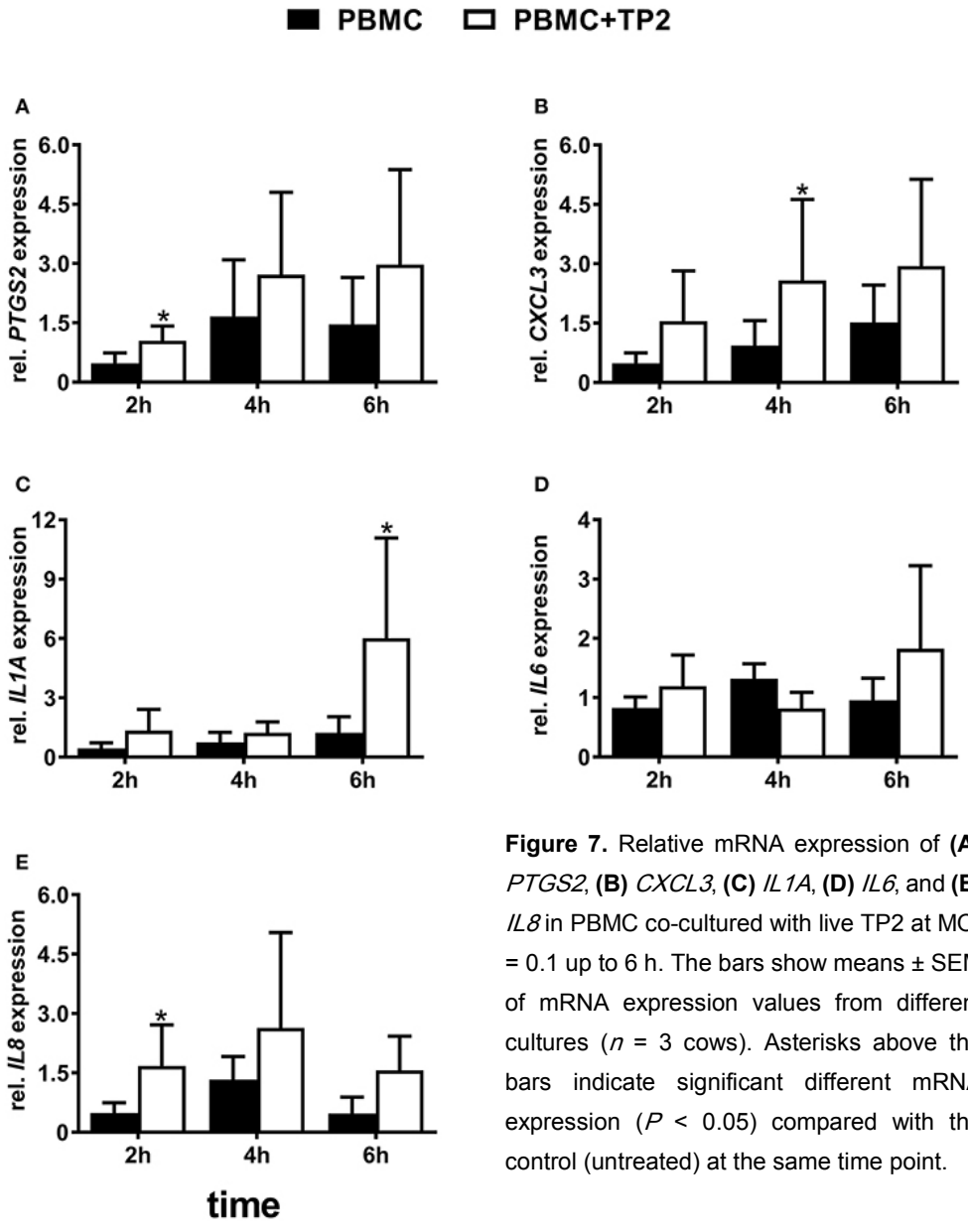

Figure 7. Relative mRNA expression of **(A)** *PTGS2*, **(B)** *CXCL3*, **(C)** *IL1A*, **(D)** *IL6*, and **(E)** *IL8* in PBMC co-cultured with live TP2 at MOI = 0.1 up to 6 h. The bars show means ± SEM of mRNA expression values from different cultures ($n = 3$ cows). Asterisks above the bars indicate significant different mRNA expression ($P < 0.05$) compared with the control (untreated) at the same time point.

In comparison with the control cells, higher *IL8* mRNA expression was observed in epithelial cells co-cultured with TP2 and PBMCs after 4 h. After 8 h, endometrial epithelial cells co-cultured with TP2 showed a 30-fold increase in *IL8* mRNA expression ($P < 0.001$); this increase was further amplified to 70-fold in the presence of PBMCs.

mRNA Expression of Pro-inflammatory Factors in PBMCs Co-cultured with TP2 at Lower Concentrations

PBMCs co-cultured with the *T. pyogenes* strain TP2 at MOI = 0.1 showed higher mRNA expression of some selected pro-inflammatory factors. In detail, PBMCs co-cultured with TP2 showed a 2-fold increase ($P < 0.05$) of *PTGS2* mRNA expression as early as after 2 h in comparison with the controls (Figure 7A), but not after 4 or 6 h.

The presence of TP2 affected the mRNA expression of *CXCL3* in PBMCs (Figure 7B). *CXCL3* mRNA was three times more highly expressed ($P < 0.05$) in PBMCs co-cultured with TP2 in comparison with the controls after 4 h. However, this increase was not observed at 2 or 6 h.

Nearly, the same transcript amount of *IL1A* was detected in PBMCs co-cultured with TP2 and in the controls after 2 and 4 h (Figure 7C). However, a significantly (5-fold) higher *IL1A* mRNA expression was observed in PBMCs co-cultured with TP2 in comparison with untreated cells after 6 h. mRNA expression of *IL6* did not change significantly in PBMCs co-cultured with TP2 in comparison with controls at all investigated time points (Figure 7D). Similar to *PTGS2*, higher *IL8* mRNA expression ($P < 0.05$) was noted in PBMCs treated with TP2 in comparison with controls as early as after 2 h (Figure 7E). Such higher mRNA expression was not observed at later time points.

Discussion

This study followed the hypothesis that the occurrence of uterine diseases depends on bacterial virulence, as well as on the host immunity. Therefore, to assess the importance of strain-specific characteristics for *T. pyogenes* in bovine CE, two different *T. pyogenes* strains were included. One strain was isolated from the uterus of a cow developing CE (TP2) and the other strain was isolated from a healthy uterus (TP5). These strains differed in their growth characteristics, metabolic profiles, and mRNA expression pattern of virulence genes. Such findings might also explain similar observations that *T. pyogenes* strains have been found to be associated with severe uterine pathology (Sheldon et al., 2006; Lima et al., 2015), but have also been isolated from healthy uteri (Santos et al., 2010; Bicalho et al., 2012; Wagener et al., 2015).

The different initial immune reaction of bovine endometrial epithelial cells can be attributed to the differences in the metabolic profiles of the bacterial strains as revealed by FTIR spectroscopy. Notably, the most prominent differences were detected in the polysaccharide composition of both strains. Polysaccharides are an important component of bacterial peptidoglycans (PGN) and can initiate an innate immune response in host epithelial cells through toll-like receptors (TLR). It has been reported previously that bovine endometrial epithelial cells express TLR 1 to 7 and 9 (Davies et al., 2008). In addition, bovine endometrial epithelial and stromal cells can detect and respond to bacterial lipopetides found in gram-positive bacteria through TLR1, TLR2, and TLR6 (Sheldon et al., 2014; Turner et al., 2014). However, differences in such PGN patterns may lead to different recognition by TLR2 and

subsequently different immune responses. This supports our observation that endometrial epithelial cells showed a higher pro-inflammatory response in the presence of TP2, which resulted after 8 h in increased *PTGS2* and *IL8* mRNA expression.

Furthermore, FTIR spectroscopy revealed differences in the protein region, which may be reflected in the different mRNA expression pattern of certain virulence factors between the two strains. The virulence factors cbpA, nanH, fimE, and fimG, which mRNA was more highly or exclusively expressed in TP2 compared with TP5, are involved in adhesion to host cells (Jost et al., 2002; Esmay et al., 2003; Jost and Billington, 2005). Therefore, TP2 might be able to attach better to epithelial cells by expressing more cell wall-associated virulence factors. Indeed, some *T. pyogenes* can invade the host cells, but usually *T. pyogenes* lives extracellular due to its ability to adhere to the host epithelium (Jost and Billington, 2005). This may reflect the importance of these virulence genes in TP2 pathogenicity.

These observations are consistent with antibodies detected against certain fimbrial proteins of *T. pyogenes* in cows with uterine *T. pyogenes* infection, reflecting the importance of fimbrial proteins for the development of postpartum uterine diseases (Bisinotto et al., 2016). *T. pyogenes* strains, which harbor a gene that encodes for *fimA*, have been found to be associated with metritis (Santos et al., 2010) and clinical endometritis (Bicalho et al., 2012). However, no relationship was found in another study between the presence of eight virulence genes of *T. pyogenes* and the development of clinical metritis (Silva et al., 2008). The ability of *T. pyogenes* to induce endometritis might be related to differential mRNA expression of their virulence genes, rather than simple presence in the bacterial genome. In the same context, most of the selected virulence factors in this study were expressed in a time-dependent manner in TP2.

PLO is the main virulence factor of *T. pyogenes* (Jost et al., 1999), but it may not be the only determinant factor for the pathogenicity of *T. pyogenes*. Both strains investigated caused the death of >90% of endometrial epithelial cells within 16 h of co-culture as living bacteria. This indicates that both strains are able to produce PLO, which causes cytolysis of endometrial cells. This goes together with our observation that *PLO* mRNA was expressed in both strains at different time points of bacterial growth without any significant differences.

Interestingly, the BFF and HI of the two strains did not influence the viability of endometrial epithelial cells up to 72 h, or the pro-inflammatory response. This may be due to the presence of a lower and sublytic concentration of PLO in the BFF compared with that produced by live bacteria. However, the BFF of 12 different *T. pyogenes* isolates caused cytolysis of endometrial cells in a previous study (Amos et al., 2014), which used a higher concentration of *T. pyogenes* (MOI = 1 vs. MOI > 10). The results of this study are also in line with previous findings showing that PLO in BFF or recombinant PLO did not stimulate a pro-inflammatory response in endometrial cells (Amos et al., 2014). However, endometrial cells generated a pro-inflammatory response to heat-killed *T. pyogenes* (Borges et al., 2012; Amos et al., 2014). This may be caused by the greatly higher number of bacteria used in these studies (equivalent to MOI = 1,000) compared with this study (equivalent to MOI = 1). This study intended to use similar starting material for a better comparison and also to reflect the situation at the beginning of a bacterial infection.

Furthermore, we investigated if culturing of endometrial epithelial cells with distinct peripheral immune cells influences epithelial cellular responses to *T. pyogenes* to gain insights into the

role of host immunity for *T. pyogenes*-related uterine infections. Our study revealed that PBMCs alone did not influence the viability or pro-inflammatory response of epithelial cells. However, the presence of PBMCs amplified the pro-inflammatory response in bovine endometrial epithelial cells to pathogenic *T. pyogenes*. This pro-inflammatory response originates from epithelial cells rather than from any traces of other immune cells, the presence of which was monitored by *CD45* mRNA expression.

An important characteristic of the innate immune response is the fast reaction to a bacterial infection. However, the endometrial epithelial cells alone did not show a reaction to live *T. pyogenes* strain TP2 up to 6 h of co-culturing in this study compared with an early reaction after 2 h to *Bacillus pumilus* (Gärtner et al., 2016). In contrast, endometrial epithelial cells in the presence of PBMCs responded to *T. pyogenes* strain TP2 as early as after 4 h by increasing the mRNA expression of *PTGS2, CXCL3, IL6*, and *IL8*. Furthermore, immune cells have low thresholds in the response to bacteria and their patterns (Shaykhiev and Bals, 2007). In this study, PBMCs reacted to *T. pyogenes* at a level as low as MOI = 0.1 through the increased mRNA expression of *PTGS2* and *IL8* after 2 h. These data suggest a mechanism whereby leukocytes within the endometrium may sensitize the epithelial cells to initial bacterial invasion by up-regulating the uterine innate immune response. As PBMCs alone did not influence the epithelial cells, we hypothesize that the effect of immune cells on epithelial cells may be mediated by the release of soluble factors from bacteria-activated immune cells rather than by cell-cell contact (Panja et al., 1994). Interestingly, *IL6* mRNA expression in endometrial epithelial cells was 4-fold higher in the presence of *T. pyogenes* strain TP2 and PBMCs compared with TP2 alone after 8 h. This higher induced expression may be mediated by the release of soluble factors, such as *IL1A* from *T. pyogenes*-activated PBMCs. This is supported by our findings that PBMCs co-cultured with *T. pyogenes* strain TP2 showed higher *IL1A* mRNA expression. *IL1A* released from damaged endometrial cells bound to the *IL1* receptor 1 on nearby endometrial cells, stimulating further secretion of *IL6* (Healy et al., 2014). However, this study assumes that *IL1A* derived from bacteria-activated immune cells rather than from damaged endometrial cells leads to up-regulation of a uterine inflammation. Indeed, measuring protein level of IL1A in culture supernatants will be interesting to follow the signaling cascade within the endometrium to attract immune cells. However, the limitation of the current set up was that the bacteria killed the epithelial cells within 16 h.

Uterine infections are associated with disturbed corpus luteum (CL) function due to the disturbance of PGE_2:$PGF_{2\alpha}$ ratios (Miller et al., 2007; Herath et al., 2009). Intrauterine infusion of live *T. pyogenes* has been shown to diminish the life span of the CL (Kaneko et al., 2013; Lima et al., 2015) or prolong its life span (Farin et al., 1989). This study indicates that PBMCs might up-regulate the mRNA expression of *PGTS2*, which is a key enzyme of PG synthesis, in endometrial epithelial cells in response to *T. pyogenes*. This up-regulation might be mediated by *IL1A* because *T. pyogenes* also induce higher *IL1A* mRNA expression in PBMCs. This is in line with earlier findings that PGE_2 and $PGF_{2\alpha}$ synthesis in bovine endometrium throughout the estrous cycle is modulated by *IL1A* (Tanikawa et al., 2005). These findings suggest that *T. pyogenes* may disturb the CL function through up-regulation of a key enzyme involved in PG synthesis.

Conclusion

This study supports the hypothesis that the pathogenicity of *T. pyogenes* can be attributed to certain strain characteristics. Notably, the clinical *T. pyogenes* strain included in this study shows not only a higher expression of known virulence factors compared with a *T. pyogenes* strain isolated from a healthy cow, but also distinct differences in the metabolic profile and growth characteristics. Therefore, certain strains of *T. pyogenes* could be an important factor for the development of endometritis in dairy cows after parturition. In addition, the presence of immune cells amplifies the pro-inflammatory response in endometrial epithelial cells to pathogenic *T. pyogenes*. All mRNA expression data in this *in vitro* model reflect the *in vivo* situation by up-regulation of such pro-inflammatory factors in cases of subclinical/clinical endometritis (Gabler et al., 2009; Fischer et al., 2010; Peter et al., 2015). However, further studies are needed to elucidate the detailed mechanisms and factors involved in communication between immune cells and endometrial cells during an infection; also, the exact role of cell wall-related factors in the host–pathogen interplay need to be deciphered.

Author Contributions

MI: Contributed ideas, performed experiments, analyzed data, and wrote the manuscript; SP: Performed experiments and edited the manuscript; KW: Performed experiments, analyzed data, and wrote the manuscript; MD: Contributed ideas, contributed to the clinical studies, provided bacterial strains, and edited the manuscript; RE: Contributed ideas and edited the manuscript; ME-S: Contributed ideas, undertook bacterial strain analysis, and edited the manuscript; CG: Conceived the study, designed experiments, analyzed data, and wrote the manuscript.

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Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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5. Discussion

The epidemiological triangle of uterine diseases consists of three sides — the host, the pathogen, and the environment (Galvão and Santos, 2014; Sheldon, 2014; Machado and Bicalho, 2015). The current project focused on studying host uterine innate immunity, the pathogen causing the disease, and the interaction between them, in order to understand the molecular mechanisms associated with development of endometritis in dairy cattle.

5.1. Innate immune response inside the bovine uterus

Proper comprehension of uterine innate immunity will help understanding of the link between uterine inflammation and infertility in dairy cows (Sheldon et al., 2009b). Since endometrial epithelial cells are the first sentential cells that encounter threats, the current project focused specifically on the immune response of uterine epithelial cells *in vivo* and *in vitro*.

The endometrium is distinct from other tissues, because the uterine tissue undergoes drastic changes during the estrous cycle and during the postpartum period, which could also imply changes in uterine immunity (Seals et al., 2003; Mitko et al., 2008; Singh et al., 2008).

During the estrous cycle in animals, steroidal hormones undergo fluctuations, accordingly influencing innate uterine immunity (Lewis, 2004). During estrogen dominant phases, uterine immunity is elevated due to increased proliferation of the epithelial layer, increased mucus secretion (which contains AMP and immunoglobulins), increased expression of cytokines including chemokines around ovulation, and increased infiltration of immune cells into the stroma of the endometrium (Wira and Sandoe, 1980; Kanda and Tamaki, 1999; Beagley and Gockel, 2003; Henderson et al., 2003; Fischer et al., 2010). On the other hand, during progesterone dominant phases, uterine immunity is low and the uterus is more susceptible to infections, due to many factors including decreased synthesis of prostaglandins and leukotrienes, decreased expression of some pro-inflammatory molecules and AMP, and decreased infiltration and killing activity of immune cells (Beagley and Gockel, 2003; Lewis, 2003; Seals et al., 2003; Lewis, 2004; Fischer et al., 2010; Wira et al., 2015). In the same context, the present study reported that the mRNA expression of selected AMP (*DEFB4A*, *DEFB5*, *LAP*, *TAP*, *BPI*, *S10A11*, and *S100A12*) was estrous cycle-dependent, with higher expression around ovulation compared with luteal phases **(Ibrahim et al., 2016).** It has also been reported that the mRNA expression of cytokines including chemokines such as *IL1B*, *IL8* and *CXCL5* in endometrial cells is higher around ovulation compared with luteal phases (Fischer et al., 2010; Oliveira et al., 2013). IL1B activates vascular endothelial cells and promotes the infiltration of inflammatory cells (Dinarello, 2002), while IL8 and CXCL5 are responsible for the recruitment of PMN (Hoch et al., 1996). This is correlated with PMN infiltration in the endometrial tissue around ovulation (Butt et al., 1991; Cobb and Watson, 1995). Together these data provide evidence that the innate immune response in the bovine uterus is activated around ovulation. This activation comprises complex and orchestrated cellular mechanisms that involve increased expression of various cytokines including chemokines, increased recruitment of immune cells, and increased expression of AMP in order to act against any pathogen that may invade the uterus during mating while the cervix is opened, or to facilitate sperm transport and subsequent fertilisation and fertility (Zhou et al., 2004; Bromfield, 2014).

However, it is not clear if these changes in the uterine immune response during the estrous cycle are solely under the influence of steroidal hormones, or whether other factors may also be involved. It has been reported that *in vitro*, endometrial tissue and cellular responses to bacteria or their PAMP were not influenced by the stage of the estrus cycle, or by the addition of exogenous estradiol or progesterone (Saut et al., 2014). These conflicting results may be found because the *in vitro* model could not replicate certain factors that are present *in vivo*, such as immune cells that interact with endometrial cells (Wicherek, 2008). Immune cells have ovarian steroid receptors; therefore, they may be involved in the regulation of steroidal hormone influences on uterine innate immunity, as reported in humans (Wira et al., 2005; Rodriguez-Garcia et al., 2013a; Rodriguez-Garcia et al., 2013b; Wira et al., 2015). It has been reported that presence of immune cells amplifies the pro-inflammatory response of bovine endometrial cells to *T. pyogenes in vitro* **(Ibrahim et al., 2017).**

During and after parturition, the uterus is under significant risk of trauma and uterine infection (Sheldon et al., 2017). Although some cows have a robust immune response, can recover, and eliminate uterine infections, other cows manifest an inappropriate immune response, and develop postpartum uterine diseases (Sheldon et al., 2009a). In the same context, the current study found that during the later stage of the selected postpartum period (Day 45–51), all candidate AMP, except for *BPI* and *S100A11*, showed higher mRNA expression in inflamed endometrium compared with healthy endometrium **(Ibrahim et al., 2016)**. This may indicate that cows with an insufficient immune response will not be able to resolve the inflammation and therefore develop chronic endometritis, which is manifested in sustained higher expression of AMP, pro-inflammatory mediators and immune cells as reported previously (Chapwanya et al., 2009; Peter et al., 2015). Cows with an appropriate immune response are able to produce a robust but controlled pro-inflammatory response. This was evident during early puerperium (D 24–30 pp), when the expression profile of AMP **(Ibrahim et al., 2016)**, and cytokines including chemokines (Peter et al., 2015), in healthy cows was comparable with that of diseased cows. However, at a later stage of puerperium, healthy cows undergo spontaneous recovery, hence the elevated expression of pro-inflammatory mediators and AMP is no longer needed (Chapwanya et al., 2012). Thus, we could conclude that endometrial epithelial cells have a mechanism by which they are able to scale their response to meet the challenge of the threat. Once the threat is contained, the inflammation will be resolved resulting in a healthy uterus; otherwise a chronic inflammation will develop with a diseased uterus, leading to subfertility. It was previously reported that repeat breeder cows showed higher mRNA expression of pro-inflammatory factors compared with normal cows (Wagener et al., 2017). The mRNA expression of these pro-inflammatory factors was higher in repeat breeder cows, which were at the follicular stage of their estrous cycle compared with repeat breeder cows at the luteal stage of the estrous cycle (Wagener et al., 2017). It is already documented that estrous cycle stage affect the mRNA expression of certain pro-inflammatory factors (Gabler et al., 2009; Fischer et al., 2010) and AMP **(Ibrahim et al., 2016)**.

We evaluated the mRNA expression of AMP throughout a prolonged postpartum period (D24–D51) and found that the transcription level of at least one or two of the candidate AMP was significantly higher in cows with inflamed endometrium compared with cows with healthy endometrium at particular points in the selected postpartum period **(Ibrahim et al., 2016).** This may reflect that AMP are expressed cooperatively to provide a protective umbrella throughout the postpartum period. Since the AMP mRNA expression profile was associated with uterine

health over a prolonged postpartum period, AMP can also be used as diagnostic markers for uterine inflammation. Some AMP are secreted under disease condition into body fluids and excretion, therefore, they have been used as diagnostic markers for many diseases in humans such as inflammatory diseases, intrauterine infections, and cancer (Heine et al., 1998; Young et al., 2003; Jentsch et al., 2004; Gollwitzer et al., 2013). Therefore, their use as diagnostic markers for the uterine health status of cows could be promising.

The mRNA expression of AMP and pro-inflammatory factors was influenced by the stage of the estrous cycle or the postpartum uterine health status, which suggest that AMP belong to a functional network of an activated immune system in the bovine endometrium that work to keep the microenvironment of the uterus ready for its different functions. Although the mRNA expression of most of the candidate AMP was affected by the stage of the estrous cycle or the postpartum uterine health status, constitutive transcription was observed for *S100A8*, *S100A9*, and *S100A12* during the estrous cycle, and for *BPI* and *S100A11* during the postpartum period, regardless of the uterine health status **(Ibrahim et al., 2016)**. These findings indicate that these AMP may be involved in a steady-state immune response, regardless of the stimulus. This could be a mechanism that provides a threshold of protection, which may be essential for some cellular processes inside the uterus, for example in order to keep uterine microflora under control, as reported previously in the human uterus (King et al., 2002).

To understand uterine innate immunity *in vivo*, an *in vitro* model was used that comprised endometrial epithelial cells co-cultured with bacteria isolated from the postpartum uterus. We used *B. pumilus* to investigate the mRNA expression of AMP **(Ibrahim et al., 2016).** The usage of a potentially pathogenic uterine bacterium such as *B. pumilus* in co-culture experiments is useful to uncover its role in uterine diseases, and to reveal immunological processes inside the uterus, since *B. pumilus* does not trigger endometrial cell death in co-cultures as quickly as some bacteria associated with endometritis such as *T. pyogenes*, which induced death of endometrial epithelial cells within 16 h **(Ibrahim et al., 2017)**. *T. pyogenes* produces pyolysin, which induces osmotic cell death (Jost et al., 1999; Amos et al., 2014) and hence a severe pathology of uterine disease (Bonnett et al., 1991; Lima et al., 2015).

The endometrial epithelial cells were not responsive to *B. pumilus in vitro* in terms of defensin expression **(Ibrahim et al., 2016)** compared with IL as reported previously (Gärtner et al., 2016). *B. pumilus* might have a defence mechanism, which inhibits defensins at the transcriptional level, as reported for many bacterial species (Menendez and Brett Finlay, 2007; Mogensen, 2009). In contrast, the transcription of *S100A8* and *S1009* was higher in the presence of *B. pumilus*. The ability of *B. pumilus* to stimulate mRNA expression of members of the *S100A* family rather than the *DEFB* family may be attributed to the ability of *B. pumilus* to activate pathways involved in the transcription of *S100A8* and *S100A9*. It has been reported that uterine epithelial cells were responsive to LPS found in Gram-negative bacteria but not to LTA found in all bacteria, in terms of mRNA expression of the *DEFB* family. This may indicate that up-regulation of defensin expression in endometrial cells depends on the activation of pathways triggered by Gram-negative but not Gram-positive bacteria. This hypothesis is supported by the fact that bovine endometrial epithelial and stromal cells can detect and respond to bacterial lipopetides found in Gram-positive bacteria through TLR1, TLR2, and TLR6, and to LPS of Gram-negative bacteria through TLR 4 and a MYD88-dependent signalling pathway (Cronin et al., 2012; Turner et al., 2014).

The different response of endometrial cells in terms of AMP expression between *in vivo* and *in vitro* conditions could be attributed to the fact that the postpartum uterus is colonised by many bacterial species, which likely provoke a more noticeable response than individual bacterial species. The *in vivo* response involves a complex network of interactions among different cell types within the endometrium, such as epithelial, stromal, and immune cells, which was not available in the *in vitro* model.

A fundamental feature of innate immunity is that the intensity of its response is scaled based upon the severity of the infection and tissue damage (Sheldon et al., 2017). To assess whether endometrial cells could scale their immune reaction according to the strain of the bacteria, we used an *in vitro* model of endometrial epithelial cells co-cultured with two different strains of *T. pyogenes*. One strain was isolated from the uterus of a cow developing CE (TP2) and the other strain was isolated from a healthy uterus (TP5). Our results showed that bovine endometrial epithelial cells scaled their pro-inflammatory response according to the strain of *T. pyogenes*, with higher mRNA expression of *PTGS2* and *IL8* in the presence of live TP2 compared with TP5 **(Ibrahim et al., 2017)**. The endometrial epithelial cells scaled their pro-inflammatory response according to quantity of bacteria. For example, in the current study, endometrial cells did not respond to heat-inactivated (HI) *T. pyogenes* at a multiplicity of infection (MOI) of 1. However, in earlier studies, endometrial cells generated a pro-inflammatory response to HI *T. pyogenes* at a MOI of 1000 (Borges et al., 2012; Amos et al., 2014). We found also that endometrial cells produced a pro-inflammatory response to live *T. pyogenes* but not to HI *T. pyogenes* or bacteria-free filtrate (BFF) *T. pyogenes* **(Ibrahim et al., 2017).** In a previous study, the presence of BFF *T. pyogenes* did not also provoke any pro-inflammatory response in endometrial cells (Amos et al., 2014). These data suggest that endometrial cells scaled their response according to the magnitude of the threat. In addition, as discussed previously, the *in vivo* experiment showed that endometrial cells scaled up their response at different points during the postpartum period, whether this upregulation was controlled, as in healthy endometrium, or not, as in the inflamed endometrium **(Ibrahim et al., 2016)**. All of these data provide evidence that bovine endometrial cells have a sophisticated mechanism by which they are able to scale up their pro-inflammatory responses to counter challenges to the tissues.

Indeed, many *in vivo* studies have shown that presence of *T. pyogenes* is a risk factor for the development of CE in cows (Wagener et al., 2014b; Wagener et al., 2015). However, the presence of *T. pyogenes* in postpartum cows does not cause the development of CE in some cows (Silva et al., 2008; Bicalho et al., 2012). This could be for many reasons such as a robust uterine immune response in these cows, or as shown here in the *in vitro* experiments, that different *T. pyogenes* strains could induce different pro-inflammatory responses in uterine epithelial cells **(Ibrahim et al., 2017).**

Uterine epithelial cells are the first line of defence inside the uterus. They recognise different pathogens, and respond by producing different pro-inflammatory mediators, which attract immune cells to the site of infection. This means that the host-pathogen interaction inside the uterus involves interactions between endometrial cells, the pathogen, and immune cells. Uterine infection with *T. pyogenes* has been associated with chronic endometritis and the infiltration of PMN and lymphocytes. Many *in vitro* models have evaluated the effect of *T. pyogenes* on endometrial cells. However, these models did not include immune cells as an essential part of that interaction. To gain insights into the role of host immunity in *T. pyogenes*-

related uterine infections, we investigated the influence of PBMC on the pro-inflammatory response of bovine endometrial epithelial cells in response to *T. pyogenes in vitro*. Uterine inflammation is associated with transmigration of PMN; however, in this study we used only PBMC, because *T. pyogenes* infection is associated with chronic inflammation, which involves infiltration of lymphocytes **(Ibrahim et al., 2017)**. Earlier studies also detected antibodies against *T. pyogenes* (Watson, 1989; Bisinotto et al., 2016). Therefore, we hypothesised that there is an active interaction between *T. pyogenes* and lymphocytes (B-cells). The presence of PBMC sensitised bovine endometrial epithelial cells to *T. pyogenes* early in the course of infection, and amplified the pro-inflammatory response to *T. pyogenes* later in the infection. This cross talk and influence of immune cells on endometrial cells is also evident in the human endometrium (Germeyer et al., 2009). It is believed that cross communication between uterine cells and immune cells is essential during important events such as implantation and parturition, or during uterine infections (Hansen, 2007; Wicherek, 2008; Germeyer et al., 2009; Oreshkova et al., 2012; Lee et al., 2015). Interestingly, the presence of PBMC alone did not provoke any response in endometrial cells in contrast with PBMC and *T. pyogenes* **(Ibrahim et al., 2017).** Therefore, we speculate that the communication between endometrial cells and immune cells is not via cell-cell contact but through the production of soluble factors from bacterially activated immune cells (Panja et al., 1994). In the present study, we did not separate immune cells and endometrial cells because our aim was to simulate the *in vivo* conditions that involve direct contact between endometrial epithelial cells and immune cells **(Ibrahim et al., 2017)**. Bovine endometrial explants have been successfully used to study host-pathogen interactions inside the bovine uterus (Borges et al., 2012). However, in this study, we used purified populations of endometrial epithelial cells to study the specific response of this cell type that are in contact with the external environment, rather than studying a mixed cellular response. We confirmed that the response is originated from endometrial epithelial cells and not contributed by contaminating PBMC by monitoring mRNA expression of *CD45* as a marker of PBMC. We also found that the mRNA expression of prostaglandin E synthase (*PGES*) was not detected in PBMC. However, higher mRNA expression of *PGES* was evident in endometrial epithelial cells co-cultured with *T. pyogenes* and PBMC, compared with *T. pyogenes* alone (data not shown). Therefore, the results suggest that the response originated from the endometrial epithelial cells and not from PBMC. The greater response of endometrial cells to *T. pyogenes* and immune cells compared with *T. pyogenes* alone, could be due to the release of soluble factors like IL1A from the bacterially activated PBMC. IL1A may further stimulate the endometrial cells to scale up their pro-inflammatory response (Healy et al., 2014). The greater response of endometrial cells to the presence of *T. pyogenes* and PBMC rather than to *T. pyogenes* alone may indicates two things. First, endometrial cells have the capacity to scale their response according to surrounding circumstances. Second, host uterine immunity depends on non-specific and specific sentential cells, and the active interaction between them. The presence of commensal bacteria inside the bovine uterus also supports the idea that endometrial cells could scale their response according to the surrounding environment. For example, *Lactobacillus buchneri* was isolated from bovine uterus and did not significantly evoke pro-inflammatory responses in bovine endometrial cells *in vitro*, even when used at a MOI of 10 (Gärtner et al., 2015). This is in contrast to the presence of *T. pyogenes* at MOI of 1, which induced a pro-inflammatory response in bovine endometrial epithelial cells and caused their death within 16 h *in vitro* **(Ibrahim et al., 2017).**

5.2. *Trueperella pyogenes* as a pathogenic bacterium causing postpartum uterine diseases

In the present study, we found that uterine endometrial epithelial cells scaled their pro-inflammatory response according to the strain of *T. pyogenes* **(Ibrahim et al., 2017)**. However, previous studies have reported that full genome sequencing of different uterine isolates of *T. pyogenes* showed that they were highly similar to each other (Goldstone et al., 2014; Machado and Bicalho, 2014). Therefore, we hypothesised that the determinants of pathogenicity of the *T. pyogenes* strains used in the present study might be attributed to strain-specific characteristics such as the metabolic profile, growth rate, and degree of mRNA expression of virulence genes.

5.2.1. Identification of the metabolic profile of *T. pyogenes* strains using Fourier-transform infrared (FTIR) spectroscopy

FTIR spectroscopy is a metabolome-based method, which has the capacity to differentiate a wide range of microbiota (Ehling-Schulz et al., 2005; Wagener et al., 2015). Moreover, it can be used for bacterial strain characterisation, biotyping, and differentiation at the inter-species and intra-species levels (Grunert et al., 2013; Vaz et al., 2013; Wagener et al., 2014a; Wagener et al., 2014b). The method depends on subjecting bacterial cells to an infrared (IR) beam, which causes the vibration of chemical bonds upon their excitation by the absorption of IR. These vibrations are transformed using an equation (Fourier transform) to generate a spectrum specific to the bacterial strain (Helm et al., 1991). Therefore, the spectra reflect the biochemical composition i.e. the phenotypic characteristics of the bacterial sample (Helm et al., 1991). However, FTIR can only be used to identify bacterial species that can be readily isolated by routine bacteriological culture techniques (Wagener et al., 2015), whereas genotyping methods [e.g. 16S ribosomal ribonucleic acid (16S rRNA) gene sequencing] can also identify non-cultivable bacteria (Santos et al., 2011). Yet, FTIR is a rapid, easy to perform, and inexpensive method (Rodriguez-Saona et al., 2001; Baker et al., 2014). Moreover, FTIR spectroscopy could have greater discriminatory power than genotyping methods. Full sequencing of different uterine isolates of *T. pyogenes* did not reveal prominent differences between these isolates (Goldstone et al., 2014; Machado and Bicalho, 2014), whereas the two strains of *T. pyogenes* used in the current study could be discriminated by FTIR. Cluster analysis based on FTIR data showed that TP2 belongs to a completely different cluster than TP5, which means that the strains are biochemically distinct **(Ibrahim et al., 2017).** Comprehensive spectral analysis showed notable differences in the amide/protein and polysaccharide regions of both strains. Polysaccharides are a key component of bacterial peptidoglycans (PGN). Indeed, PGN are recognised by TLR2 on host cells and induce the release of pro-inflammatory factors (Timmerman et al., 1993; Schwandner et al., 1999; Stewart et al., 2003). This may explain why endometrial epithelial cells have a different pro-inflammatory response to each bacterial strain.

5.2.2. Growth rate of different strains of *T. pyogenes*

After parturition, pathogenic bacteria can be isolated from the uteri of most animals. However, not all animals have mucopurulent or purulent vaginal mucus (Williams et al., 2005). Therefore, it has been suggested that bacterial growth density rather than just the presence of an organism could be a determinant for development of uterine diseases (Williams et al., 2005). In the bovine uterus, bacterial growth densities for pathogenic bacteria (e.g. *T. pyogenes*) have

been associated with purulent vaginal mucus and severity of endometritis (Mateus et al., 2002b; Williams et al., 2005). In the human genital tract, the microbial load was also associated with the degree of inflammation (Kasper et al., 2010; Kacerovsky et al., 2012). Therefore, we hypothesised that the *T. pyogenes* strain (TP2) isolated from a cow with muco-purulent vaginal discharge at day 21 pp would have a higher growth rate than the strain (TP5) isolated from the uterus of a cow with no abnormal vaginal discharges at day 21 pp, and thus, endometrial cells could scale up their pro-inflammatory response to that strain. As expected, we found that TP2 had a higher growth rate at 6 h compared with TP5 **(Ibrahim et al., 2017).** A bacterial strain with a higher growth rate will shed and release more PGN during growth and multiplication (Cloud-Hansen et al., 2006). PGN is a PAMP, which induces a potent pro-inflammatory response in host cells (Takeuchi et al., 2002). Therefore, TP2, which grew at a faster rate than TP5, might release more PGN and thus induce a more pathogenic effect in endometrial cells. It was expected that the *T. pyogenes* strain growing at a faster rate would deplete nutrients from endometrial cells *in vitro* and induce more rapid death of the cells. However, both strains induced the death of more than 90% of the endometrial cells after 16 h, with no significant difference between the strains. This may be because the *in vitro* medium contains sufficient nutrients for the growth of both the endometrial cells and the bacterial cells.

5.2.3. Virulence gene transcription in different *T. pyogenes* strains

The degree of virulence is related directly to the ability of the microorganism to cause disease despite host resistance mechanisms. The clinical course of a disease often depends on the interaction of virulence factors with host responses (Peterson, 1996; Casadevall and Pirofski, 2001). Previous studies have evaluated the association between the presence of certain virulence genes in the genome of uterine isolates of *T. pyogenes* and the occurrence of uterine disease (Silva et al., 2008; Bicalho et al., 2012). However, these studies evaluated only the presence of the virulence genes, which may be misleading because the presence of genes in the genome does not necessarily mean that expression of the genes occurs. In the current study, the transcription of virulence genes in two strains of *T. pyogenes* (TP2 versus TP5) was evaluated **(Ibrahim et al., 2017)**. Transcription of *cbpA* and *fimE* was detected in TP2 but not in TP5. Moreover, higher mRNA expression of *nanH* and *fimG* was evident in TP2 compared with TP5. These virulence factors are involved in adherence to host cells (Jost et al., 2002; Esmay et al., 2003; Jost and Billington, 2005). Furthermore, bacterial cell wall-associated virulence proteins can induce a pro-inflammatory response in host cells (Hajishengallis et al., 2002; Ashkar et al., 2008; Mossman et al., 2008) and stimulate adaptive immunity and the production of specific antibodies against them (Bisinotto et al., 2016). Therefore, TP2, which expressed more cell wall-associated virulence factors, might be able to better attach to endometrial cells and induce a stronger pro-inflammatory response. This may explain why this strain is more pathogenic to the endometrium, and why it is associated with CE. The difference in mRNA expression profiles of virulence factors between TP2 and TP5 may suggest the potential efficacy of immunisation of cows against these virulence factors, which were associated with *T. pyogenes* strain isolated from cows with CE as reported previously (Machado et al., 2014). Immunisation of cows against *T. pyogenes* can be beneficial for many reasons. *T. pyogenes* is resistant to many antibiotics (Santos et al., 2010), and immunisation can avoid the dilemma of antibiotics resistance, besides potentially protecting against CE associated with *T. pyogenes* (Wagener et al., 2014b; Wagener et al., 2015).

6. Conclusion

Considering the host, this study offers evidence that bovine uterine epithelial cells have sophisticated mechanisms by which they scale their initial immune responses according to the surrounding circumstances. They increase the expression of candidate AMP around ovulation and during inflammatory processes in the bovine endometrium. In addition, endometrial epithelial cells responded to a potential pathogenic Gram-positive *B. pumilus* by increasing mRNA expression of distinct AMP. Moreover, endometrial epithelial cells responded to TP2 by increasing the mRNA expression of some pro-inflammatory factors. This response was further amplified in the presence of immune cells.

Considering the pathogen, this study showed that TP2 has a distinct metabolic profile, was able to grow faster, and expressed different virulence factors compared with TP5, the strain isolated from a healthy cow. Therefore, the strain of *T. pyogenes* is likely to be a determinant factor for the development of postpartum uterine diseases in cows.

7. Summary

Nearly half of dairy cows develop uterine diseases in the first weeks after parturition, the most common manifestation being endometritis. Such diseases profoundly affect reproductive performance and reduce the profit potential of dairy farms, with an associated yearly cost of €1.4 billion within the European Union.

Bacteria that enter into the uterus after calving trigger an innate immune response that results in the release of cytokines and antimicrobial peptides (AMP). There are different classes of AMP, which exert their antimicrobial action through different mechanisms. The beta-defensin (DEFB) family consists of cationic AMP that can permeabilize bacterial membranes. This family includes DEFB1, DEFB4A, and DEFB5, lingual AMP (LAP), and tracheal AMP (TAP). The bactericidal/permeability-increasing protein is also a cationic AMP that bind to bacterial lipopolysaccharides (LPS) that eventually results in the death of bacteria. Another AMP family is the S100 calcium-binding protein (S100A) family including the following members: S100A8, S100A9, S100A11, and S100A12. These AMP exert their antimicrobial action through chelation of several ions. The host immune response and release of AMP is essential for the removal of invading bacteria. A variety of bacterial strains have been discovered in the uterus so far. These include *Escherichia coli* and *Trueperella pyogenes,* which are the most important bacterial species associated with endometritis.

The aim of this research project was to better understand the innate immune response in the context of postpartum uterine diseases. In the current project, the mRNA expression pattern of selected AMP (DEFB1, DEFB4A, DEFB5, LAP, TAP, BPI, S100A8, S100A9, S100A11, and S100A12) was evaluated in bovine endometrial cells collected (1) at different stages of the oestrous cycle; (2) during the puerperium depending on uterine health status (healthy, subclinical, or clinical endometritis) starting on Day 24 to 30 postpartum for 3 weeks on a weekly basis; and (3) in cell cultures in presence of *Bacillus pumilus* at three different multiplicities of infection (MOI 1, 5, and 10) up to 6 hours. Furthermore, to gain insights into bacterial factors contributing to the host-pathogen interactions, two strains of *T. pyogenes* were included in this study: one strain (TP2) was isolated from the uterus of a postpartum dairy cow developing CE and a second strain (TP5) was isolated from a uterus of a healthy cow. The two strains were compared in terms of their metabolic fingerprints, growth rate, virulence gene transcription, and effect on bovine endometrial epithelial cells *in vitro*. Finally, the effect of the presence of immune cells on the response of endometrial cells to *T. pyogenes* was evaluated. The endometrium samples were either collected at the abattoir (oestrous cycle) or from postpartum cows on a dairy farm using the cytobrush technique. For *in vitro* experiments, endometrial epithelial cells isolated from different animals were co-cultured with live *B. pumilus* at MOI of 1, 5, and 10, or *T. pyogenes* (TP2 or TP5) in form of live, heat-inactivated (HI) or bacteria-free filtrate (BFF) at a MOI of 1, or live TP2 and/or PBMC at a ratio of 1:1 to epithelial cells. Total RNA was isolated from endometrial cells and subjected to a reverse transcription-polymerase chain reaction for quantification of the mRNA expression of selected AMP or pro-inflammatory mediators. The results showed that the mRNA expression of all candidate AMP, except *DEFB1*, *S100A8,* and *S100A9*, was oestrous cycle-dependent. Higher mRNA expression was observed around ovulation compared with luteal phases. The mRNA expression of almost all candidate AMP was uterine health status-dependent, with higher mRNA expression in inflamed than in healthy endometrium, especially during the late stage of

the puerperium (week 5–7). In cell culture experiments, the presence of *B. pumilus* had no significant effect on mRNA expression of the DEFB family but higher mRNA expression of *S100A8* and *S100A9* was observed in the presence of *B. pumilus*.

TP2 grew at a faster rate, expressed more virulence factors such as collagen-binding protein (*cbpA*), neuraminidase H cell wall-bound protein (*nanH*), and fimbrial protein subunit E and G (*fimE* and *fimG*), and elicited a higher mRNA expression of pro-inflammatory factors such as prostaglandin-endoperoxide synthase 2 (*PTGS2*), chemokines (C-X-C motif) ligands 3 (*CXCL3*), and interleukin (*IL) 8* in bovine endometrial epithelial cells compared with TP5. The presence of PBMC amplified the mRNA expression of pro-inflammatory factors (*PTGS2, CXCL3, IL1A, IL6*, and *IL8*) in bovine endometrial epithelial cells co-cultured with live TP2 compared with untreated cells, especially as early as after 4 h.

In summary, it can be assumed that AMP are important components of the innate immune system in the uterus that create an environment that is free of pathogenic bacteria. Higher mRNA expression of the candidate AMP around ovulation or in inflamed endometrium during the puerperium suggests their crucial role in uterine innate immunity to defend against invading pathogenic bacteria. TP2 has distinct differences in the mRNA expression of certain virulence factors, a distinct metabolic profile, and different growth characteristics compared with TP5. Therefore, the strain of *T. pyogenes* could be a crucial factor for the development of endometritis in dairy cows after parturition. Moreover, the pro-inflammatory response of endometrial cells was amplified in the presence of immune cells. Therefore, communication between endometrial cells and immune cells might be important for bacterial clearance.

8. Zusammenfassung

Untersuchungen auf zellulärer Ebene zur Entstehung von Endometritis in Kühen

Annähernd die Hälfte der Milchkühe entwickelt Krankheiten des Uterus in den ersten Wochen nach der Abkalbung. Die häufigste Uteruserkrankung postpartum ist die Endometritis. Solche Erkrankungen beeinflussen nachhaltig die Fruchtbarkeit und reduzieren den Gewinn von Milchkuhbetrieben in der Europäischen Union um jährlich 1,4 Milliarden Euro.

Bakterien, die nach der Kalbung in den Uterus aufsteigen, bewirken eine Antwort des angeborenen Immunsystems, was zur Freisetzung von Cytokinen und antimikrobiellen Peptiden (AMP) führt. Es gibt verschiedene Klassen von AMP, welche ihre antimikrobielle Wirkung durch unterschiedliche Mechanismen erzielen. Die beta-Defensin (DEFB)-Familie besteht aus kationischen AMP, die bakterielle Membranen permeabilisieren können. Diese Familie beinhaltet DEFB1, DEFB4A, DEFB5, linguales AMP (LAP) und tracheales AMP (TAP). Das „bactericidal/permeability increasing protein“ (BPI) ist auch ein kationisches AMP, das an bakterielles Lipopolysaccharid (LPS) bindet und dadurch den Tod von Bakterien erwirken kann. Eine andere AMP-Familie ist die S100-Calcium-bindende Protein-Familie (S100A), welche die folgenden Mitglieder enthält: S100A8, S100A9, S1100A11 und S100A12. Diese AMP entfalten ihre antimikrobielle Wirkung durch Chelatierung von verschiedenen Ionen. Die Immunantwort des Wirtsorganismus und die Freisetzung von AMP sind grundlegend für die Entfernung der eingewanderten Bakterien. Bisher wurde eine Vielzahl von Bakterienstämmen im Uterus entdeckt. Diese beinhalten *Escherichia coli* und *Trueperella pyogenes*, welche die wichtigsten Bakterienstämme sind, die mit Endometritis assoziiert sind.

Das Ziel dieses Forschungsprojekts war, die angeborene Immunantwort im Rahmen von postpartalen Uteruserkrankungen besser verstehen zu können. In dem aktuellen Projekt wurden das mRNA-Expressionsmuster ausgewählter AMP (DEFB1, DEFB4A, DEFB5, LAP, TAP, BPI, S100A8, S100A9, S100A11 und S100A12) in bovinen Endometriumszellen untersucht. Die Endometriumszellen wurden gewonnen (1) während verschiedener Stadien des Sexualzykluses, (2) während des Puerperiums in Abhängigkeit vom Gesundheitsstatus des Uterus (gesund, subklinische oder klinische Endometritis) ab Tag 24-30 postpartum in einem wöchentlichen Rhythmus für die folgenden drei Wochen; und (3) in Zellkulturen in Anwesenheit von *Bacillus pumilis* in drei verschiedenen Gehalten (Muliplicities of infection (MOI) von 1, 5 und 10). Des Weiteren wurden zwei *T. pyogenes* Stämmen in diese Studie aufgenommen, um zu untersuchen, wie bakterielle Faktoren zu Wirts-Pathogen-Interaktionen beitragen. Ein Stamm (TP2) wurde aus dem Uterus einer Kuh postpartum gewonnen, die an klinischer Endometritis erkrankte und der zweite Stamm (TP5) wurde aus einem gesunden Uterus isoliert. Diese beiden Stämme wurden verglichen in Bezug auf ihre metabolischen „Fingerabdrücke“, Wachstumsraten, Transkription von Virulenzgenen und ihren Einfluss auf endometriale Epithelzellkulturen *in vitro*. Ebenso wurde der Effekt der Anwesenheit von Immunzellen auf endometriale Epithelzellen *in vitro* untersucht. Endometriumsproben wurden entweder am Schlachthof (Sexualzyklus) oder im Milchviehbetrieb mittels Cytobrush-Technik gewonnen. Für die *in vitro*-Experimente wurden endometriale Epithelzellen aus verschiedenen Tieren isoliert und mit lebenden *B. pumilus* in einer MOI von 1, 5 und 10 oder mit *T. pyogenes* (TP2 und TP5) in Form von lebenden, hitze-inaktivierten (HI) oder bakterien-freien Filtraten (BFF) in einer MOI von 1 inkubiert. Ebenso wurden Epithelzellen und/oder mit PBMC in einem

Verhältnis von 1:1 mit lebenden TP2 ko-kultiviert. Gesamt-RNA wurde aus Endometriumszellen isoliert und mittels reverser Transkriptions-Polymerase Kettenreaktion wurde die Quantifizierung der mRNA-Expression ausgewählten AMP oder pro-inflammatorischen Mediatoren durchgeführt.

Die Ergebnisse zeigten, dass die mRNA-Expression aller Kandidaten-AMP, außer *DEFB1*, *S100A8* und *S100A9*, abhängig vom Sexualzyklus beeinflusst war. Um die Ovulation herum war im Vergleich zur Lutealphase die mRNA-Expression höher. Die mRNA-Expression fast aller untersuchter Kandidaten-AMP war abhängig vom Gesundheitszustand des Uterus mit erhöhter mRNA-Expression bei entzündetem Endometrium verglichen mit einem gesundem Endometrium, besonders im späteren Stadium des Puerperiums (Woche 5-7). In Zellkulturversuchen hatte die Gegenwart von *B. pumilus* keinen signifikanten Effekt auf die mRNA-Expression der DEFB-Familie. Aber eine höhere mRNA-Expression von *S100A8* und *S100A9* konnte in Gegenwart von *B. pumilus* beobachtet werden.

TP2 wuchs schneller und exprimierte mehr Virulenzfaktoren [collagen-binding protein (*cbpA*), neuraminidase H cell wall-bound protein (*nanH*), und fimbrial protein subunit E und G (*fimE* und *fimG*)] als TP5 und rief eine höhere mRNA-Expression von pro-inflammatorischen Faktoren [(prostaglandin-endoperoxide synthase 2 (*PTGS2*), chemokines (C-X-C motif) ligands 3 (*CXCL3*), interleukin (*IL)-8*)] in bovinen endometrialen Epithelzellen hervor verglichen mit TP5. Außerdem war die mRNA-Expression von pro-inflammatorischen Faktoren *PTGS2, CXCL3*, *IL1A, IL6 und IL8* in bovinen endometrialen Epithelzellen in Ko-Kultur mit lebenden TP2 durch die Anwesenheit von Immunzellen verstärkt, verglichen mit unbehandelten Zellen. Dies war schon nach 4 Stunden beobachtbar.

Zusammenfassend kann festgestellt werden, dass AMP wichtige Komponenten für die angeborene Immunabwehr im Uterus sind, die ein Milieu erzeugen, welches frei von pathogenen Bakterien ist. Eine erhöhte mRNA-Expression der Kandidaten-AMP um die Ovulation herum oder in entzündetem Endometrium während des Puerperiums weisen auf eine entscheidende Rolle bei der angeborenen Immunabwehr des Uterus bei der Bekämpfung der einwandernden pathogenen Bakterien hin. TP2 zeigte deutliche Unterschiede in der mRNA-Expression bestimmter Virulenzfaktoren, im metabolischen Profil und bei dem Wachstum im Vergleich zu TP5. Demzufolge könnte der *T. pyogenes*-Stamm ein entscheidender Faktor für die Entstehung von Endometritis bei Milchkühen nach dem Abkalben sein. Darüber hinaus wurde die pro–inflammatorische Antwort der Endometriumszellen in Anwesenheit von Immunzellen amplifiziert. Aus diesem Grund könnte die Kommunikation zwischen Endometriumszellen und Immunzellen wichtig für die Beseitigung von Bakterien sein.

9. References

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10. Publications and scientific activity

Publications

- **Ibrahim M,** Peter S, Wagener K, Drillich M, Ehling-Schulz M, Einspanier R, Gabler C Bovine endometrial epithelial cells scale their pro-inflammatory response *in vitro* to *Trueperella pyogenes* infections in a strain specific manner. *Front. Cell. Infect. Microbiol*. 7:264. doi: 10.3389/fcimb.2017.00264
- **Ibrahim M,** Peter S, Gärtner MA, Michel G, Jung M, Einspanier R, Gabler C. Increased mRNA expression of selected antimicrobial peptides around ovulation and during inflammatory processes in the bovine endometrium postpartum. *Theriogenology* 2016; 86:2040-2053
- Peter S, Michel G, Hahn A, **Ibrahim M,** Lübke-Becker A, Jung M, Einspanier R, Gabler C. Puerperal influence of bovine uterine health status on the mRNA expression of pro-inflammatory factors. *J. Physiol. Pharmacol.* 2015; 66:449-462

Conference talks and posters

- **Ibrahim, M.,** Wagener, K., Drillich, M., Ehling-Schulz, M., Einspanier, R., *Gabler*, C. *Trueperella pyogenes* isolated from a cow with clinical endometritis showed a higher growth rate and increased mRNA expression of virulence factors compared with a strain isolated from a healthy cow. Oral presentation at the 20th Annual Conference of European Society for Domestic Animal Reproduction (ESDAR). Lisbon, Portugal. October 27-29, 2016
- **Ibrahim, M.,** Peter, S., Wagener, K., Drillich, M., Ehling-Schulz, M., Einspanier, R., *Gabler*, C. Presence of bovine peripheral blood mononuclear cells amplified mRNA expression of pro-inflammatory genes in bovine endometrial epithelial cells co-cultured with *Trueperella pyogenes*. Oral and poster presentation at the 18th International Congress of Animal Reproduction. Tours, France. June 26-30, 2016
- **Ibrahim, M.,** Peter, S., Wagener, K., Drillich, M., Ehling-Schulz, M., Einspanier, R., *Gabler*, C. *T. pyogenes* strain isolated from a cow with clinical endometritis elicits higher pro-inflammatory response in endometrial cells than the strain isolated from a healthy cow. Poster presentation at the 22nd Meeting of the Physiology and Biochemistry Division of the German Veterinary Medical Society (DVG). Berlin, Germany. March 30 – April 1, 2016
- **Ibrahim, M.,** Peter, S., Wagener, K., Drillich, M., Ehling-Schulz, M., Einspanier, R., *Gabler*, C. The pro-inflammatory response of bovine endometrial cells depends on the *Trueperella pyogenes* strain. Oral presentation at the 49th Annual Conference of Physiology and Pathology of Reproduction. Leipzig, Germany. February 10-12, 2016
- **Ibrahim, M.,** Peter, S., Wagener, K., Drillich, M., Ehling-Schulz, M., Einspanier, R., *Gabler*, C. Bovine endometrial pro-inflammatory response differs depending on the strain of *Trueperella pyogenes*. Oral presentation at the 2nd International Conference on Biology and Pathology of Reproduction in Domestic Animals. Gdansk, Poland. September 28-30, 2015
- **Ibrahim, M.,** Einspanier, R., *Gabler*, C. Cow's molecular endometritis. Ph.D. Symposium of Biomedical School, Dahlem Research School, Freie Universität Berlin. Berlin, Germany. March 20, 2015

- **Ibrahim, M.,** Gärtner, M., Michel, G., Jung, M., Einspanier, R., *Gabler*, C. S100 proteins expression regulated by health status and stage of estrous cycle of the bovine endometrium. Poster presentation at the 18th Annual Conference of European Society for Domestic Animal Reproduction (ESDAR), Helsinki, Finland. September 11-13, 2014. Abstract published in Reproduction in Domestic Animals (Vol.49 Suppl 3:1-102. doi: 10.1111/rda. 12409)
- **Ibrahim, M.,** Holder, C., Einspanier, R., *Gabler*, C. Potential immunoregulatory role of distinct antimicrobial peptides during the estrous cycle in the bovine endometrium. Poster presentation at the 47. Annual Conference of Physiology and Pathology of Reproduction, Gießen, Germany. February 27-28, 2014. Abstract published in Reproduction in Domestic Animals (Vol.49 Suppl 1:1-52. doi: 10.1111/rda.12292)
- **Ibrahim, M.,** Michel, G., Jung, M., Einspanier, R., *Gabler*, C. mRNA expression of distinct antimicrobial peptides was increased postpartum in the endometrium of subclinical cows compared to healthy cows. Poster presentation at the 17th Annual Conference of European Society for Domestic Animal Reproduction (ESDAR). Bologna, Italy. September 12-14, 2013. Abstract published in Reproduction in Domestic Animals (Vol.48 Suppl 1:1-124. doi: 10.1111/rda.12228)

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12. Selbständigkeitserklärung

Hiermit bestätige ich, **Mohammad Abdelwahab Ali Ibrahim** dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, den **07.12.2017** **Mohammad Abdelwahab Ali Ibrahim**

Detailed assessment of contribution of Mr. **Mohammad Abdelwahab Ali Ibrahim** to the research projects presented under this cumulative doctoral thesis

Contribution	Research project #1 (Antimicrobial peptides in the bovine endometrium)	Research project #2 (Role of *Trueperella pyogenes* strains in endometritis)
Study design	30%	30%
Data collection	45%	75%
Data analysis	75%	80%
Manuscript writing	85%	85%
Manuscript editing	55%	55%

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